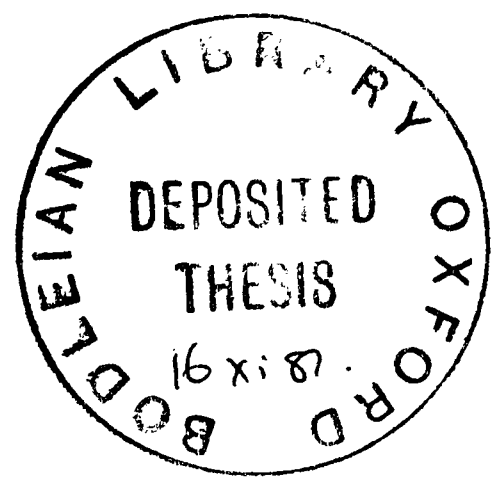
A STUDY OF TYROSINE HYDROXYLASE ACTIVITY

IN NORADRENERGIC NEURONES

IN THE RAT BRAIN



A dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Oxford.

Susanna Graham-Jones

Somerville College

Hilary Term 1981

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ABSTRACT

Tyrosine hydroxylase is the enzyme which controls the rate-limiting step in the synthesis of noradrenaline . In order to discover whether the activity of tyrosine hydroxylase might serve as an indicator of noradrenergic function in the brain, two preparations for the direct measurement of TH activity in rat brain regions by a tritium-release method were employed : synaptosomal suspensions prepared from pinched-off nerve terminals , and partially solubilised enzyme preparations prepared from frozen homogenates or synaptosomal suspensions and assayed at saturating concentrations of cofactor and tyrosine.

There was evidence of an increase in tyrosine hydroxylase activity in hippocampal synaptosomes of rats killed immediately after a mild electrical footshock. Activation of synaptosomal enzyme activity was also found after single doses of clonidine and parachloroamphetamine, and after repeated handling; and single doses of morphine and of yohimbine appeared to lower tyrosine hydroxylase activity . Repeated administration of drugs such as clonidine, desipramine and 2-deoxyglucose, however, did not affect tyrosine hydroxylation rate.

A preliminary finding , suggesting differences in synaptosomal tyrosine hydroxylase activity related to experience with different reinforcement schedules (continuous reward vs. partial reward) in a runway experiment , was not substantiated in later experiments; nor was there any difference between the synaptosomal tyrosine hydroxylase activity of naive controls and rats given repeated daily shocks for a week .

The saturated TH assay performed on solubilised enzyme was, as predicted, unresponsive to the short term stimulation effects detected with the synaptosomal assay. However, other changes, such as a reduced maximal hydroxylation rate after repeated desipramine administration , and an increased rate several weeks after a course of electrical stimulation of the septal area, were established with the saturated assay. Although the changes in stimulated rats were associated with increased behavioural tolerance to stress, e.g. resistance to extinction of a running response in a runway, other experiments in which the behavioural stress-tolerance was induced by behavioural methods alone showed no accompanying changes in TH activity.

Measures of synaptosomal and saturated soluble TH activity appear to constitute independent indicators of noradrenergic function. It seems that synaptosomal tyrosine hydroxylase activity is not , as anticipated, controlled by the firing rate of locus coeruleus neurones ; but it may be subject to local regulation in noradrenergic terminals. The results are discussed in the context of theoretical aspects of the regulation of noradrenaline synthesis in the brain , and the mechanisms underlying physiological responses to stress and behavioural tolerance to stress.

EXTENDED ABSTRACT

Tyrosine hydroxylase (TH) is the enzyme which controls the rate-limiting step in the synthesis of noradrenaline from the amino acid tyrosine, in noradrenergic neurons. Since noradrenaline synthesis can accelerate when the rate of firing of noradrenergic neurones is increased, whilst noradrenaline levels remain relatively stable, the activity of tyrosine hydroxylase may serve as an indicator of noradrenergic function in the brain. Following an introductory chapter reviewing recent work on noradrenaline in the brain, chapters 2 & 3 of this thesis are concerned with the background for the choice of tyrosine hydroxylase (TH) activity as such an indicator , with special emphasis placed on the many different modes of regulation of noradrenaline synthesis.

Most previous work on tyrosine hydroxylase in the central nervous system has been concerned with the activity of the solubilised enzyme from the striatum, which contains dopaminergic terminals. Two preparations were employed in most of the experiments in this study, focussing on noradrenergic mechanisms: synaptosomal suspensions prepared from pinched-off nerve terminals (in which the hydroxylation of labelled exogenous tyrosine at a final tyrosine concentration of 20 μM was measured at pH7.4) ; and partially solubilised enzyme preparations, prepared from frozen homogenates or synaptosomal suspensions and assayed at the optimal pH 6.2, at saturating concentrations of cofactor and tyrosine. Brain regions containing noradrenergic terminals, such as the hippocampus and hypothalamus , were assayed , as was the locus coeruleus, which contains cell bodies. Details of the assays are given in Chapter 4, and the results of experiments are to be found in sections 1 to 9 of Chapter 5.

Tyrosine hydroxylase activity was elevated in synaptosomes prepared from the hippocampus of rats killed immediately after a mild electrical footshock (current intensity 0.3 mA; see Chapter 5,section 1). Single doses of saline, desipramine, and chlordiazepoxide did not alter synaptosomal tyrosine hydroxylase activity (Chapter 5, section 3). But activation of synaptosomal enzyme activity was found after administration of single doses of clonidine and parachloroamphetamine; and single doses of morphine and of yohimbine appeared to lower tyrosine hydroxylase activity . These results , and the lack of correlation between TH activity measurements in different noradrenergic terminal regions, suggest local regulation of the rate limiting step in noradrenaline synthesis in brain regions which receive projections from the locus coeruleus. There is little correspondence of the synaptosomal TH results with previous reports of alterations in the firing rate of noradrenergic cells in the anaesthetized rat after systemic or iontophoretic administration of these drugs.

The shock-induced changes in synaptosomal tyrosine hydroxylase activity dissipated rapidly; but rats handled for 1-2 weeks showed a persistently raised activity in the synaptosomal assay (Chapter 5, section 2). Repeated administration of drugs such as clonidine, desipramine and 2-deoxyglucose, however, were without lasting influence on the synaptosomal TH activity in noradrenergic brain regions.

A preliminary finding of a difference in hippocampal synaptosomal tyrosine hydroxylase activity between groups of rats run in different reinforcement schedules (continuous reward vs. partial reward) in the acquisition phase of a runway experiment was not substantiated in later experiments (Chapter 5, section 7); nor was there any difference between the synaptosomal tyrosine hydroxylase activity of naive controls and rats given repeated daily shocks for a week .

The saturated TH assay performed on solubilised enzyme was, as predicted, unresponsive to the short term stimulation effects detected with the synaptosomal assay. However, other changes, such as a reduced maximal hydroxylation rate after repeated desipramine administration (Chapter 5, section 5), and an increased rate several weeks after a course of electrical stimulation of the septal area, were established with the saturated assay (Chapter 5, section 9). Although the changes in septal-stimulated rats were associated with increased behavioural tolerance to stress, e.g. resistance to extinction of a running response in a runway, other experiments (in which the behavioural stress-tolerance was induced by behavioural methods alone) showed no accompanying changes in TH activity.

Thus measures of synaptosomal and saturated soluble TH activity appear to constitute independent indicators of noradrenergic function. No evidence was found for control of noradren aline synthesis by the rate of firing of locus coeruleus neurones. The results are discussed in the context of theoretical aspects of the regulation of noradrenaline synthesis in the brain , and the mechanisms underlying responses to stress and behavioural tolerance to stress.

CONTENTS

ABSTRACT

EXTENDED ABSTRACT
LIST OF FIGURES

CHAPTER 1 1
NEUROBIOLOGY OF NORADRENERGIC NEURONS

1. role of NA neurons in the brain.
2. anatomy of NA neurons in the brain.
3. physiological studies of NA neurons.
4. noradrenergic effects on target cells.
5. noradrenaline and behaviour.
6. noradrenaline and stress.
7. rationale for experiments.
8. the need for a sensitive measure of noradrenergic function.

CHAPTER 2 18
TYROSINE HYDROXYLASE AND THE SYNTHESIS OF NORADRENALINE

1. the catecholamine synthesis pathway.
2. tyrosine hydroxylase in the brain and in the periphery.
3. early studies of tyrosine hydroxylase: isolation,purification and absolute requirements.
4. the rate-limiting step in catecholamine synthesis.
5. subcellular localization of tyrosine hydroxylase.
6. enzymology of tyrosine hydroxylase.
7. methods for assaying TH activity in brain regions.

CHAPTER 3 48
REGULATION OF TYROSINE HYDROXYLASE

1. in vitro expression of TH activity.
2. enzyme constraints: substrate availability.
3. availability of reduced cofactor.
4. regulation by end product inhibition of TH.
5. activation of TH by calcium ions.
6. depolarization by electrical stimulation
7. cyclic AMP and phosphorylation.
8. tyrosine hydroxylation in noradrenergic synaptosomes.
9. TH regulation : in vitro effects of psychotropic drugs ?
10. changes in TH activity after in vivo stimulation.
11. measurement of noradrenaline synthesis and tyrosine hydroxylase activity: effects of stimulation and stress.
12. long term changes in TH activity in the CNS.
13. regulation of noradrenaline synthesis: models and mechanisms.

CHAPTER 4 105
METHODS
1. rats
2. purification of tyrosine
3. TYROSINE HYDROXYLASE ASSAY
preparation of samples
preparation of the p_2 synaptosomal pellet
preparation of homogenates
incubation stage : synaptosomal assay
incubation stage : soluble assay
Dowex columns
scintillation counting
protein assay
calculation of results
range of blank and control values
4. NORADRENALINE ASSAYS
A.fluorimetric
B.radioenzyme method
5. STATISTICAL ANALYSIS OF RESULTS.

CHAPTER 5 125
EXPERIMENTS
Section 1. Single footshock . 128
Section 2. Repeated footshock. 150
Section 3. Single doses of clonidine,morphine, desipramine,and chlordiazepoxide. 163
Section 4. Single doses of yohimbine and 2-deoxyglucose . 176
Section 5. Long term drug administration. 196
Section 6. Single dose of parachloroamphetamine. 252
Section 7. Partial reinforcement acquisition training. 269
Section 8. Partial punishment acquisition and conflict. 287
Section 9. Hippocampal theta driving. 300

CHAPTER 6
EVALUATION : TYROSINE HYDROXYLASE ACTIVITY AS AN INDEX OF NORADRENERGIC FUNCTION. 318

APPENDIX I 342

APPENDIX II 350

APPENDIX III 357

APPENDIX IV 364

REFERENCES

LIST OF FIGURES

CHAPTER 1.

FIG. 1.1 Distribution of noradrenaline-containing pathways in rat brain. (from Livett, 1973)

FIG. 1.2 Metabolic pathways for noradrenaline.

FIG. 1.3 Schematic diagram of a noradrenergic neurone indicating noradrenergic receptor sites.

CHAPTER 2

FIG. 2.1 Biosynthesis of noradrenaline from tyrosine.

FIG. 2.2 Structure of a monoamine neurone (from Cooper,Bloom and Roth, 1974)

FIG. 2.3 Stimulation of tyrosine hydroxylase activity in vitro by tetrahydrofolate and DMPH4 (purified enzyme from adrenal medulla) (from Nagatsu, 1964a)

FIG.2.4 a) Tyrosine hydroxylase activity; velocity of reaction plotted against substrate concentration

b) Lineweaver-Burk reciprocal plot of 1/velocity of reaction against 1/substrate concentration. Intercept on ordinate is 1/Vmax; intercept on abscissa is 1/Km.

CHAPTER 3

FIG. 3.1 Model of a noradrenergic terminal.
(from Salzman and Roth, 1979)

FIG. 3.2 Activation of soluble tyrosine hydroxylase by various methods, demonstrated by kinetic changes on Lineweaver-Burk plots.
(from Roth et al, 1975)

FIG. 3.3 Post-stimulation activity of tyrosine hydroxylase after in vivo stimulation of the locus coeruleus.
(from Salzman and Roth, 1980a)

FIG. 3.4 Cortical norepinephrine levels at times following termination of locus coeruleus stimulation.
(from Salzman and Roth, 1980a)

FIG. 3.5 Accumulation of DOPA in cortex and hippocampus during in vivo stimulation of the locus coeruleus in the presence of a DOPA decarboxylase inhibitor.
(from Salzman and Roth, 1980a)

FIG. 3.6 Induction of tyrosine hydroxylase in adrenal medulla after reserpine administration.
(from Thoenen et al, 1969)

FIG. 3.7 Immunopreciptation of tyrosine hydroxylase in locus coeruleus from reserpine-treated and control rats.
(from Reis et al, 1974)

FIG. 3.8 A comparison of the time courses of the changes in tyrosine hydroxylase activity in different brain regions after a single injection of reserpine.
(from Zigmond, 1979)

FIG. 3 9 A further comparison of changes in tyrosine hydroxylase activity in different brain regions after reserpine.
(from Black, 1975)

FIG. 3.10 Kinetic analysis of tyrosine hydroxylase in the locus coeruleus from reserpine-induced, oxotremorine-activated, and control rat homogenates.
(from Reis et al, 1975)

FIG. 3.11 Changes in activity of tyrosine hydroxylase in locus coeruleus at different times after administration of oxotremorine.
(from Lewander et al, 1975)

FIG. 3.12 Immunotitration of tyrosine hydroxylase in locus coeruleus at 3 days after oxotremorine.
(from Lewander et al, 1975)

FIG. 3.13 The relationship of impulse traffic,noradrenaline synthesis and release, and post-synaptic receptor activity.
(from Fillenz, 1980)

FIG. 3.14 Metabolic adaptation to antidepressant drugs.
(from Mandell et al, 1975)

CHAPTER 4

FIG. 4.1 Plot of tyrosine hydroxylase activity (in cpm) in partially solubilised preparation of rat striatum with increasing concentration of substrate.
(Boarder and Stewart, unpublished data)

FIG 4.2 Lineweaver-Burk double reciprocal plot of data from FIG. 4.1.
(Boarder and Stewart, unpublished data)

CHAPTER 5

EXPERIMENTS :

(FIGURES ARE LABELLED ACCORDING TO SECTION)

FIG. 1a — Mean change in synaptosomal TH (fmol/min/mg P_2 protein) in shocked rats compared to TH in paired controls; data from a series of 9 experiments.

FIG. 1b — Difference between synaptosomal TH (fmol/min/mg P_2 protein) of shocked and paired control rats, plotted against TH of controls; each mark represents one pair of rats.

FIG. 3a — Noradrenergic receptor agonists and antagonists.

FIG. 5a — Effect of DMI administration on beta-receptor density in rat cerebral cortex. (from Wolfe et al, 1978)

FIG. 6a — Serotonergic pathways in the rat brain (from McGeer et al, 1978)

FIG. 6b — Possible interconnections between the locus coeruleus and the raphe nuclei . Note the short serotonergic neurons linking the anterior, middle and posterior raphe nuclei. 5HT neurones from the middle raphe are shown synapsing on possible cholinergic neurones which in turn synapse on noradrenergic neurons in the locus coeruleus. (from Morgane and Stern, 1974)

CHAPTER 5

PARTIAL REINFORCEMENT

(FIGURES ARE IN APPENDIX II)

FIG. 7a — Experiment B: Running speeds in acquisition at one trial per day.

FIG. 7b — Experiment B: Running speeds in acquisition at 6 trial per day.

FIG. 7c — Experiment C : Running speeds in acquisition and extinction, showing the partial reinforcement extinction effect at one trial per day (Preston, unpublished data).

FIG. 7d — Experiment D: Runway times in acquisition at one trial per day; partial reward and partial punishment.

FIG. 7e — Experiment E: Runway times in acquisition; partial reward and partial punishment. (No trial on day 10).

CHAPTER 5

PARTIAL PUNISHMENT

(FIGURES ARE IN APPENDIX III)

FIG. 8a — see FIG. 7d

FIG. 8b — see FIG.7e

FIG. 8c — Experiment G: Running speeds in runway and goal sections during acquisition at one trial per day. (Davis, 1979)

FIG. 8d — Experiment G: Running speeds in start, runway and goal sections during conflict phase, showing partial punishment effect. (Davis, 1979)

FIG. 8e — Experiment H; Runway times in runway and goal sections at one trial per day during acquisition Effect of chlordiazepoxide (CDP). (Davis, 1979)

FIG. 8f — Experiment H: Runway time in goal section during conflict phase at one trial per day. Effect of chlordiazepoxide during acquisition and conflict phases. (Davis, 1979)

CHAPTER 5

THETA DRIVING

(FIGURES ARE IN APPENDIX IV)

FIG. 9a — Experiment A : Latency from 3rd to 5th bar press; group means of logarithmically transformed latencies, over days of acquisition training.
(Holt, unpublished data)

FIG. 9b — Experiment A : Latency from 3rd to 5th bar press; group means over days of extinction.
(Holt, unpublished data)

FIG. 9c — Experiment E : Mean suppression ratio in CER training after previous theta-driving in the hippocampus by electrical stimulation of the septal area.
(Holt, unpublished data)

CHAPTER 1

THE NEUROBIOLOGY OF NORADRENALINE NEURONES

1. ROLE OF NA NEURONS IN THE BRAIN.
2. ANATOMY OF NA NEURONS IN THE BRAIN.
3. PHYSIOLOGICAL STUDIES OF NA NEURONS.
4. NORADRENERGIC EFFECTS ON TARGET CELLS.
5. NORADRENALINE AND BEHAVIOUR.
6. NORADRENALINE AND STRESS.
7. RATIONALE FOR EXPERIMENTS.
8. THE NEED FOR A SENSITIVE MEASURE OF NORADRENERGIC FUNCTION.

CHAPTER 1

1. THE ROLE OF NORADRENERGIC NEURONES IN THE BRAIN.

Although a great deal of research effort has been expended in the study of noradrenergic neurons in the brain, there is still no adequate theory of the role played by this well-defined system of neurons , either in terms of specific physiological or psychological functions subserved by particular noradrenergic pathways , or , since this is a widely divergent system, in the broader terms of some more holistic regulatory role in the brain. The classical methods of investigation in brain research have yielded a curiously fragmentary body of knowledge about noradrenaline in the brain, different methods often producing conflicting rather than complementary results. There is , for example, a curious lack of confidence in the speculations about the function of the locus coeruleus and its main ascending projection, the dorsal noradrenergic bundle, in recent reviews of the literature (Amaral and Sinnamon, 1977; Clark, 1979; Moore and Bloom, 1979; McNaughton and Mason, 1980) .

2. ANATOMY OF NORADRENERGIC NEURONS IN THE BRAIN

The anatomy of the noradrenaline-containing pathways in the brain was first described in the rat by Dahlstrom and Fuxe (1964) using histochemical fluorescent staining. Most of the noradrenergic cell bodies are in the pons and medulla oblongata. Ungerstedt (1971) emphasised the ascending projections which will be of interest here (see FIG 1.1). The dorsal bundle, ascending from the locus coeruleus (A6) in the floor of the fourth ventricle , projects widely all over the forebrain and diencephalon . The cerebral cortex,

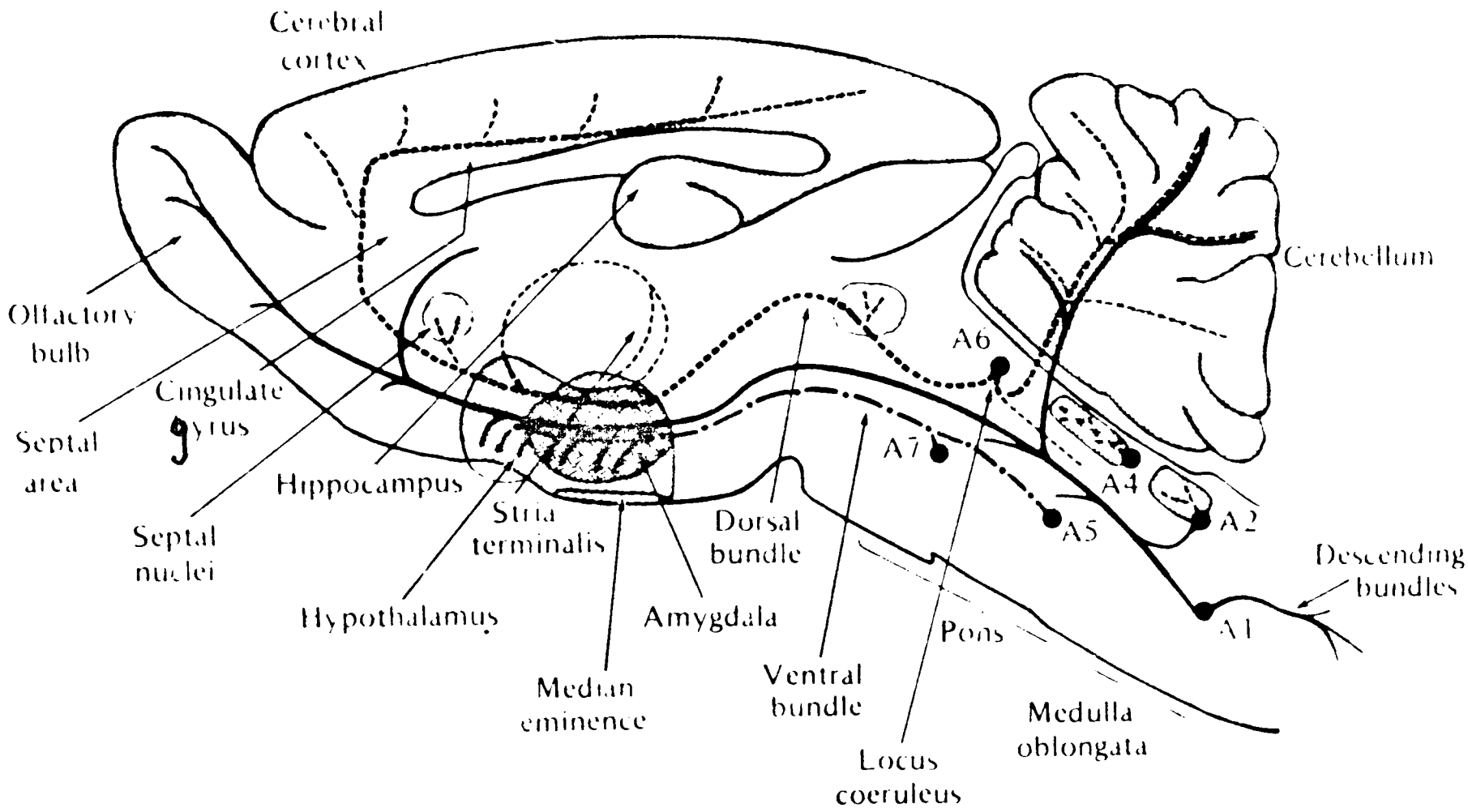


FIG. 1.1 Distribution of noradrenaline-containing pathways in rat brain. (from Livett, 1973)

hippocampus, amygdala, cingulate gyrus , septal area, cerebellum , thalamus and hypothalamus all receive noradrenergic fibres from the locus coeruleus, which contains only about 1500 cells in the rat. This is therefore a widely branching system, with collaterals of the same cell body possibly innervating different structures in the brain (Olson and Fuxe, 1971). The reviews cited above cover the large number of studies of the anatomical details of this system, and of the ventral ascending bundle from the other noradrenergic nuclei in the pons and medulla, including the locus subcoeruleus (A5). Here I shall point out a few implications from these studies, rather than making any attempt to summarise.

The wide distribution of noradrenergic terminals (estimated at 100,000 per neuron,according to Moore and Bloom, 1979) arising from a small nucleus such as the locus coeruleus, the possibility of collaterals innervating different brain regions, and the fine structure of the terminals themselves make it unlikely that the role of this system is the transmission of specific information between brain regions, as seems to be the case in the highly specific, topographically organized projections of the principal ascending sensory pathways. Only about 3 % of the varicosities on noradrenergic terminals in the rat neocortex are associated with specialised synaptic junctions (Descarries et al, 1977) ; it appears that noradrenaline (NA) release from these varicosities results in a diffuse availability of NA at postsynaptic sites, rather than involving precise transmission of information from a given noradrenergic terminal across a specialised junction to a defined target site. The noradrenergic innervation of many parts of the brain, such as the neocortex, is sparse; morphologically it resembles the branching plexus of terminals characteristic of the sympathetic innervation of smooth muscle (Hartman and Udenfriend ,1972). The ascending noradrenergic pathways form part of the reticular formation , along with other poorly-defined networks of as yet unidentified fibres, and it has been proposed that noradrenaline acts as

a neuromodulator in the brain, rather than as a specific neurotransmitter (Amaral and Sinnamon,1977) . This change in terminology awaits clarification, however; the difference between these two neurochemical concepts is by no means clear (see Schildkraut, 1971).

Recent studies have emphasised that there may after all be some specificity in the provenance and distribution of NA-containing fibres in the brain. Morphological differences between dorsal bundle projections (from cell bodies in the locus coeruleus) and lateral tegmental and ventral noradrenergic bundle projections (from cell bodies in the medial and lateral medulla as well as other non-coeruleus groups in the pons) have been found (see Moore and Bloom, 1979), and some areas such as the hypothalamus and lateral septum have a dual noradrenergic innervation, in contrast to areas such as the neocortex and hippocampus which receive noradrenergic fibres only from the locus coeruleus-dorsal bundle pathway. Segal (1980) defines two ascending noradrenergic systems, which roughly correspond to the dorsal bundle and lateral tegmental projections, as having different relationships between terminal areas and the nuclei of origin. He points out that the neocortex, hippocampus and cerebellum do not project back to the locus coeruleus (LC); whereas there are two-way monosynaptic connections between other noradrenergic nuclei of the lateral tegmental system and their terminal areas such as the amygdala. The significance of these 'open loop' and 'closed loop' connections will be discussed later.

Within the LC-dorsal bundle system itself, some topographic differentiation has recently been discovered. Although Olson and Fuxe (1971) showed that some LC cells could be activated antidromically from both cerebellum and neocortex, Segal and Landis (1974) showed differences in the distribution of terminals from the ventral and dorsal poles of the locus coeruleus. Mason and Fibiger (1979) used horse radish peroxidase retrograde

tracing techniques and found that the distribution of labelling in the LC differed according to the terminal region injected. McNaughton and Mason (1980) comment that 'the locus coeruleus can no longer be regarded as a homogeneous nucleus'.

Further evidence of specialisation of noradrenergic function , as opposed to a diffuse and uniform influence in the brain, comes from studies showing different morphological characteristics of cortical and hippocampal noradrenergic terminals. Koda et al (1977) have shown that 18 % of the noradrenergic terminals in the dentate gyrus of the hippocampus make specialised synaptic contacts, in contrast to the figure of 3 % reported by Descarries et al (1977) for the neocortex. However, there is as yet little information as to the functional significance of this morphological finding; though Crutcher and Davis (1980) speculate that there may be a different type of postsynaptic receptor distribution in association with the specialized synapses.

3. PHYSIOLOGICAL STUDIES OF NORADRENERGIC NEURONS

Noradrenaline has been recognized as a potential neurotransmitter in the central nervous system since the work of Vogt (1954). It fulfils the criteria laid down by Werman (1966) for the identification of transmitter substances. Noradrenaline is synthesised in the terminals and varicosities of neurons projecting from the locus coeruleus; the steps in the synthetic pathway and the responsible enzymes are discussed in Chapter 2 (and see FIG. 2.1). The earlier stages in synthesis take place in the cytoplasm; the intermediate compound, dopamine, is taken up into vesicles in the terminals and converted to NA which is then stored in filled vesicles. Vesicular content can be measured and differentiated from total NA in homogenates of brain tissue. It appears that

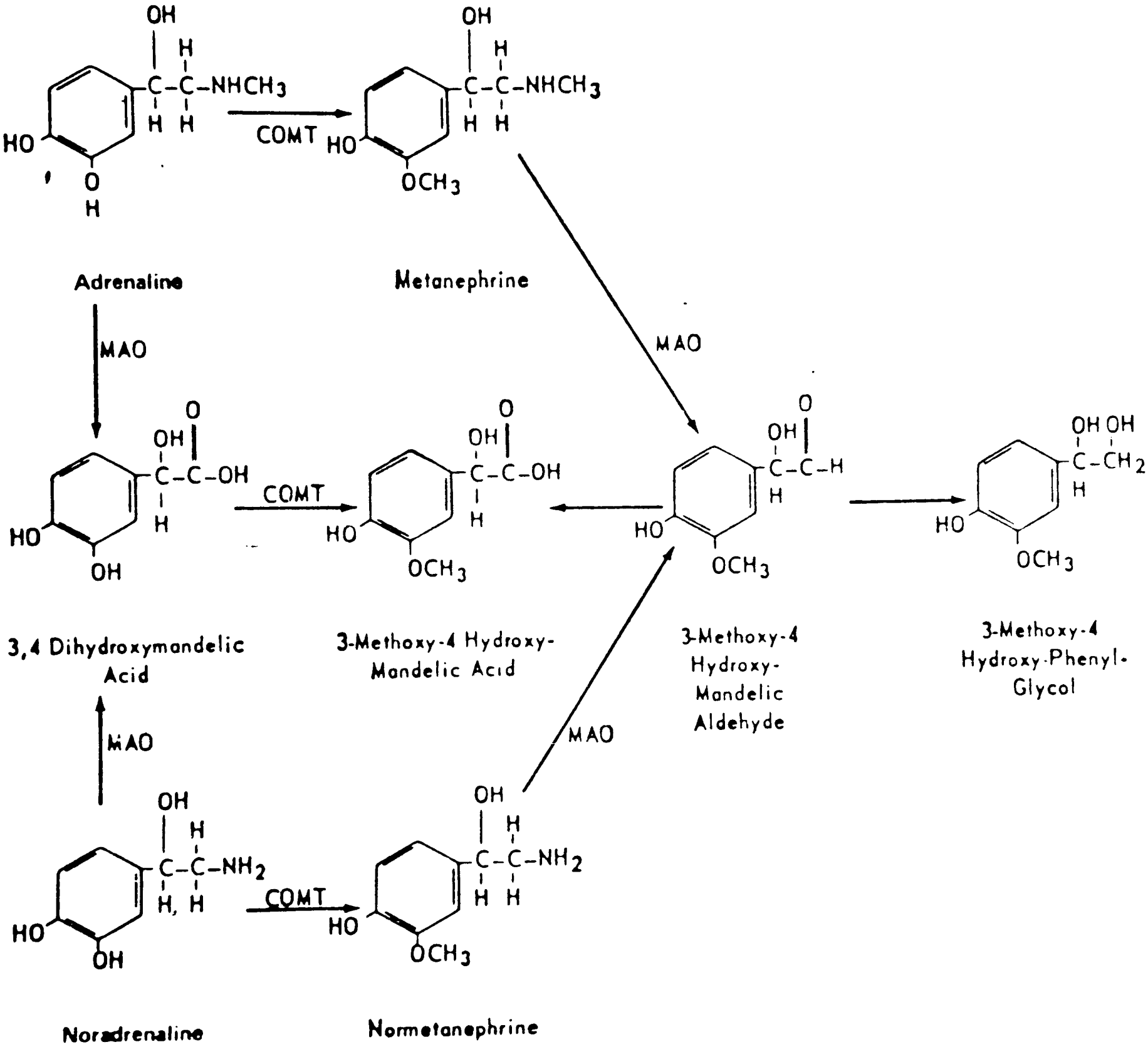


FIG. 1.2 Metabolic pathways for noradrenaline.

very little NA remains in the cytoplasm, because of intraneuronal metabolism by the enzymes monoamine oxidase and catechol-O-methyl tranferase (COMT: see FIG 1.2). Cytoplasmic NA can be transported out of the terminals by a carrier-mediated mechanism; but release of NA in response to firing of the noradrenergic neurone takes place by exocytosis from vesicles, which is a calcium-dependent process (see Fillenz, 1977 for review). The amount of NA released is proportional to the NA content of the vesicles (West and Fillenz, personal communication). Inactivation of released NA is largely brought about by a high-affinity re-uptake mechanism at the presynaptic membrane, although extraneuronal metabolism by COMT is also possible.

Noradrenaline exerts its effects on post synaptic target neurones in the brain by occupying noradrenergic receptor sites (FIG 1.3). In hippocampus and cortex, these are mostly thought to be of the beta-noradrenergic type and to involve the action of a NA-dependent adenylate cyclase in the postsynaptic membrane which mediates the electrophysiological effects of NA on the postsynaptic cell. An additional postsynaptic receptor type, the $alpha_1$ noradrenergic receptor, is found in the periphery and possibly in the brain also. A further receptor type, the $alpha_2$ noradrenergic receptor, is located on the presynaptic terminals in many brain regions, and serves to regulate NA release, according to the NA concentration around the terminal and the rate of firing of the presynaptic neuron (Starke, 1977). Yet another receptor type , designated as an autoreceptor (Aghajanian, 1978), is located on the soma and dendrites of locus coeruleus neurons, shares some of the characteristics of the $alpha_2$ receptors, and seems to play a part in regulating the rate of firing of these cells. In view of the extensive collateral network of NA fibres around the LC, it has been suggested that these autoreceptors mediate inhibition of the firing of LC cells by recurrent collaterals (Aghajanian et al, 1977).

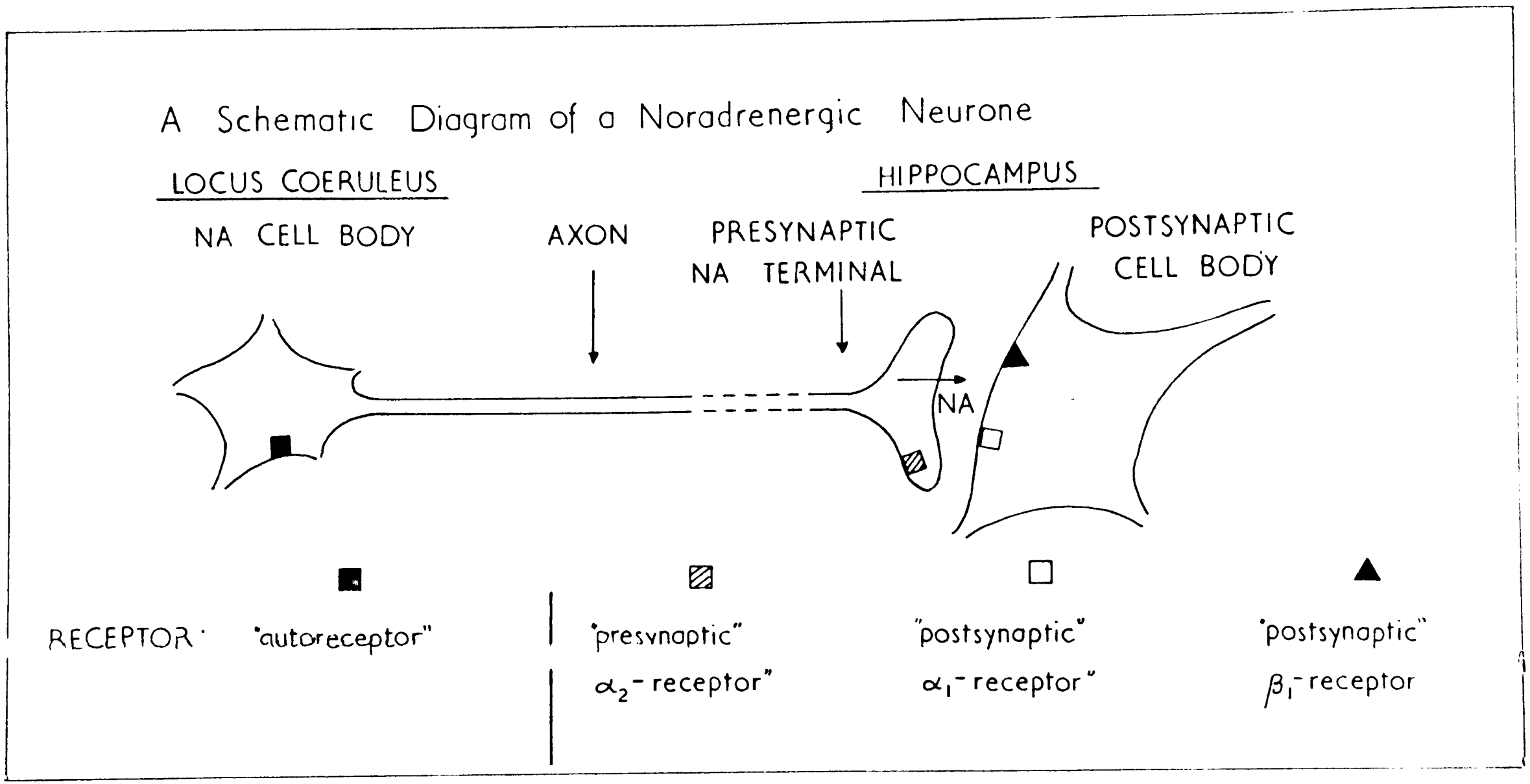


FIG. 1.3 Schematic diagram of a noradrenergic neurone indicating noradrenergic receptor sites.

Conduction velocity in NA neurons in the brain is slow; and both orthodromic and antidromic responses to stimulation of LC neurons have latencies of more than 30 msec (see McNaughton and Mason , 1980, for review). Stimuli which activate NA neurons , as shown with single unit recordings in the LC , include iontophoretic application of acetyl choline, substance P, and glutamate; inhibition of firing is produced by NA itself, adrenaline, enkephalins, opiates and GABA (see Moore and Bloom,1979, for review). Studies in anaesthetized rats show a resting tonic firing rate of 1-5 Hz in LC cells , with a burst of firing occurring in response to painful sensory stimulation such as pinching the leg of the anaesthetized animals (Segal, 1979). The studies of Bloom and associates in cats (Chu and Bloom, 1973), and of Segal and Bloom (1976) in rats, have revealed that in unanaesthetized animals the rate of firing of LC cells is related to the level of arousal and the sleep-wake pattern, and confirms that a damped burst of firing is seen in response to a noxious or novel stimulus of relatively high intensity. Redmond (1979) suggests that an increased rate of firing in the LC in monkeys is related to fear and responses to threatening stimuli. These results are in agreement with the picture of the locus coeruleus as part of the arousal system, sensitive to changes in arousal and to sensory inputs representing harmful or novel stimuli; the 'damped burst' pattern of firing may be accounted for by collateral inhibition.

4. NORADRENERGIC EFFECTS ON TARGET CELLS.

Segal (1980) and Mason and McNaughton (1980) have recently reviewed the effects of noradrenergic stimulation on the hippocampus. Recordings from pyramidal cells in the hippocampus show little change in response to a single stimulating pulse in the LC; but the effect of trains of pulses at about 10 Hz is a marked inhibition of hippocampal firing. Iontophoresis of NA onto hippocampal cells likewise causes inhibition. The

hippocampal responses are of the 'slow onset, slow offset' variety. The inhibition mediated by NA is different from that mediated by GABA in that it consists of hyperpolarization without a reduction in membrane resistance. Although many of the investigations in this field have been confined to the hippocampus, cerebellum or cerebral cortex , it is probably safe to assume that LC stimulation produces inhibition of the firing of postsynaptic cells in most projection areas; several studies agree that the inhibitory response is likely to be mediated by beta-receptors, since it is inhibited by beta-blockade with such drugs as sotalol (Bevan et al, 197?).

However, recent work with chronically implanted animals has allowed the monitoring of hippocampal unit activity in awake animals, and this has revealed more interesting and characteristic patterns (Segal and Bloom , 197?). Some cells in the hippocampus (probably pyramidal cells) are sensitive to LC stimulation in the awake rat (Segal, 1980); whereas other cells are not affected by LC stimulation (see Moore and Bloom, 1979). The study of evoked responses to stimuli has shown a facilitatory effect of LC priming stimuli given at subthreshold current; and the hippocampal response (inhibition of the firing rate of pryamidal units) to a loud tone and to LC stimulation was reduced by the use of drugs which interfere with noradrenergic transmission (Segal and Bloom, 1976). Recent formulations of the significance of this work have moved away from the notion that NA simply inhibits postsynaptic neurons regardless of other simultaneous inputs to the post-synaptic neuron. (Freedman et al, 1977) According to McNaughton and Mason (1980, p.167),

> 'the function of the inhibition could as easily be to improve the response to other afferents by reducing the noise to signal ratio...such noise-signal reduction could be the primary function of the NA input and the observed inhibition, a secondary, high intensity effect.'

Thus the importance of the noradrenergic innervation in certain areas of the forebrain may be a capacity to facilitate transmission of non-noradrenergic

impulse traffic when the locus coeruleus is activated.

5. NORADRENALINE AND BEHAVIOUR

The classical methods for investigating the function of brain regions in terms of their role in the behaviour of the whole organism have been of three kinds: stimulation, activating the given area or pathway; ablation, with lesions of the region under investigation; and administration of drugs with a known effect on neurons similar to those under investigation. In some cases this approach has given rewarding results, as in the elucidation of the role of the neurones of the nigro-striatal dopaminergic pathway in disorders of extrapyramidal motor function such as Parkinsonism. But in the case of the LC-dorsal bundle noradrenergic pathway, no such 'necessary and sufficient' role has emerged despite extensive use of all the three methods outlined above. Many hypotheses have been generated as to a role for noradrenaline in regulation of the sleep-wake cycle (Pujol et al, 1973) , sexual behaviour, intake of food and drink (see Clark, 1979), and in 'higher' functions of the central nervous system such as selective attention (Mason,1980), the learning of reinforced (rewarded) responses (Crow and Arbuthnott, 1972) and the extinction of non-rewarded or punished responses (see McNaughton and Mason,1980 , for a recent review).

Noradrenaline has also been implicated in various psychiatric disorders such as anxiety (see Redmond, 1979, for a review), depression (Schildkraut, 1965; Maas, 1978) and schizophrenia (Stein, 1971) . But the data amassed in these investigations , both in animal and clinical research, do not yet give unambiguous support to any of these speculations. The animal research is complicated by marked species differences both in the anatomy of the noradrenergic neurons and in the behavioural results of interference with

noradrenergic neurones (Clark, 1979). Clark 's overall conclusion is that the LC-dorsal noradrenergic bundle 'is not associated with specific behaviours'. McNaughton and Mason (1980), whilst making a plea for 'better theory at the purely behavioural level' , concluded that although the delineation of specific functions for the dorsal bundle remained problematic, their interpretation of the behavioural literature indicated that the dorsal bundle 'appears essential for the correct elaboration of a fairly wide range of behaviour'.

Summing up at the end of a volume devoted to the study of the catecholamines and behaviour, Friedhoff (1975) comments,

> Although there is widespread acceptance of the idea that catecholamines are important in the regulation of emotional state, definitive evidence for this involvement is lacking. (p.214) ...The most serious deficit in our knowledge is at the level of behaviour. We need a more explicit definition of the relationships of biogenic amine neuronal systems to emotional responsivity, attention, arousal, and mood. (p.216)

Improvements in technique will enable us to refine the tactics of the experimental approach to the study of noradrenergic pathways in relation to behaviour. But we need to know more about how noradrenergic neurones work at a biochemical and physiological level in order to be able to interpret the apparently contradictory evidence already available . It seems quite likely that the notion of 'one neuronal pathway, one behavioural function' represents a gross misunderstanding about the way the brain works, just as the notion of noradrenaline as an 'inhibitory' transmitter may have obscured a facilitatory role of noradrenaline in the transmission of signals about novel or noxious stimuli in the environment.

The poverty of our understanding of noradrenergic neurones is emphasised when we take into account other aspects of function, such as the

relationship of changes in noradrenergic neurones to changes in other neurochemical systems. Brodie and Shore (1957) suggested that the noradrenergic and serotonergic neurons in the brain might bear some relationship to the sympathetic and parasympathetic divisions of the autonomic nervous system the periphery. Biochemical evidence for interactions between changes in the two systems has been reviewed recently (Samanin and Garattini, 1975; Pradhan, 1978; and see section 6 of Chapter 5). Although some tentative speculation as to the nature of the relationships between the central monoamine systems has been put forward from time to time (e.g. reciprocal inhibitory control of 5HT neurons by noradrenergic neurons , and vice versa: Pujol et al , 1973; Kostowski 1975; or a reciprocal relationship between the activity of noradrenergic and dopaminergic neurones, as envisaged by Antelman and Caggiula, 1977), no coherent explanatory theory which deals with the available evidence has emerged. As with the noradrenergic system itself, there is a dearth of information about the time courses of changes in different indices of activity in the neurones . This leads to consequent difficulty in defining associated changes in two or more transmitter or neuromodulator systems.

6. NORADRENALINE AND STRESS

It is now well established that noradrenergic neurones can react rapidly to noxious or novel environmental stimuli in an intact animal, and that prolonged or repeated stimulation of the same kind calls forth a different kind of response. Fillenz (1977) has reviewed the evidence for short and long-term control of noradrenaline release in the periphery, and there is evidence that similar, if not identical, mechanisms operate in the central nervous system (see Chapter 3).

The responses of noradrenergic neurones to environmental stimuli are

different from the responses generated in ascending sensory pathways which run in parallel to the ascending traffic in the ascending reticular activating system. From the evidence cited earlier as to the characteristics of noradrenergic neurones, and from the analogy drawn with the peripheral sympathetic nervous system, the possibility arises that central noradrenergic neurones play a major part in enabling the animal to react appropriately to stress, both in the short and long term (see Stone, 1975, for review). The short term reaction consists of rapid arousal in times of danger, with preparation for 'fight or flight' , as in Selye's 'alarm reaction' (1951). The long term reaction, on the other hand, may involve setting up a new equilibrium state in the neuron, and/or preparation for a different kind of response to future stresses. Van Dongen (1980) suggests that central noradrenergic cells ' prepare the central nervous system to cope with a crisis situation ' .

7. RATIONALE FOR EXPERIMENTS.

The notion that noradrenergic activity is concerned with reactions to stress , and preparation for future stress , has served as the point of departure for the studies reported here. However, in contrast to studies which emphasise an intensive attack on noradrenergic neurones (severe stress, resulting in depletion of noradrenaline; lesions or electrical stimulation of noradrenergic neurones) , I have assumed that it should be possible to trace changes in the noradrenergic system without such drastic methods , precisely because such knowledge of the system as we do have suggests that noradrenergic neurons may play a part in enabling the animal to adapt successfully to stress. If a system has a basically homeostatic or adaptive role, then it is arguable whether its function will be more apparent under conditions when adaptation to a stimulus is successful, or when a stimulus overwhelms the adaptive capacity of the organism. Whilst borrowing copiously from the results of the latter type of investigation, I have attempted to look at the

former situation. To give a specific example; it has long been assumed that noradrenergic neurons are activated by stressful stimuli such as electric shock (Maynert and Levi, 1964; Bliss et al,1966). Rather than studying noradrenergic function after what Stone (1973) has called 'exhaustive stress', I have preferred to look for a response to very low-intensity footshock. A principal reason for this policy was that the longer-term experiments described in this thesis were designed to allow for manifestation of behavioural tolerance to footshock stress in operant behaviour-shaping experiments , and this was clearly impossible at high shock intensities.

Behavioural tolerance to stress can be manifested in several behavioural paradigms . In the partial reinforcement paradigm, a reward is presented to subjects on a proportion (usually 50 %) of acquisition trials. The other trials are not rewarded. The effect is to increase the probability of performance of the learned response during the extinction phase (when reward is never presented) compared to the performance of subjects continuously rewarded during acquisition. This increase is known as the partial reinforcement extinction effect, which, as Mackintosh (1974) says, 'is one of the most studied as well as most robust phenomena of animal learning'. Two theoretical mechanisms have been proposed to explain the partial reinforcement extinction effect when different inter-trial intervals are employed. Capaldi's theory (1967) accounts best for short term conditioning , in that the subject is said to associate the immediate after-effect of non-reward on one trial with reward on a subsequent trial. Amsel's (1962) theory, on the other hand, deals best with the partial reinforcement extinction effect at a long intertrial interval (such as 24 hours, in a one-trial per day paradigm).

The non-reward hypothesis was amplified by Gray (1979) to suggest that noradrenaline might be involved in the 'conditioned frustration' mechanism proposed by Amsel (1962) to account for the increased resistance

to extinction . Gray (1975) argued that frustrative non-reward and associated stimuli are aversive, and that the effects of non-reward are in some respects similar to those of electric shock. Brown and Wagner (1964) showed cross-tolerance between non-reward and shock . In fact Gray has advanced the proposition that a common physiological mechanism, involving the dorsal noradrenergic bundle and the septo-hippocampal system, is concerned in the development of tolerance to all forms of stress (see, for example, Gray, 1967, and 1979) .

In support of this hypothesis , work in this laboratory (Owen et al,1977; Owen, 1979) showed that resistance to extinction on some schedules in partially rewarded rats was abolished by lesions of the dorsal noradrenergic bundle . The effect of the dorsal bundle lesion was to increase resistance to extinction in the continuously rewarded (CR) group, and to decrease resistance to extinction in the partially rewarded (PR) group; this accorded with the predictions of the non-reward hypothesis of NA function. Although this evidence suggests that noradrenaline pathways may act as a substrate for the maintenance of responding (in extinction conditions) by animals with some experience of non-reward, the different results obtained as a function of length of training and of inter-trial interval complicate the issue (see section 7 of Chapter 5). Direct evidence as to the role of noradrenaline in intact animals is also lacking, since the risk of damage to other structures and systems after lesions of the dorsal bundle is considerable.

Another behavioural method of inducing tolerance to stress arose from experiments which showed that although performance on certain tasks is impaired after a session of moderately severe footshock or cold swim ('helplessness',or 'learned helplessness' - see Gray, 1981, for review), the impairment can be reduced or prevented by repeated exposure to stress before the test session. This phenomenon has been termed 'toughening up' by Miller

et al, 1976, and a connection with noradrenergic mechanisms was made when it was found that NA levels were depleted after the impaired performance and that this acute depletion was prevented by the repeated exposure to stress.

The development of a partial punishment paradigm in this laboratory (see section 8, Chapter 5) provided another opportunity to examine the effects of footshock in behavioural experiments on noradrenergic function. Partial punishment was first introduced in a runway experiment by Miller (1960). The design used in this laboratory is closely analogous to the partial reinforcement design. The partial punishment group is subjected on half the acquisition trials to a low-intensity footshock as well as food reward. The shock is, of course, aversive; but it has been established that rats will tolerate some level of shock and reach asymptotic running speeds in the runway that are not very dissimilar to those of the continuously rewarded group. In the second phase of the experiment, shock and food are paired on every trial; this is called the 'conflict' phase. The effect of partial punishment training is to maintain the running response during the conflict phase at higher speeds than in continuously rewarded rats not previously shocked. Resistance to 'conflict' is analogous to 'resistance to extinction' in the PR design.

There is some evidence for noradrenergic involvement in this form of tolerance to stress, although the results of dorsal bundle lesions on this effect are not yet clear; as in the partial reinforcement extinction effect, the requirement for intact noradrenergic pathways may vary according to the extent of training and other factors. But the partial punishment paradigm seemed another interesting arena in which to look for noradrenergic changes associated with behavioural training on different reinforcement schedules.

Gray (1972) has suggested that hippocampal theta rhythm in a specific frequency range (see section 9 of Chapter 5) represents a

physiological component of the processes underlying behavioural responses to non-reward. He showed that artificial 'driving' of the theta rhythm in this frequency range was accompanied by increased resistance to extinction in a runway experiment analogous to those referred to above. Other work in this laboratory (McNaughton et al, 1977) provides evidence of a noradrenergic involvement in the characteristic 'theta-driving' relationship between stimulus frequency and the threshold current intensity for theta-driving.

The hypothesis that noradrenergic mechanisms are involved in the development of behavioural tolerance to stress was explored, in the experiments reported in sections 7,8 and 9 of Chapter 5, using the partial punishment and partial reinforcement experimental paradigms as developed in this laboratory. Further descriptions of the background to these experiments is given in the relevant sections of Chapter 5. The broad aim was to discover whether the development of 'coping behaviour' was accompanied by changes in central noradrenergic function; the rats were never exposed to severe stress.

8. THE NEED FOR A SENSITIVE MEASURE OF NORADRENERGIC FUNCTION.

It follows from the above discussion that a major hurdle is that of finding a sensitive method for detecting changes in noradrenergic function. 'Exhaustive stress' was presumably employed as a research method because only high-intensity stimulation resulted in changes in noradrenaline (NA) levels in the brain (see Chapter 5, section 1) . Noradrenaline levels, then, may not provide a sensitive index of noradrenergic function, although activation of noradrenergic neurones can only be effectively expressed, so far as we know, by release of NA. I have instead looked to the synthesis of noradrenaline as a more likely pointer to function, since synthesis must be increased in noradrenergic nerve terminals to keep up with release of NA. The rate-

limiting step in the synthesis of noradrenaline is catalysed by the enzyme tyrosine hydroxylase (TH), and the experiments described later (Chapter 5, sections 1-9) all employed assays of the activity of this enzyme as a measure of noradrenergic function. Chapters 2 and 3 provide an account of the background for this choice and the assumptions involved; the details of the methods used are given in Chapter 4.

Although early experiments with this technique suggested that it would allow us to distinguish between groups of rats trained on different reinforcement schedules, this promise was not borne out in further investigations (see Chapter 5, section 7). Later experiments were therefore designed to clarify the effects of stimuli such as footshock on tyrosine hydroxylase activity in noradrenergic neurones in the brain, using simpler paradigms than those used in the behaviour-shaping experiments. For example, behaviourally naive rats were subjected to single footshock, or repeated footshock , to investigate the tyrosine hydroxylase response to the aversive stimulus per se. Other methods used to explore the usefulness of tyrosine hydroxylase activity as a measure of noradrenergic function included administration of various drugs , chosen because of their documented effect on monoamine systems in the brain, and administered in single or repeated doses .

For ease of exposition, the results of these experiments are presented, in Chapter 5, in the following order, corresponding, roughly, to increasing complexity of the stimuli used to elicit changes in noradrenergic function: investigations of the effect of single and repeated mild footshock in behaviourally naive rats (sections 1 & 2); investigations of the effects of single and repeated doses of drugs (sections 3, 4, 5 & 6); investigations of the effects of behaviour-shaping procedures which result in tolerance to stress (sections 7 & 8); and investigations of the effects of brain stimulation (section 9) which results in a similar form of tolerance to stress to that described in the behaviour-shaping experiments.

CHAPTER 2

TYROSINE HYDROXYLASE AND THE SYNTHESIS OF NORADRENALINE

1. THE CATECHOLAMINE SYNTHESIS PATHWAY.
2. TYROSINE HYDROXYLASE IN THE BRAIN AND IN THE PERIPHERY.
3. EARLY STUDIES OF TYROSINE HYDROXYLASE: ISOLATION,PURIFICATION AND ABSOLUTE REQUIREMENTS.
4. THE RATE-LIMITING STEP IN CATECHOLAMINE SYNTHESIS.
5. SUBCELLULAR LOCALIZATION OF TYROSINE HYDROXYLASE.
6. ENZYMOLOGY OF TYROSINE HYDROXYLASE.
7. METHODS FOR ASSAYING TH ACTIVITY IN BRAIN REGIONS.

CHAPTER 2

TYROSINE HYDROXYLASE

1. THE CATECHOLAMINE SYNTHESIS PATHWAY

The catecholamines noradrenaline (NA) and dopamine (DA) are formed from the precursor amino acids phenylalanine and tyrosine. The synthetic pathway was revealed by the work of Blaschko (eg Blaschko,1939) in the 1930's, well before the discovery of the significance of amines other than adrenaline in the periphery or the central nervous system . The formation of noradrenaline proceeds by a series of enzyme-catalysed reactions; tyrosine is hydroxylated to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase; DOPA is decarboxylated by DOPA decarboxylase to form dopamine, which is then hydroxylated by dopamine-beta-hydroxylase to NA (see FIG 2.1). Tyrosine hydroxylase (TH) was the last of the NA-synthesising enzymes to be discovered; there is far less of it in NA-containing tissues than in dopaminergic neurones, and TH is always present in much smaller amounts than DOPA decarboxylase and dopamine-beta-hydroxylase (Bacopoulos and Bhatnagar, 1977; and Fuxe et al, 1978).

2. TYROSINE HYDROXYLASE IN THE BRAIN AND IN THE PERIPHERY.

The richest sources of catecholamines and their synthesising enzymes in the body are the adrenal medulla and the caudate nucleus in the brain. Sensitive techniques were needed to study noradrenergic innervation in the brain; the histofluorescent techniques of Falck and Hillarp (1962) demonstrated the monoamine pathways as a necessary preliminary to regional studies, and radiochemical techniques have proved sensitive enough to allow measurement of very low concentrations of amines in the brain. McGeer (1963) was the first to show that injection of high specific-activity radioactively labelled tyrosine into the striatum resulted in the formation of labelled catecholamines.

TH is involved in the hydroxylation of tyrosine to form DOPA in all catecholaminergic sites, but it is becoming increasingly clear that the characteristics and properties of the enzyme vary with its regional and subcellular localization. We do not yet know much about the functional significance of the differences that have been found, for example between noradrenergic and dopaminergic areas of the brain; however, an attempt will be made here to draw as much as possible on studies of the enzyme in noradrenergic areas to avoid erroneous generalizations from studies of dopaminergic tyrosine hydroxylase.

3. EARLY STUDIES OF TYROSINE HYDROXYLASE: ISOLATION,PURIFICATION AND ABSOLUTE REQUIREMENTS.

Much of the early work on TH was done on enzyme from homogenates of the adrenal medulla; Nagatsu and his collaborators isolated the enzyme (1964a, 1964b) and published several accounts of its properties, comparing it with phenylalanine hydroxylase, which in some respects it closely resembles.

Homogenates from adrenal medulla or from brainstem, prepared in 0.32 M sucrose from fresh or frozen tissue, demonstrated some activity in a radiochemical assay using aromatic amino acid decarboxylase inhibition to maximise accumulation of labelled DOPA ; this was formed from labelled tyrosine added to the homogenate to give a final concentration of 20 μM before incubation at 37°C. The optimum pH for the reaction was 6.2. Activity was also found in the 'particulate' fraction of the homogenate from brain and peripheral organs; the particulate fraction was obtained by centrifuging the sucrose homogenate at 15,000 x g for 20 minutes. In the brain, the brainstem was found to yield more activity than cortex or cerebellum; and very little activity was found in the supernatant of the 15,000 x g homogenate.

Purification of TH by solubilization and precipitation with ammonium sulphate resulted, in the earlier attempts, in loss of activity.At this stage it was not clear whether this was due to loss of essential cofactors or to instability of the enzyme. However, using the analogy of phenylalanine hydroxylase, it was possible to proceed with the definition of the essential cofactors, namely those additives required to restore activity to the enzyme preparation. The first essential was the addition of mercaptoethanol, an anti-oxidant. Absolute requirements for molecular oxygen and a pteridine cofactor were discovered,and activity was further enhanced by addition of ferrous ions. For maximum activity, the cofactor had to be maintained in the reduced

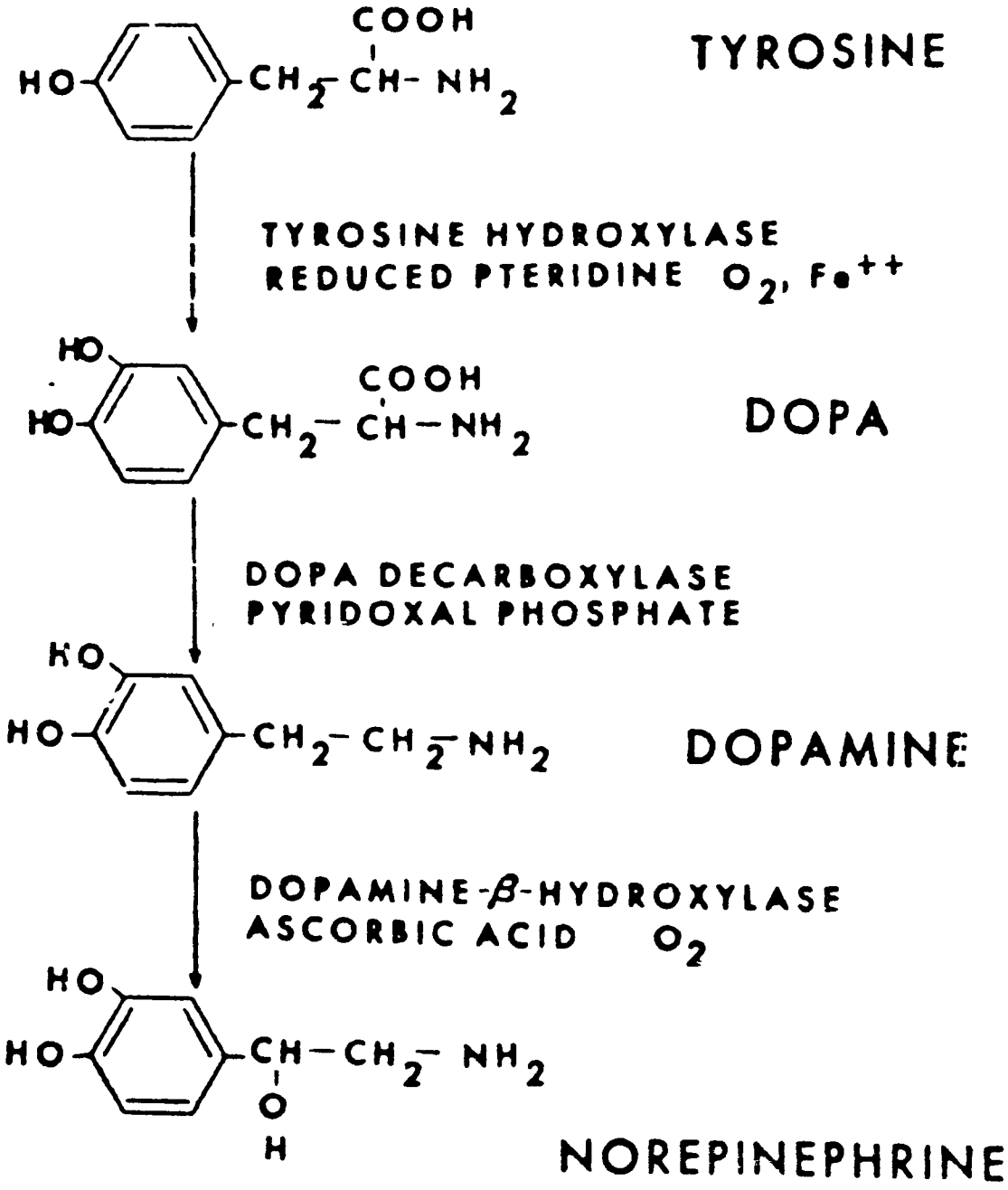
state,and various mixtures were devised to fulfil this requirement in the conditions of the in vitro system using solubilized TH. For example, one assay system for measuring TH activity in Nagatsu's studies used mercaptoethanol as antioxidant for both enzyme and cofactor, ferrous iron,and saturating levels of tetrahydrofolate as cofactor. Other authors have used catalase to maintain the cofactor in the reduced state, to inhibit peroxide formation and the non-enzymatic hydroxylation of tyrosine. These and other assay systems will be discussed later (section 7 of this Chapter).

4. THE RATE-LIMITING STEP IN CATECHOLAMINE SYNTHESIS.

The conversion of tyrosine to DOPA is the rate-limiting step in the biosynthesis of noradrenaline and dopamine. This was demonstrated in noradrenergic tissue by Levitt et al (1965) and Udenfriend et al (1966) and confirmed by other workers subsequently. The main strands of evidence were as follows. Addition of various NA precursors to a preparation of perfused heart resulted in increased NA production; but saturation could only be demonstrated when tyrosine was the exogenous precursor used. This suggested that the tyrosine-DOPA conversion limits the overall NA synthesis rate.The fact that only tiny amounts of L-DOPA are found free in noradrenergic tissues also suggests that the NA synthetic process can 'mop up' excess DOPA via the decarboxylase reaction but that excess tyrosine is less readily disposed of.

Further evidence comes from the use of inhibitors of the various enzymes in the NA synthesis pathway (see FIG 2.1). Inhibition of TH by alpha-methyl-tyrosine lowers NA levels in peripheral tissues much more drastically than does inhibition of DOPA decarboxylase or dopamine-beta-hydroxylase. Udenfriend et al (1966) calculated the degree of synthesis inhibition after alpha-methyl-tyrosine and showed that it coincided with the observed inhibition; this was a direct demonstration of the rate-limiting function of TH in NA synthesis.

FIG. 2.1 Biosynthesis of noradrenaline from tyrosine.



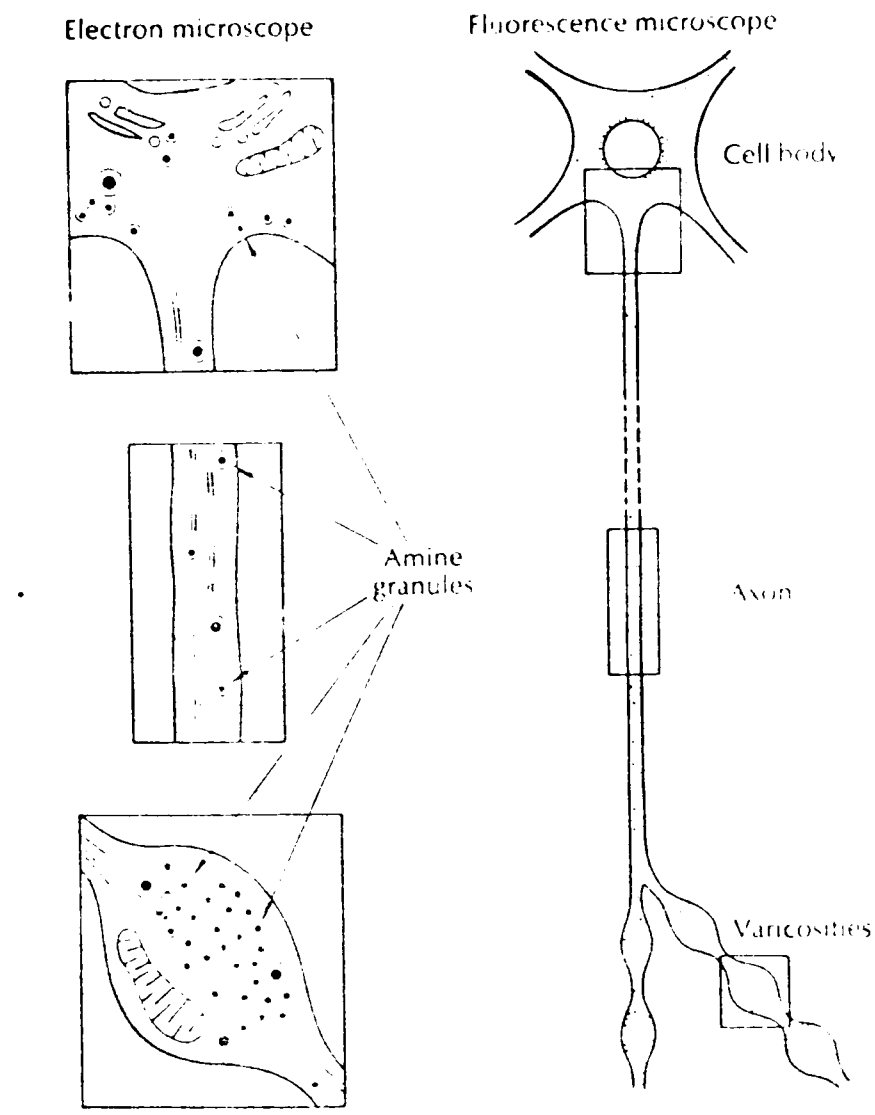
5. SUBCELLULAR LOCALIZATION OF TYROSINE HYDROXYLASE.

i. Localization of enzyme protein.

Further purification of tyrosine hydroxylase so that antibodies could be raised to it resulted in the immunocytochemical technique of Joh,Geghman and Reis (1973) allowing visualization of enzyme by means of the marker antibody in sections of brain. These methods have been further developed recently so that they now provide for quantitation of the enzyme protein in a given area (Benno et al, 1979). So despite the low concentrations of the enzyme in noradrenergic tissue, it is now clear that the enzyme is present in all parts of the neuron, from cell body and dendrites down the axon to the terminals (see FIG.2.2). At the subcellular level, TH in cell bodies is associated with endoplasmic reticulum and Golgi bodies as well as being found free in cytoplasm or near ribosomes. In axons , TH is associated with microtubules which may be involved in the transport of the enzyme from the cell body , where it is manufactured , to the terminals. Here TH has been found associated with vesicles, both small and large. However, since subcellular fractionation studies show TH to be located in cytoplasm, this finding may be an artefact of tissue fixation.

TH as found in tissue homogenates is not a single homogeneous protein. Fractionation on a Sephadex G-150 column shows up the different molecular weight of enzyme from different tissues. TH from NA neurons has a molecular weight (MW) of 200,000 whereas TH from sympathetic ganglia has a MW of 130,000. The enzyme from different parts of the NA neurons (eg cell body enzyme and terminal enzyme) is of the same MW, indicating that the different tissues contain different 'species' of TH. Further purification to homogeneity is possible, and gives a single species with MW 62,000.This may be the basic catalytic unit of the TH molecules,and may be the template for

FIG. 2.2 Structure of a monoamine neurone
(from Cooper,Bloom and Roth, 1974)



antibody formation. Treatment with trypsin breaks the molecule down further to a 35,000 MW moiety but there is doubt as to whether this is functional. Although the TH found in NA neurons in the locus coeruleus has not been completely characterized, Reis and Joh and collaborators have shown that it has a MW of 130,000 and is associated with an RNA subunit of MW 60,000 ; it has a biphasic immunotitration curve against antibody. The TH from dopaminergic neurons has two forms,with MW 200,000 and 65,000. Different results are obtained by fractionating on sephadex from those obtained with a sucrose density gradient (Lovenberg, 1978).

It may be that the forms with higher MW are associated with a regulatory, non-catalytic subunit. Since this may be one mode of regulation of the enzyme activity, it seems even more important that analysis of catalytic activity of the TH in different areas is carried out with reference to enzyme of the appropriate type. In a recent review, Weiner (1979) quotes estimates of specific activity of purified enzyme from different laboratories over an enormous range from 64 to 360 nmols/minute/mg protein.

ii. Localization of enzyme activity.

There has been considerable controversy over the past 15 years as to the localization of TH catalytic activity in 'particulate' and 'soluble' fractions of homogenates. This has partly been resolved but the multiplicity of different techniques still used by different laboratories is one consequence which adds to the difficulties of comparing results. Nagatsu's finding (1964a) that most of the TH activity in brain and adrenal medulla homogenates was 'particulate' (i.e. present in the fraction sedimenting at 15,000 x g) was probably due to entrapment of TH in synaptosomes during homogenisation in sucrose. High-speed centrifugation and the use of hypotonic solutions (in which synaptosomes are easily lysed) were found to yield higher concentrations of enzyme in the supernatant fractions.

Kuczenski and Mandell (1972) differentiated between TH from striatum and TH from midbrain using percentage of activity remaining in the 'particulate' fraction after homogenising either in sucrose or in hypotonic buffer.The striatal enzyme, in the 'particulate' fraction from nerve terminals, had more activity and a greater affinity for cofactor than the 'soluble ' enzyme which accounted for most of the activity in the midbrain region , which was assumed to contain a preponderance of cell bodies. It is not clear, however, whether the TH in this region is located principally in noradrenergic or dopaminergic cell bodies. Segal and Kuczenski (1974) continued this type of analysis in noradrenergic areas of the brain, namely the locus coeruleus, hippocampus and cortex, and hypothalamus ; the 'whole homogenate' in hypotonic buffer was separated into particulate and soluble fractions by centrifugation, and a separate estimation of 'total' enzyme activity was made by assaying a detergent-treated aliquot of the original homogenate.

The TH activity was indeed differently distributed in different regions; the ratio of soluble to pellet-bound activity was high (14.25) in the locus coeruleus, and lower (0.91) in the terminal regions of hippocampus-cortex. The pellet-bound enzyme in this experiment could not have been entirely in synaptosomes because of the hypotonic buffer concentration. From further experiments in which synaptosomes were formed and then disrupted by hypotonic shock, it appears that 60% of synaptosomal activity can be solubilised; the rest is presumably genuinely membrane bound (see Musacchio, 1975).

Mandell et al (1972) have demonstrated alterations of kinetic properties of TH with apparent shifts from the 'soluble' to the 'particulate' type of enzyme with various preparations of dopaminergic neurons, in vitro; but it has yet to be established that this is a mechanism which operates in vivo, or that it occurs at all in noradrenergic neurons. Lloyd (1977) showed

increased activity of TH in noradrenergic synaptosomes of hippocampus with ethanolamine, though not with phosphatidyl serine, which activates the enzyme in striatal synaptosomes; but it is not clear what the mechanism of action is, nor whether it is related to membrane binding. Katz et al (1976) suggest that activation in vitro by agents such as salts, polyanions and polysaccharides may simply be due to electrostatic interaction.

6. ENZYMOLOGY OF TYROSINE HYDROXYLASE.

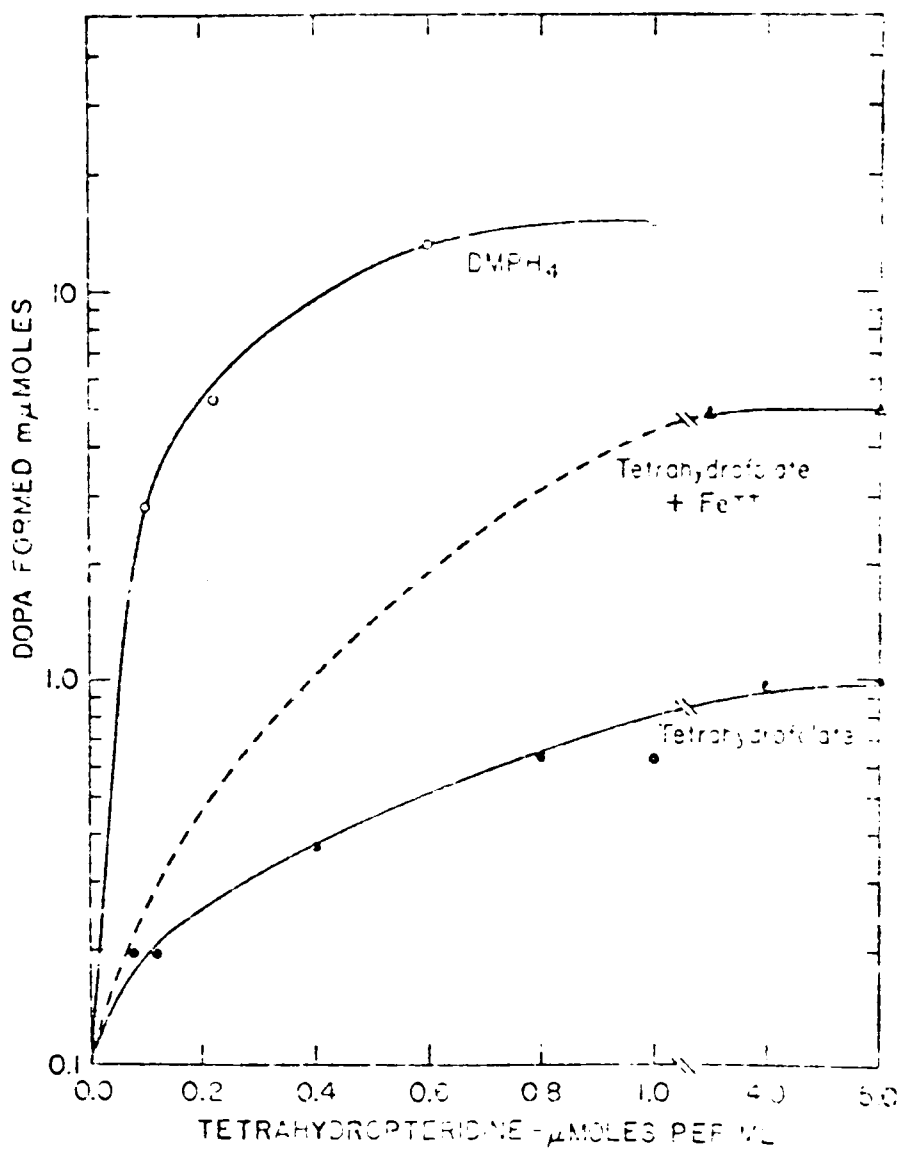
The substrate specificity of purified TH was discussed by Udenfriend (1966). Phenylalanine and L-tyrosine are the only substrates; D-tyrosine, tyramine and tryptophan are not hydroxylated by TH. Substrate availability as a constraint on expressed enzyme activity is discussed in Chapter 3. Oxygen,which is the second substrate of tyrosine hydroxylase, is also an absolute requirement, and is also briefly discussed in Chapter 3.

The absolute requirement for a pteridine cofactor was discovered early on (Nagatsu,1964a); more difficulty was encountered in discovering the likely 'natural ' cofactor. After early studies with tetrahydrofolate, which gave some stimulation and was easy to obtain, dimethyltetrahydropteridine (DMPH4) came into common use (FIG.2.3). The antioxidant mercaptoethanol, or a cofactor-regenerating system such as crude sheep-liver pteridine reductase, was required to maintain the cofactor in the reduced,active form. The combination of mercaptoethanol and beef liver catalase proved adequate in some studies , obviating the need for the reductase enzyme and the addition of reduced pyridine nucleotide (Shiman and Kaufman,1971) .

Catalase was also found to eliminate the need for ferrous iron, at any rate for the purpose of breaking down peroxide formed as an inhibitory by-product in the reaction; however, Waymire (1972) and others expressed doubt as to whether this is a complete account of the role of ferrous iron. Hoeldtke and Kaufman (1977) have now completed further purification and characterisation of the enzyme and have shown that iron is a constituent of the enzyme molecule and as such is necessary for catalytic activity.

Kaufman and Fisher (1974) initiated further debate on the appropriateness of DMPH4 as the cofactor in studies of TH; Shiman and

FIG. 2.3 Stimulation of tyrosine hydroxylase activity in vitro by tetrahydrofolate and DMPH4 (purified enzyme from adrenal medulla)



Kaufman (1971) had shown some anomalous characteristics of the reaction (for example, the greater rate of disappearance of DMPH4 than the rate of production of DOPA), and Nagatsu (1964a) showed that using tetrahydrobiopterin, the maximal activity obtained was five times that with DMPH4. Kaufman investigated various possible pteridines and showed that although tetrahydrobiopterin (BH4) is the cofactor of choice and most resembles the 'natural' cofactor, 6MPH4 is an acceptable alternative for experimental purposes. The affinity of TH for 6MPH4 is lower than that for BH4, but the reaction characteristics are not anomalous. Nevertheless, many studies have continued to use DMPH4.

Cofactor concentration and availability are currently receiving more attention as experimental variables, now that measurement techniques have been developed. Gal and Sherman (1977) suggest that although the Km of TH with respect to cofactor is $5x10^{-5}$M,which is considerably higher than the levels of endogenous reduced cofactor, the lack of a pool of BH4 is not a limiting factor ; the reduction of BH2 to BH4 is very rapid in vivo because of the high activity of dihydropteridine reductase (Turner et al, 1974). However, this remains an unresolved question; in Chapter 3, the availability of reduced cofactor as a constraint on TH activity is discussed further.

Tyrosine hydroxylase is a mixed function oxidase. That is, it catalyses the insertion of an oxygen atom into the substrate molecule. Two theories of the mechanism of the reaction have been put forward to date; the 'ping-pong' theory of the 1960's (Ikeda et al,1966) , and the sequential mechanism,involving formation of a quaternary complex as intermediate, proposed by Kaufman and Fisher (1974). This latter proposal has also received support from other workers (Weiner,1979). It seems likely that the hydroxylase binds oxygen, tyrosine, and pterin cofactor in a semi-ordered

sequence, resulting in the formation of oxidised quinonoid cofactor, water of reaction, and DOPA. The Km for tyrosine in brain varies from 10 µM to 75 µM, varying with incubations conditions and with different pteridine cofactors; the Km for pteridine cofactors vary between 200 and 1000 µM (see Lovenberg, 1978; and Kaufman, 1975; but these figures are quoted for caudate and adrenal , rather than for noradrenergic brain regions).

Inhibitors of TH : Nagatsu (1964a) found little inhibition of TH activity with 'general inhibitors' such as dinitrophenol. Substances which chelate iron, such as alpha-alpha-dipyridyl , were inhibitory. During the early purification work it was clear that there must be considerable inhibition, or 'masking', in vivo, since estimates of Vmax in vitro were up to 40 times the in vivo NA synthesis rate (Mc Geer,1978). Endogenous inhibitors included peroxide formed in the reaction, which probably oxidises both TH and BH4 if oxidation is not controlled by catalase or ferrous iron (Shiman et al, 1971); and catecholamines and DOPA were also found to inhibit TH activity. The importance of this end-product inhibition mechanism is still under dispute, but it has been proposed as the principal mode of regulation of TH (see Weiner,1970). Kinetic studies showed that the catechols competed with cofactor for binding to the enzyme; they did not compete with tyrosine (Nagatsu et al, 1972). It is unlikely that DOPA has much inhibitory effect in vivo, as it is rapidly decarboxylated to dopamine.

Tyrosine itself can act as an inhibitor in high concentrations; this is found when BH4 is used as cofactor, but not when the synthetic pteridines DMPH4 and 6MPH4 are employed. (Shiman et al, 1971). However, it is not known whether this inhibition operates to a significant degree in the brain in vivo.

Inhibition by high and low concentrations of oxygen , the co-substrate for TH, outside the normal physiological range , have also been suggested (Mandell,1978); this is not likely to be critical except under extreme hypoxic conditions (Davis, 1977), as the concentration is maintained near 5% in vivo.

The inhibitor generally used for experimental purposes is another amino acid, alpha-methyl-L-para-tyrosine (Moore and Dominic, 1971). This is a specific competitive inhibitor of TH at low concentrations and can be administered in vivo, although it is toxic to liver and kidney. 3-iodo-tyrosine is another inhibitor commonly used in the assay system for soluble TH. Other aromatic acids may also compete with tyrosine.

Naphthoquinones including some antibiotics have some capacity for inhibiting TH but are not frequently used.

7.METHODS FOR ASSAYING TH ACTIVITY IN BRAIN REGIONS

Methods for assaying TH activity involve measuring some aspect of the hydroxylation reaction in which tyrosine is converted to DOPA. Thus DOPA itself can be collected and isolated for measurement after incubation of the enzyme with tyrosine as substrate for a given time under standardised conditions. Measurement of DOPA can be carried out by fluorimetric, colorimetric, spectrophotometric assay or by liquid chromatography. In noradrenergic regions especially, the activities measured are low, and in general radiochemical detection has been the preferred method for assaying DOPA; labelled tyrosine is used as substrate (see Salzman and Roth, 1979, for a discussion of uptake of labelled tyrosine) ; after the incubation period, labelled DOPA is isolated from the remaining labelled substrate and radioactivity in the DOPA fraction is counted (see Nagatsu et al, 1964a) . A commonly used adaptation of this method (e.g. Nagatsu et al, 1964b) is measurement of the labelled water of reaction formed when ditritiotyrosine is used as substrate; instead of counting the tritium label on the DOPA formed in the reaction, the tritiated water formed is isolated by ion exchange (this is easier than separating DOPA from tyrosine). The amount of labelled water is stoichiometrically equivalent to the labelled DOPA formed in the TH-catalysed reaction.

Other , indirect methods of measuring TH activity include assessment of oxygen consumption during the hydroxylation of tyrosine, and measures of the oxidation of the pteridine cofactor . These methods have not been extensively used in the work discussed in this introduction nor in our own studies, and will not be discussed further.

With regard to the radiochemical method outlined above,however, it will be clear from the discussion of the properties of TH (sections 3 and 6 above) that the standardization of conditions for the TH assay is critical.

The early studies of TH (Nagatsu 1964a) demonstrated that there was measurable activity in brain regions homogenised in 0.25 M sucrose to which labelled tyrosine was added to a final concentration of 20 μM,for incubation at 37°C. With this preparation, no absolute requirement for cofactors is demonstrated, though various additives raised the measured activity significantly. This indicates that the homogenate contained intact synaptosomes,or pinched-off nerve terminals which contain the necessary ingredients, enzyme and cofactors for the reaction, and are capable of taking up labelled tyrosine from the reaction mixture and converting a proportion of it to labelled DOPA without further additives. This was confirmed by Mc Geer et al (1965) and Karobath (1971).

This is the prototype for the SYNAPTOSOMAL assay described in Chapter 4; this is best performed on freshly prepared synaptosomes which have many of the characteristics of intact noradrenergic cells (Gray and Whitaker 1962). They are able to take up amines and precursors against a concentration gradient, and to synthesise, store and release amines under appropriate conditions. It should be emphasised that the synaptosomal assay we have used requires uptake of precursor as well as its subsequent conversion to DOPA. There is evidence to suggest that uptake of tyrosine is not a limiting factor under the conditions used in this assay, but some authors use the term 'synaptosomal conversion rate of labelled tyrosine' instead of 'synthesis rate of NA ' in order to avoid this assumption.

Some examples of synaptosomal assay methods used by various authors are outlined in Table A.

TABLE A

SYNAPTOSOMAL STUDIES OF TYROSINE HYDROXYLATION RATE.

Author	Sample Preparation	Incubation	Tyrosine Concentration
Nagatsu et al 1964a	Homogenised in 0.32 M sucrose and centrifuged at 15000 x g for 20 minutes ; 'unfortified particles'	KPO_4 buffer pH 6.2 + DDCI	20 µM
Mc Geer et al 1965	Homogenised in 0.32 M sucrose and centrifuged at 900 x g for 10 minutes; supernatant recentrifuged at 20000 x g for 20 minutes; 'synaptosomes'.	KPO_4 buffer 0.1 M pH 6.4	0.32 µM
Karobath 1971	Homogenised as in Nagatsu 1964a but with teflon-glass. 14000 x g for 15 mins, then recentrifuged on density gradient.	NaCl 100 mM KCl 5 mM glucose 10 mM sucrose 50 mM ascorbate 2 mM EDTA 2 mM pH 6.6	15-25 µM
Harris and Roth 1971	Homogenised in 0.25 M sucrose	KPO_4 buffer + DDCI	50 µM
Patrick and Barchas 1974	Homogenised in 0.32 M sucrose Centrifuged 20000 x g for 20 minutes. Preincubation 5-10 mins.	NaCl 125 mM KCl 5 mM $MgCl_2$ 1 mM $CaCl_2$ 1 mM glucose 10 mM Tris-HCl 50 mM pH 7.4	10 µM
Lloyd 1977	Homogenised in sucrose	Na Cl 150 mM KCl 2mM $MgCl_2$ 3 mM $MgSO_4$ 1.5 mM KPO_4 1.3 mM glucose 25 mM sucrose 0.3 mM pH 7.0	20 µM
Boarder and Fillenz 1978	Homogenised as in Karobath 1971; centrifuged 16000 x g for 20 minutes,after discarding pellet of nuclear debris.	as in Patrick, 1974 ascorbate 1 mM $NaPO_4$ 15 mM pH 6.2 or 7.2	20 µM

TABLE A (continued)

Brain Region	Tyrosine Hydroxylation Rate	
guinea pig whole brain	12 ng/g/hr	(3.4 pmol/min/mg protein)
beef caudate	160 pmol/hr/sample	
rat brain	5.19 pmol/min/mg protein	
rat cerebral cortex	1.8 nmol/g/hr	(510 fmol/min/mg protein)
rat cortex	0.23 nmol/g/hr	(65 fmol/min/mg protein)
brainstem	0.36 nmol/g/hr	(102 fmol/min/mg protein)
striatum	11.0 nmol/g/hr	(3.11 pmol/min/mg protein)
rat striatum	33.7 pmol/30 min	(560 fmol/min/mg protein)
hippocampus	3.35 pmol/30 min	(55 fmol/min/mg protein)
rat striatum	6.18 pmol/min/mg protein	
hippocampus	220 fmol/min/mg protein	
hypothalamus	810 fmol/min/mg protein	

(figures in brackets are approximate conversions to standard units)

Having demonstrated the activity in sucrose homogenates of brain regions and other noradrenergic tissues, Nagatsu and other workers were able to stabilise and purify the soluble enzyme from high-speed supernatants of homogenates, separating the enzyme from the cofactors present in the synaptosomes. The purified enzyme was then incubated under varying conditions and with different additives as described above (section 3, this Chapter) to discover the optimum conditions at which maximal velocity (Vmax) of the hydroxylation reaction was reached. These would then be defined as the saturating, optimum assay conditions. Cofactor type and concentration, substrate concentration, pH, cofactor regenerating system,buffer concentration and temperature were among the defined conditions.

Enzyme from different regions or species could then be compared by means of this assay; or it could be used as a measure of maximum activity which might be surpassed in enzyme from experimental animals treated so as to increase the enzyme activity still further. The increase in activity could either come about by a change in the characteristics of the enzyme itself , or by an increase in the amount of active enzyme contributing to the hydroxylation of tyrosine. In kinetic terms, this change would be represented as an increase in Vmax . To determine whether the actual amount of enzyme had increased under conditions producing an increase in Vmax, a different assay would be needed, essentially measuring the amount of enzyme protein ; this is the immunotitration technique of Joh and coworkers (1973).

Examples of the SATURATING ASSAYS used in various laboratories are given in Table B.

TABLE B

SATURATING ASSAYS OF TYROSINE HYDROXYLASE ACTIVITY

Author	Method
Nagatsu et al, 1964a	Enzyme from adrenal medulla was homogenised with mercaptoethanol, centrifuged at 105,000 x g and purified with ammonium sulphate. Incubation mixture for this early 'fortified' assay contained acetate buffer, tetrahydrofolate or DMPH4 cofactor, ferrous sulphate ($FeSO_4$), mercaptoethanol (ME) and a DOPA-decarboxylase inhibitor (DDCI), at pH 6.0. Tyrosine concentration was 100 µM and the labelled DOPA formed in the reaction was measured by alumina column chromatography.
Shiman et al, 1971	Adrenal TH was solubilised and purified, and incubated with catalase, and either ME or dihydropteridine reductase (DPR) and TPNH to prevent oxidation of the cofactor DMPH4, at pH 6.2 in a phosphate buffer. Tyrosine concentration was 500 µM and the labelled DOPA formed was extracted over a Dowex-50 ion-exchange resin.
Waymire et al,1971	Rat brain was homogenised in water and incubated with DMPH4,ME, and $FeSO_4$ in an acetate buffer at pH 6.1. Tyrosine concentration was 100 µM and the labelled DOPA was estimated in various ways; the favoured method was the coupled decarboxylase method, using a DDCI.
Kuczenski and Mandell, 1972	Enzyme from rat striatum was purified and dialysed after centrifugation at 45,000 x g . Incubations contained ME, DMPH4, and $FeSO_4$ in Tris buffer at pH 6.1. The saturating tyrosine concentration was 500 µM. Labelled water was counted after elution from a Dowex-50 column.
Cicero et al 1973	This study used a very similar method but the concentrations of cofactor, Fe SO_4 and ME were different. Tyrosine concentration was 100 µM and pH was 6.0.
Lloyd and Kaufman, 1974	Purified enzyme from bovine caudate tissue was assayed at pH 6.1 in a phosphate buffer in the presence of catalase, ME, and 6MPH4, another synthetic pteridine cofactor. Tyrosine concentration was 50 µM, and pH 6.1.

TABLE B (continued)

Author	Method
Acheson et al, 1979	This is a recent study of the pH-dependence of TH activity . Solubilized enzyme from a high-speed supernatant of various brain regions was assayed at pH 6.0 with catalase, DPR and 6MPH4. The tyrosine concentration was 75 µM.

The activity measured in these assays varies a great deal according to mode of preparation and purification as well as the cofactor and substrate concentrations and the source of the enzyme itself . The variation in activity will be discussed in the next chapter. However, the following examples of results in controls give some indication of the different units used in different studies , which hinder comparison (see Shiman et al, 1971). The rates refer to the amount of labelled DOPA (or equivalent labelled H_2O) formed in a given incubation period (usually 10-60 minutes) from a sample of known weight or protein content.

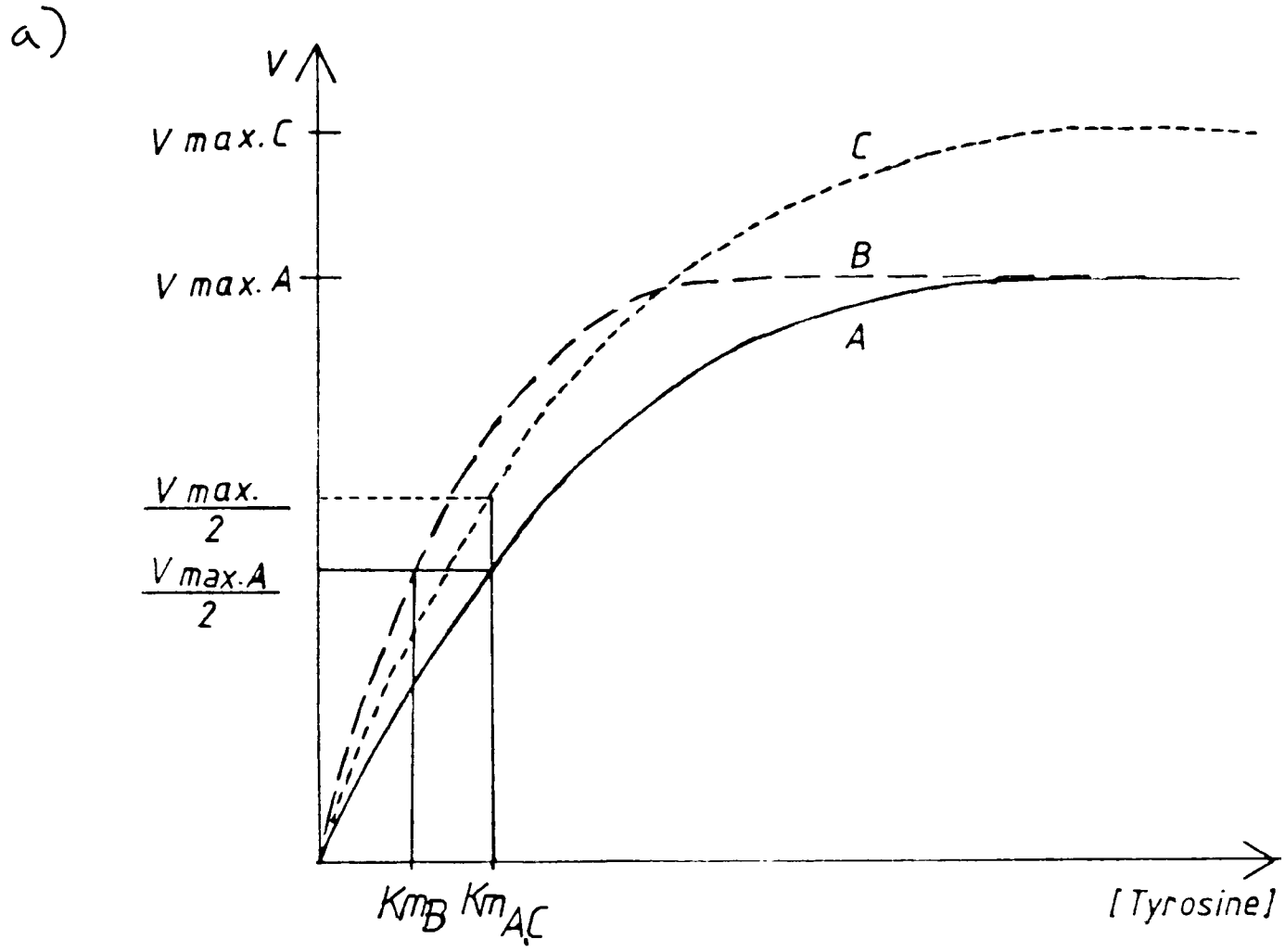
Author	Source	TH Activity
Nagatsu 1964	adrenal medulla	13 nmol/15min/2 mg
Shiman 1971	adrenal medulla	10 nmol/15 min/sample
Waymire 1971	rat brain	0.2 ng/g/min
Kuczenski and Mandell 1972	rat striatum	0.33 nmol/20 min
Cicero 1973	rat caudate hypothalamus pons-medulla	1498 nmoles/g/hr 172 nmol/g/hr 116 nmol/g/hr
Lloyd and Kaufman 1974	bovine caudate	0.015 nmol/mg

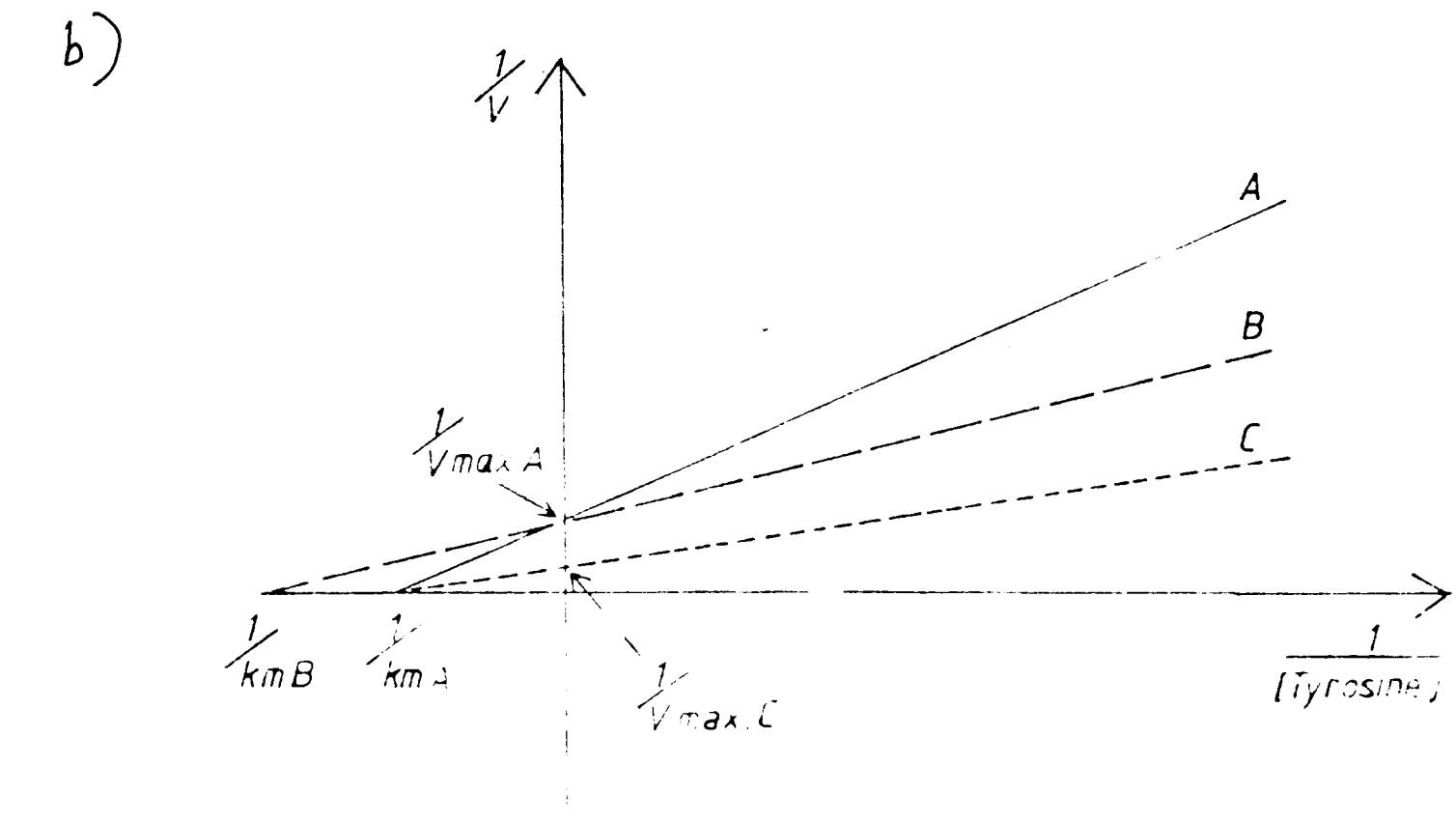
On the other hand, changes in activity under ideal conditions for tyrosine hydroxylation determined as above may be difficult both to obtain and to interpret. Methods used to 'activate' tyrosine hydroxylase will be discussed in Chapter 3; but given our knowledge so far of the conditions under which the enzyme operates in vivo, we might assume that changes in synthesis rate in vivo which arose from changes in TH activity would, if transferable to the soluble assay system at all, be manifested as changes in the reaction rate under suboptimal conditions.

Assays of TH activity at subsaturating cofactor and substrate concentration have been increasingly used to show up 'activation' of the enzyme, i.e. increases in the reaction rate measured under standardised but suboptimal conditions. If the assay is performed with a range of concentrations of substrate, cofactor, or inhibitory substances as well as under the standardised conditions , holding the antioxidant and cofactor regenerating systems at constant concentrations, then any change in the kinetic properties of the enzyme such as the Michaelis constant (Km) for cofactor or substrate, or for inhibitors (Ki) , can be described . FIG.2.4 illustrates the possible effects of activation of the enzyme on the velocity of the reaction (in terms of molecules of DOPA formed per minute) at different substrate concentrations. The velocity, V, rises with increasing substrate concentration to a maximum, Vmax, where the enzyme is saturated. At the stage where half the enzyme sites are involved, the velocity is Vmax/2. The substrate concentration at which this velocity is achieved is the Km for substrate. The dashed line in the figure shows one possible outcome of enzyme activation; the reaction reaches maximal velocity at a lower substrate concentration than before. In this case the affinity of the enzyme for substrate is said to be increased and the Km is decreased.

FIG.2.4 a) Tyrosine hydroxylase activity; velocity of reaction plotted against substrate concentration (see text)

b) Lineweaver-Burk reciprocal plot of 1/velocity of reaction against 1/substrate concentration. Intercept on ordinate is 1/Vmax; intercept on abscissa is 1/Km.





(a)

The dotted line in Fig 2.4 shows another possibility; the activation in this case results in an increase in the number of enzyme molecules. If the other factors, e.g. cofactor supply, are not limiting, then the Vmax will increase . The Km may remain constant , or affinity may be altered at the same time.

The double-reciprocal plot in Fig 2.4 (b) shows these changes in a different way; a change in Km is shown as a shift on the abscissa, and changes of V max as shifts along the ordinate.

Since the definition of suboptimal conditions is necessarily rather arbitrary, different groups working on TH have used different assay conditions and different units of measurement, and it is therefore laborious and sometimes impossible to make direct comparisons between experimental results obtained in different laboratories. Table C shows some of the 'subsaturating' assay conditions used to assay TH in homogenates or supernatants prepared from neural tissue .

TABLE C

TYROSINE HYDROXYLASE ACTIVITY MEASURED

UNDER 'SUBOPTIMAL' ('SUBSATURATING') CONDITIONS.

Author	Method
Hendry and Iversen 1971	Tissue from mouse superior cervical ganglion was homogenised in water. The incubation mixture contained $NaPO_4$ buffer,ME,a DDCI,and DMPH4 at 1.25 mM. TYROSINE concentration was 7.5 µM and labelled DOPA was measured by alumina chromatography.
Segal and Kuczenski 1974	Rat brain regions homogenised in 2mM potassium phosphate buffer were assayed in an incubation mixture containing ME, ferrous sulphate. a Tris-acetate buffer, DMPH4 at 1 mM, and TYROSINE at 3 µM. Tritiated H_20 was measured after separation on a Dowex-50 column.
Phillipson and Sandler 1975	Chick embryo sympathetic ganglia were homogenized in H_20 and assayed in an incubation mixture containing phosphate buffer, ME, a DDCI, DMPH4 , pH 6.0, with TYROSINE at 6 µM. Labelled DOPA was measured by alumina chromatography.
Morgenroth et al 1975	Brain regions were homogenised in hypotonic tris-acetate buffer and centrifuged at 104,000 x g. The supernatant was assayed for soluble TH activity in an incubation medium containing catalase, DPR,a DDCI, and DMPH4 at 100 µM, at a pH of 6.0. The TYROSINE concentration was 10 µM. Labelled H_20 was measured after separation on a Dowex-50 column.
Roth et al 1975	A very similar method to the above, but DMPH4 as well as BH4, the natural cofactor, was used at a concentration of 100 µM. TYROSINE concentration was 10 µM.
Salzman and Roth 1980	Another very similar method, using BH4 at 170 µM and increased concentrations of NADPH (for the DPR system) and catalase. TYROSINE concentration was 10 µM.

TABLE C (continued)

Examples of TH activity measured in rat brain regions under subsaturating conditions.

Author	Source	TH Activity
Segal and Kuczenski 1974	locus coeruleus hippocampus hypothalamus	2.56 pmol/20min/mg tissue 270 fmol/20 min/ mg tissue 4.02 pmol/20 min/mg tissue
Morgenroth et al 1975	cerebral cortex medulla-pons	3.6 pmol/min/mg protein 8.2 pmol/min/mg protein
Roth et al 1975	hippocampus	7.9 pmol/min /mg protein
Salzman and Roth 1980	hippocampus	7.0 pmol/min/mg protein

Another example of the use of suboptimal assay conditions concerns pH. There is ample evidence that the pH optimum for soluble TH from brain regions is 6.0-6.2 . But it is equally clear that in the brain the hydroxylation of tyrosine is likely to be occurring at a pH around 7.4. Recently, therefore,several groups have tried looking for evidence of activation by performing the reaction at a range of pH values, and have indeed found that 'activated' TH in some experiments exhibited a broader pH optimum range than the 'normal' TH in control experiments , so that activation is manifested as an increased velocity at a higher-than-optimum pH (Acheson et al, 1979; Simon and Roth, 1979; Bahkit and Gibb, 1979).

Another source of variation between different laboratories is the method of preparing the soluble enzyme. As discussed above (section 5), TH is found mainly in the cytoplasm in the nerve cell body and in terminals; the activity found in the particulate fraction of homogenates is partly due to soluble enzyme trapped in synaptosomes. Nevertheless,some activity may also be due to enzyme bound to membrane fragments even after lysis of synaptosomes, and this activity would be lost if the supernatant only was assayed. Some workers (Kuczenski and Mandell,1972) suggest that enzyme activation in vitro in dopaminergic neurones is brought about by a shift of the enzyme from the soluble to the membrane bound state.

In the experiments to be described in this thesis, the method of 'partial solubilization' has been adopted; synaptosomal preparations, which contain most of the total enzyme activity, are separated from the 15,000 g supernatant,suspended in incubation medium as for a synaptosomal assay, and then frozen, thawed,and vortexed with buffer containing 0.2% (final concentration) Triton X-100 to lyse the synaptosomal membranes and bring most of the TH into solution without losing membrane-bound activity. This method can also be used with homogenates, using a hypotonic buffer

containing Triton X-100. In other respects, the 'SATURATING SOLUBLE' and 'SUBSATURATING SOLUBLE' assays described in Chapter 4 (Methods) resemble those used in other laboratories . Table D shows some examples of different assay methods using Triton-solubilised enzyme.

TABLE D

ASSAYS OF TYROSINE HYDROXYLASE ACTIVITY

AFTER TRITON-SOLUBILIZATION OF SAMPLES.

Author	Method
Coyle 1972	After homogenising with Tris-HCl buffer, the tissue was centrifuged at 10,000 x g and the supernatant was used for the assay, with an incubation mix containing catalase, DPR, DMPH4 at 6.4 mM, and TYROSINE at 200 µM.
Zivkovic 1974	Homogenised in Tris-acetate buffer with 0.2 % Triton; the supernatant after centrifugation at 15,000 x g was incubated at pH 6.2 with catalase, DPR and NADPH, DMPH4 at 200 µM, and TYROSINE at 100 µM. A DDCI was also present as labelled DOPA was measured after alumina chromatography.
Lewander 1977	Homogenised in phosphate buffer with 0.2 % Triton; brief centrifugation at 6000 x g, and incubation of supernatant with catalase (or ferrous ammonium sulphate), 1 mM DMPH4 and 200 µM TYROSINE. Labelled DOPA was isolated from alumina columns.
Saavedra 1976	Homogenate in Tris-HCl buffer with 0.2% Triton; incubated at pH 6.0 with DPR and TPNH, catalase, 40 µM 6MPH4, and 100 µM TYROSINE.
Zigmond 1979	Homogenised in hypotonic Tris buffer with 0.1% Triton and assayed at pH 6.0 with 700 µM BH4 and TYROSINE at 51 µM.
Segal and Kuczenski 1974	An aliquot of tissue was homogenised in phosphate buffer and treated with Triton 0.2 %. The incubation mixture contained ME, ferrous sulphate, and Tris-acetate buffer; and DMPH4 1mM and TYROSINE at 3 µM, pH 5.8.
Buda 1975	Samples were sonicated in phosphate buffer with 0.2 % triton. After centrifugation, the samples were incubated with catalase, ferrous sulphate, DMPH4 1mM, and TYROSINE 20 µM, at pH 6.0..
Boarder and Fillenz 1979	Samples homogenised in phosphate buffer with 0.2 % Triton, and assayed with catalase,BH4 1 mM, amd TYROSINE 55 µM, at pH 5.8.

Note: the concentrations of cofactor and substrate are reported here to show the variation amongst a small sample of different assay protocols. Other constituents are not reported in detail. DPR represents a dihydropteridine reductase system . DDCI represents a DOPA decarboxylase inhibitor in assays where labelled DOPA is eluted off alumina. In other assays the Dowex-50 column method for measuring labelled H_2O was used.

I have used the SYNAPTOSOMAL and SOLUBLE assays concurrently in several experiments in an attempt to examine the relationship between different measures of TH activity from the same tissue measured under different assay conditions. Since many factors besides the kinetic properties of the enzyme contribute to NA synthesis rates in vivo, soluble assay conditions can only demonstrate potential changes in NA synthesis rate in terminals. Some of these other factors will , however, be present as regulators of the TH activity in synaptosomes freshly prepared from brains of experimental animals (eg. cofactor concentration and availability , antioxidants , noradrenaline) . So synaptosomal synthesis rates may reflect in vivo synthesis rate of NA in noradrenergic terminals at the time of sacrifice more accurately than extrapolation from a soluble assay under arbitrary standardised conditions.

Some standardization is of course required in the synaptosomal assay too, the principal variable being the concentration of added substrate. The reaction is saturable with added tyrosine,and most synaptosomal studies in NA-containing tissue have used concentrations of 10-50 μM tyrosine, with 'apparent Km' values of 7-9 μM. Since the cofactor concentration in synaptosomes is unknown and no exogenous cofactor is added (Karobath, 1971), there is no measure of enzyme affinity for cofactor.

Concurrent measures of changes in TH activity in synaptosomal and solubilized synaptosomal preparations may give some indication of whether changes in the maximum potential for NA synthesis rate are being expressed in the intact nerve terminal, or , conversely , whether synthesis acceleration can be found in the terminals of NA neurons independently of kinetic changes in the solubilised enzyme.

In other words, the synaptosomal tyrosine conversion rate has been used as an indicator of NA synthesis which would be expected to bear some relation to results obtained from other 'in vivo' measures of NA synthesis (these are discussed in Chapter 3). Some differences are also to be expected in that all the in vivo methods measure the effects of synthesis or metabolite accumulation over a pre-set period in vivo , whereas the synaptosomal synthesis rate reflects the synthetic activity of the isolated terminals , and the measurement of synthesis rate in the test tube is confined to the time over which the reaction rate is linear (20-40 minutes; the reaction is stopped in the present experiments after 20 minutes and synthesis rate is expressed per minute).

The significance of the use of these different methods for measuring noradrenergic function, and, in particular, NA synthesis rate , is not yet clear. The choice of methodology may have considerable impact on attempts to assess whether NA synthesis rate, as measured by TH activity , can be used as an indicator of firing rates in noradrenergic neurons .

CHAPTER 3

REGULATION OF TYROSINE HYDROXYLASE ACTIVITY.

1. IN VITRO EXPRESSION OF TH ACTIVITY.
2. ENZYME CONSTRAINTS: SUBSTRATE AVAILABILITY.
3. AVAILABILITY OF REDUCED COFACTOR.
4. REGULATION BY END PRODUCT INHIBITION OF TH.
5. ACTIVATION OF TH BY CALCIUM IONS.
6. DEPOLARIZATION BY ELECTRICAL STIMULATION
   OR POTASSIUM IONS.
7. CYCLIC AMP AND PHOSPHORYLATION.
8. TYROSINE HYDROXYLATION IN NORADRENERGIC SYNAPTOSOMES.
9. TH REGULATION : IN VITRO EFFECTS OF PSYCHOTROPIC DRUGS ?
10. CHANGES IN TH ACTIVITY AFTER IN VIVO STIMULATION.
11. MEASUREMENT OF NORADRENALINE SYNTHESIS ;
    EFFECTS OF STIMULATION AND STRESS.
12. LONG TERM CHANGES IN TH ACTIVITY IN THE CNS.
13. REGULATION OF NORADRENALINE SYNTHESIS:
    MODELS AND MECHANISMS.

REGULATION OF TYROSINE HYDROXYLASE ACTIVITY

In this Chapter, studies which have reported changes in TH activity (as measured directly by the methods outlined in Chapter 2) will be reviewed. First , methods of enhancing TH activity 'in the test tube' will be discussed; then 'in vivo' stimulation of the animal resulting in activation of TH post mortem. These results will then be compared with results from other methods of measuring NA function after similar stimuli. This will lead on to the starting point for the experiments described in this thesis, which have utilised various measures of TH activity as a measure of noradrenergic function under different experimental conditions.

1. IN VITRO EXPRESSION OF TH ACTIVITY.

The focus in this review will be on studies reporting attempts to alter TH activity in noradrenergic neurons in the CNS. Most studies of the enzyme in the nervous system have been on TH in the caudate nucleus , where TH activity is higher than in other brain regions; but the differences in dopaminergic and noradrenergic TH, emphasised in Chapter 2, make one wary of extrapolating from DA to NA neurons. Apart from the crucial question of whether noradrenergic TH can be activated in the test tube, I shall consider two additional questions , relating to the probability that stimuli which activate the enzyme in vitro also play a role in vivo. First, have the stimuli been shown to accelerate synthesis in synaptosomes where the enzyme is subject to some of the constraints operating in vivo? And second, have the models based on in vitro mechanisms of activation any heuristic value for explaining in vivo changes in noradrenergic activity?

The obvious starting point for an investigation of factors in activation of TH is the early work of Nagatsu,already referred to in section 6

of Chapter 2. It is clear from studies of the purified enzyme that it is normally subject to various constraints, some of which have been investigated in kinetic studies . It is logical to look at the operation of these factors under artificially manipulated conditions,and to ask whether such changes are likely to be achieved in vivo. In the following discussion, changes in kinetic parameters such as Km and Vmax will be reported when possible, but it should be noted that these are not accurate in any absolute sense except when referring to fully purified homogenous preparations of the enzyme; the figures are often reported as 'apparent' Km or Vmax. Since the enzyme activity does not always obey Michaelis-Menten kinetics with respect to the synthetic cofactors (Kaufman and Fisher 1974), Vmax for substrate and cofactor are only to be regarded as approximations in much of the work to be described in this chapter.

2. ENZYME CONSTRAINTS: SUBSTRATE AVAILABILITY.

Obviously, the velocity of the tyrosine hydroxylation reaction will increase with substrate availability up to the Vmax which is a function of enzyme quantity, given a constant supply of cofactors. The amino acid tyrosine is present in tissues in a concentration of about 100 μM , and is taken up by a high-affinity mechanism with some specificity ; but in vivo and in synaptosomes the uptake mechanism has a higher Km than that of the enzyme with respect to the substrate (Patrick and Barchas,1974). Tyrosine concentration is not likely to be a regulatory factor in vivo, except in stressed animals, according to Gibson and Wurtman (1977,1978). This is in contrast to 5HT and cholinergic neurons, where tryptophan and choline uptake respectively may have some regulatory role in transmitter synthesis (Wurtman et al ,1977).

The concentration of tyrosine in brain is generally about 70-80 μM , and the Km of TH with respect to tyrosine in whole brain is between 10 and 25 μM (Carlsson and Linqvist,1978; Kaufman,1975), so usually the enzyme is at least 75% saturated with respect to substrate . Carlsson and Lindqvist, 1978, however, showed a reduction in TH activity when brain tyrosine concentration was reduced . Synaptosomal studies utilise the rapid uptake of exogenous labelled tyrosine, at concentrations up to 50 μM of exogenous tyrosine in the final incubation. The concentration of endogenous tyrosine in the synaptosomes is unknown; but because the reaction is generally linear up to 45 minutes (Harris and Roth, 1971; Karobath,1971; Lloyd,1977; Boarder and Fillenz,1978) the assumption is made that tyrosine concentrations remain near saturation.

Oxygen is the co-substrate for TH. Davis (1977) reported brain oxygen tension as 15 torr, and the apparent Km for oxygen in synaptosomes

from striatum in rat brain was 20 μM , equivalent to 12 torr. It may be that oxygen is subsaturating under certain circumstances (Brown et al, 1974; Davis et al, 1976). However, isolated TH has also shown to be inhibited at high concentrations of both tyrosine and oxygen (Fisher and Kaufman,1972; Shiman et al ,1971). There is little evidence that NA synthesis is regulated in vivo by substrate availability under normal conditions, although circumstances have been described, such as severe stress , when concentration of tyrosine or oxygen may conceivably be limiting.

3. AVAILABILITY OF REDUCED COFACTOR.

Given the low levels of reduced tetrahydrobiopterin (BH4) in brain (1 μM, according to Kaufman,1973, but 'less than 100 μM' according to Lovenberg, 1978) and the varying estimates of the Km for cofactor under different assay conditions (480 μM according to Roth et al,1975; but 10 μM in a study by Gal and Sherman, 1977, and 200 μM according to Lovenberg , 1978) it would seem that cofactor availability is a likely regulating factor for TH activity. Bullard notes that BH4 is not readily taken up in brain (1978). The inhibition of TH by the catecholamines is manifested as competition for the cofactor binding site at high concentrations of catecholamines (Nagatsu,1972). Other regulatory mechanisms (this Chapter, section 5 et seq.) result in changes in the Km for cofactor, which may make cofactor availability more or less limiting for synthesis of NA according to the state of the enzyme.

However, it is only the reduced form of biopterin which is used as cofactor, so the enzymatic reduction of biopterin (BH2 reduction to BH4) may provide another regulatory step. Bullard et al (1978) have investigated the relationship of pteridine reductase and cofactor concentration in striatal dopaminergic synaptosomes. There seems to be an excess of pteridine reductase activity over TH activity, and the levels of the two enzymes are not correlated. Levels of reduced cofactor are correlated with tyrosine hydroxylase activity ($r=0.86, p < 0.01$) and with tryptophan hydroxylase activity; in fact 47% of the variance in synaptosomal TH was accounted for by covariance with reduced biopterin levels. These experiments have yet to be replicated in noradrenergic tissues.

Manipulation of enzyme activity by changing exogenous cofactor levels in synaptosomal incubations appeared impossible in Bullard's study. Probably the cofactor is synthesised and reduced intraneuronally. However, Boarder and Fillenz (1978) found increased tyrosine hydroxylation in synaptosomes from hypothalamus and striatum during incubation with exogenous BH4 (Patrick, 1977, reports a similar activation of synthesis in striatal synaptosomes); however, hippocampal synaptosomes were not activated in this manner, and it was concluded that levels of reduced cofactor might be saturating in hippocampal synaptosomes but not in those from other brain regions. Very little is known about the regulation of cofactor supply to TH in noradrenergic neurons, but this certainly has the hallmark of a possible in vivo regulatory mechanism for monoamine synthesis.

4. REGULATION BY END PRODUCT INHIBITION OF TH.

High concentrations of NA have repeatedly been shown to inhibit hydroxylation rate both in synaptosomes and in soluble enzyme preparations (Karobath,1971; Nagatsu,1964a; Boarder and Fillenz,1978). Early demonstrations of this phenomenon led to the proposition (Weiner, 1970) that TH activity was regulated by the inhibitory effect of a pool of noradrenaline which reduced the catalytic activity of the enzyme .

> 'Regulation through end product inhibition is a rapidly occurring event, and the synthesis rate should be capable of almost instantaneous adjustment in response to altered physiological demands for catecholamines. Under most conditions, this type of mechanism probably accounts for the bulk of regulation necessary to maintain physiological balance with respect to catecholamines in man and animals.'
> (Dairman and Udenfriend, 1970)

The mechanism of this inhibition was shown by Nagatsu (1972) to be competition for the cofactor binding site; increases in NA concentration did not affect affinity for tyrosine.

Activation of NA neurones usually involves release of NA (Alouisi and Weiner,1966), provided calcium ions are available for excitation-secretion coupling. But it has repeatedly been shown that NA content in brain regions is not reliably diminished during increased release of NA (Thoa et al,1971; Gordon et al, 1966); the simplistic end product inhibition hypothesis seemed untenable on this evidence. The alternative proposal of a strategic , but unmeasurable pool of NA sequestered 'near' the enzyme and away from the rest of the cytoplasmic NA , has remained merely theoretical. However, recently Ames et al (1978) have succeeded in showing in vitro activation of TH in hypothalamus, adrenal, and striatum - i.e. in both noradrenergic and dopaminergic neurons - after gel filtration to remove catecholamines. Small amounts of catecholamines added afterwards reversed this activation.

End product inhibition cannot therefore be dismissed as a mode of regulation. However, since kinetic changes in 'activated' TH often consist in a reduced affinity for inhibition by end product (increased Ki) , as well as an increase in affinity for cofactor (reduced Km for pterin), it may be that the regulation is controlled more by a change in affinity of enzyme for noradrenaline and cofactor than by large changes in NA concentration intraneuronally.

Another way in which access of inhibitory catecholamines to the enzyme may be controlled in vivo is via changes in presynaptic receptors and the uptake mechanism for released NA. Inhibition of NA uptake by drugs such as desipramine results in resistance to inhibition of the enzyme TH by high concentrations of NA (Harris and Roth,1971).

ACTIVATION OF TYROSINE HYDROXYLASE .

So far, this discussion has amounted to an exploration of some of the constraints which prevent maximum expression of the activity of soluble or synaptosomal tyrosine hydroxylase. In the next few sections of this chapter (sections 5-7), another set of possible changes in TH activity will be discussed. These concern events related to or occurring during neuronal depolarization and during transmitter release. The effects on TH activity of increasing calcium concentration, phosphorylating conditions, and depolarisation by electrical stimulation or by incubation in high-potassium solutions will now be discussed. Work employing preparations of the soluble isolated enzyme and preparations retaining some structural integrity (synaptosomes and brain slices) are presented separately within each section.

5. ACTIVATION OF TH BY CALCIUM IONS.

In 1973, Gutman and Segal reported increases in TH activity of up to 200 % above control activity when Ca^{++} was added in low concentrations (50 - 200 μM) to the supernatants of homogenates of rat hypothalamus, brainstem,cortex, and striatum. Barium addition produced a smaller increase of 32% . The assay system included mercaptoethanol, ferrous ammonium sulphate, DMPH4 (100 μM), labelled tyrosine at 50 μM , and a DOPA-decarboxylase inhibitor.

Morgenroth et al (1975a) used a different assay system and undertook kinetic analysis of the changes in TH activity of the high-speed supernatant of a homogenate of rat medulla-pons in hypotonic Tris-acetate buffer. The incubation medium contained catalase, dihydropteridine reductase,NADPH, DMPH4 (100 μM), and tyrosine at 10 μM for a subsaturating assay. A dopa-decarboxylase inhibitor was included,and stoichiometry of tritiated water and

DOPA formation was tested. This showed activation of 400% in medulla-pons, and 383% in cortex supernatants in the presence of 50 μM Ca^{++} in the subsaturating assay. The calcium chelator EGTA prevented this activation; when administered alone, EGTA increased the activity of cortical TH, but had no effect on the enzyme from medulla-pons.

With high DMPH4 concentration (100 mM) in assays from medulla-pons, a decrease in Km for tyrosine from 58 to 10 μM was found after calcium addition; and at saturating tyrosine concentration (100 μM), the Km for DMPH4 was reduced from 673 to 125 μM in the presence of calcium (100 μM). The Ki for NA as inhibitor was increased from 0.34 mM to 6.27 mM. The Vmax did not change for either substrate or cofactor; the effect of the calcium ions seemed to be an increase in the binding capacity, or affinity, of the enzyme without affecting the maximum velocity of the reaction. At subsaturating cofactor concentrations, however,the calcium-activated enzyme would theoretically be able to maintain a tyrosine hydroxylation rate nearer the maximum rate than the control enzyme as a result of the kinetic changes reported; and the inhibitory feedback from end-product inhibition would be much less effective for the calcium-activated enzyme .

Roth et al (1975) showed activation of TH by addition of calcium at low concentrations to soluble enzyme from hippocampus; using the natural cofactor BH4 instead of DMPH4, the kinetic changes were even more pronounced; there was a 5-fold reduction in Km for BH4, a 30-fold reduction in Km for tyrosine substrate, and a 33-fold increase in the Ki for NA, after addition of calcium at 5 μM .

Morgenroth et al (1974) proposed that calcium might play a role in direct activation of TH in noradrenergic neurones in addition to its known role in stimulus-secretion coupling. Since neuronal depolarization is accompanied

by a large calcium influx into neurones, it was proposed that TH might be activated by the calcium influx directly. This aspect of the role of calcium was further investigated by Salzman and Roth (1979), partly in response to several failures to replicate the original work on calcium-activation of soluble TH in several other laboratories (Kuczenski and Mandell,1972; Weiner ,1979; Lerner,1977), which suggested that the phenomenon was not as robust as had been thought and might be related to particular aspects of the assay method or enzyme preparation. Salzman & Roth's study will be discussed in the section on depolarization-induced changes (section 6). By 1978, neither removal of end-product inhibition nor calcium-related activation appeared to account satisfactorily for all reported changes in the catalytic activity of noradrenergic TH in vitro.

Dopaminergic TH in soluble form is not activated by calcium ; it is activated by EGTA,which removes calcium by chelation (Morgenroth et al,1976). Calcium addition to synaptosomal suspensions from caudate nucleus resulted in a reduction of enzyme activity (Lloyd,1977). Morgenroth et al (1975a,1976) found that soluble TH from rat cerebral cortex gave a conflicting mixed picture of activation by both calcium addition and by EGTA , presumably because there are both noradrenergic and dopaminergic nerve endings. In the light of this finding, assays performed on cortical tissues must be interpreted with care; they have in the past often been characterised as 'noradrenergic areas', but this is misleading; although there are noradrenergic terminals distributed widely all over the cortex, the sparse innervation by dopaminergic fibres actually contains more TH than is present in NA fibres (Bacopoulos and Bhatnagar, 1977).

6. DEPOLARIZATION BY ELECTRICAL STIMULATION OR POTASSIUM IONS.

Peripheral nervous system.

Several studies on peripheral noradrenergic tissues have used the rodent hypogastric nerve-vas deferens preparation as a source of TH enzyme; the preparation is set up in incubating medium, stimulated electrically,and after 15-40 minutes of stimulation the tissue is homogenised. The supernatant is used for the tyrosine hydroxylase assay to see whether post-stimulation enzyme activity is different from that of unstimulated control vas TH. Thoa (1971) failed to show activation of TH in supernatant or homogenate, or any change in kinetic properties of the enzyme from stimulated tissue,using an assay based on that of Nagatsu (1964a) with ferrous sulphate and mercapto-ethanol. Morgenroth et al (1974) however obtained activation in a similar preparation, using an assay with catalase and pteridine reductase plus NADPH as cofactor regenerating system,and subsaturating DMPH4 and tyrosine concentrations. Affinity for cofactor and substrate were increased, and affinity for the inhibitory catecholamines was reduced, in soluble enzyme prepared from guinea-pig vasa deferentia which had either been subjected to electrical stimulation at low frequencies or had been incubated with high potassium concentration (52 mM). There was no change in the apparent Vmax for synthetic cofactor or for tyrosine .

Weiner (1977,1979) replicated the electrical stimulation effect in vas deferens using yet another TH assay technique, combining the coupled decarboxylase technique of Waymire (1971) with a modification of the catalase-pteridine reductase system (Shiman and Kaufman,1970; Zivkovic,1974). The enzyme prepared from stimulated tissue showed a maximal increase of 200% of control activity, and a reduction of Km for the cofactor 6MPH4. The stimulation was greatest at low concentrations of cofactor. A parallel preparation was used to measure the labelled CO_2 evolved

during stimulation in medium containing labelled tyrosine; this provided confirmation that the activation seen in post-stimulation tissue was very similar to the increase in synthesis seen during the stimulation period. Weiner (1979) investigated the relationship of the activation of TH to the duration of stimulation, and found that a 30-second period of stimulation could activate the enzyme to the same extent as a 30-minute period. The minimum period of stimulation which gave an increase in TH activity was 10 seconds, using high-frequency stimulation (25 Hz). The shorter periods of stimulation showed a small increase in Vmax. However, TH activity in unstimulated tissue appeared to be reduced with duration of incubation,especially with low cofactor concentrations, and this led Weiner (1979) to suggest a model whereby the TH is proposed to exist in two forms differing in Km for cofactor; activation of the enzyme implies conversion of some of the enzyme in the low-affinity form to the high-affinity form.

Central nervous system.

Slices of brain tissue have been incubated with potassium or subjected to electrical field stimulation to produce activation of the enzyme isolated from homogenates of the slices (Harris and Roth, 1971; Bustos et al,1978). Bustos et al used hippocampal slices,incubating in high-potassium media or setting up field stimulation for periods of 20 minutes. The soluble enzyme from stimulated slices showed a 250% increase in activity in the subsaturating assay as used by Morgenroth et al (1975 ; see above); 5 minutes stimulation was long enough to show a rise. The kinetic changes are very similar to those for activation by calcium; again a reduction in Km for tyrosine and cofactor (DMPH4) was found, with an increase in the Ki for inhibition by NA. A rough equivalence of 1000 stimulus pulses to incubation with 1 μM calcium was calculated.

Harris and Roth (1971) , having shown that synthesis of catecholamines in cortical and striatal slices was increased after incubation with 52 mM potassium, looked for post-stimulation activation in synaptosomes prepared by homogenising the slices in 0.25 M sucrose and incubating at pH 6.2 with 50 μM ^{14}-C tyrosine. There was no change in the apparent 'Vmax ' in stimulated tissue; this 'Vmax ' was obtained by measuring activity at a range of incubation times and amounts of tissue. Activity under subsaturating conditions was not reported for this experiment. These synaptosomes may well have been largely dopaminergic. Two other studies have attempted to show post-stimulation activation in synaptosomes . Patrick and Barchas (1974) attempted to show increased synaptosomal conversion of tyrosine to DOPA in cortical synaptosomes, at pH 7.4. Striatal, but not cortical synaptosomes, showed activation during incubation with high potassium. Boarder and Fillenz (1978) also examined hydroxylation rate during incubation of synaptosomes with high concentrations of potassium (55mM). They replicated the Patrick and Barchas finding of increased activity in striatal synaptosomes at pH 7.2, but found a decrease in activity at pH6.1 . Hippocampal and hypothalamic synaptosomes showed no change in activity during high-potassium incubation at pH 6.1; insufficient counts for assessment of the effect of potassium were obtained at the higher pH (tyrosine concentration 5 - 20 μM).

Although the evidence from soluble enzyme assays suggests that depolarizing stimuli can result in kinetic changes in TH from noradrenergic neurones, as shown in post-stimulus solubilization and incubation of the enzyme, there is less evidence for activation of TH in noradrenergic synaptosomes during depolarizing stimulation. Most of the work on synaptosomes has been performed on striatal or cortical tissue, and the results may not be applicable to noradrenergic neurons. Some relevant and recent work from this laboratory is summarized in section 8 of this Chapter.

7. CYCLIC AMP AND PHOSPHORYLATION.

Kakiuchi et al (1969) and Shimuzu et al (1970) showed that cyclic AMP (cAMP) is elevated in brain slices after electrical stimulation or exposure to high potassium concentrations. Although it is not proven that this rise in cAMP is even partly due to presynaptic changes (there is abundant evidence for postsynaptic cAMP associated with changes in postsynaptic noradrenergic receptors), a model has been proposed (Salzman and Roth,1980) which incorporates a cAMP-dependent step in activation of TH.

The first studies of cAMP-activation of TH used dopaminergic tissue (Harris and Roth, 1971); evidence for a role of cAMP in activation of soluble isolated TH from noradrenergic neurons in the CNS was found by Roth et al (1975). They showed that soluble (high-speed supernatant) TH from rat hippocampus was activated in vitro by incubation with cAMP in a similar manner to the activation produced by calcium ions (see above). The activation was seen at subsaturating concentrations of cofactor and substrate, and represented increased affinity for both cofactor and substrate, and decreased affinity for inhibitory catecholamines. As with calcium , use of BH4 as cofactor rather than the synthetic cofactor DMPH4 increased the activation achieved with a given concentration of cAMP (0.1mM). With BH4, there was a 24-fold reduction of Km for BH4, a 21-fold reduction of Km for tyrosine, and a 40-fold increase of Ki for NA. The activation with calcium was additive with that due to cAMP, but cAMP-induced activation was not affected by the presence of the calcium-chelating agent EGTA in the medium.

Morgenroth et al (1975b) identified further requirements for hippocampal TH activation in the presence of cAMP; magnesium ions and adenosine triphosphate (ATP) were essential components of the incubation, suggeting that phosphorylation was involved. Roth et al (1975) suggested that

activation required the operation of a cAMP-dependent protein kinase and involved phoshorylation of a protein. This could explain why some other studies had failed to find activation using cAMP alone (Lovenberg and Bruckwick,1975; Weiner et al,1979) . Also the finding that highly purified enzyme or triton-treated solubilised enzyme preparations are not susceptible to activation under some circumstances may represent loss of similar components which are required for activation or manifestation of increased activity (Weiner, 1979).

Using phosphorylating conditions, i.e. incorporating magnesium and ATP as well as cAMP into incubation of soluble enzyme from vas deferens with labelled tyrosine, Weiner (1979) found a further increase over that obtained with cAMP alone (100 μM). The Km for cofactor is reduced, but there is also a small increase in Vmax, which was not found by Roth et al (1975). Also the pH optimum range was extended from 6.2 towards a more physiological range, around pH 7. The maximal activity achieved was related to levels of cAMP. Ames et al (1978) gave further confirmation of activation of TH by phosphorylation in supernanants of triton-homogenates of hypothalamus and striatum ; the apparent Km for the synthetic cofactor 6MPH4 was significantly reduced . Ames contrasted this with the effect of removing catecholamines from the supernatants; activation was produced by both procedures in a non-additive manner. Ames therefore concluded that the principal effect of the activation by phosphorylation was the increase in the Ki for end product inhibition. The interactions of the various modes of activation need further clarification; kinetic studies are of limited use for elucidating in vivo changes, even if phosphorylation does play a part in increasing TH activity in vivo.

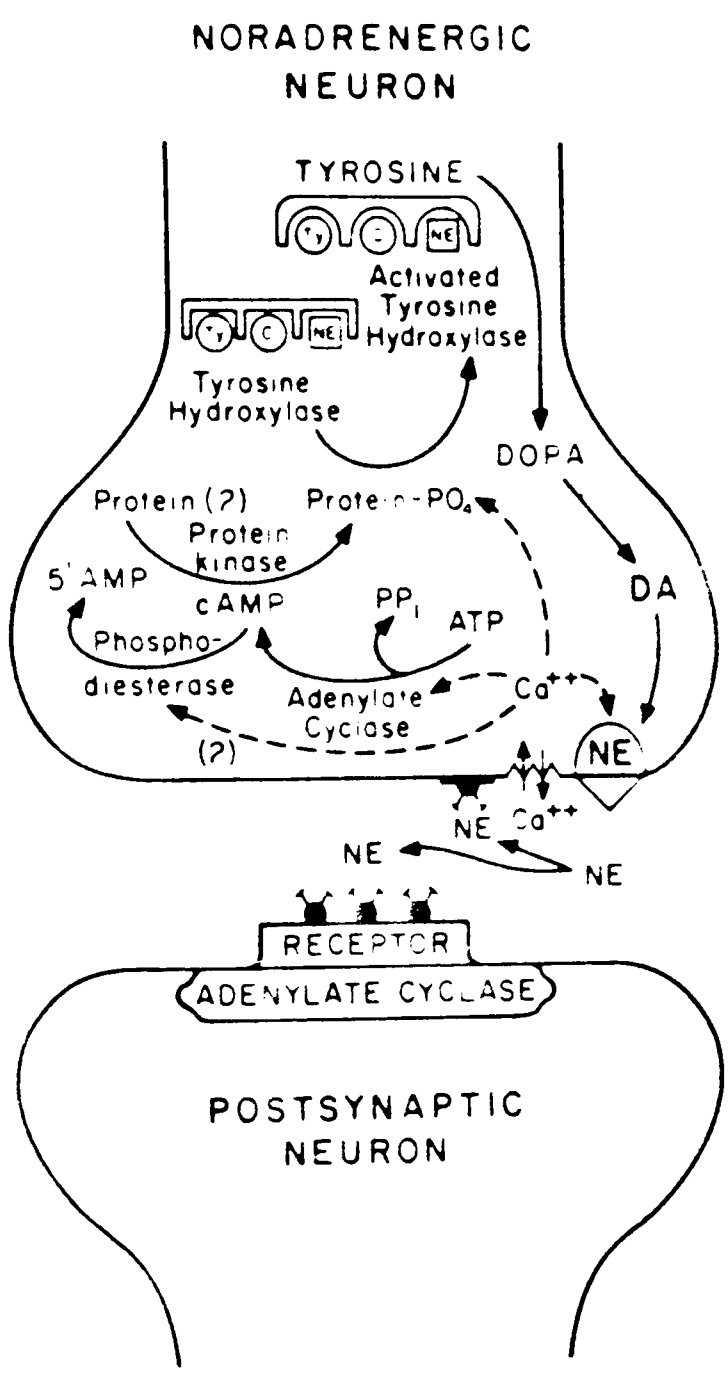
It is still not established that TH itself is the protein which is phosphorylated during the reaction with protein kinase, though recent work

seems to be pointing to this conclusion. Several early attempts to demonstrate incorporation of labelled phosphate into the enzyme molecule failed (Lloyd and Kaufman,1974); but more recently Letendre (1977) suggested that purified TH from phaeochromocytoma can be shown to incorporate ^{32}P,and a study by Yamauchi et al (1978) showed that incorporation required protein kinase. Vuillet et al (1980) have provided the missing link in this intriguing chain of evidence; they showed incorporation of ^{32}P concurrently with activation of the enzyme (also from phaeochromocytoma). The Km of the TH for cofactor 6MPH4 was reduced from 480 μM to 120 μM after phosphorylation; there was no change in Km for tyrosine (74 μM) or in Vmax.

As regards activation of tyrosine hydroxylase in synaptosomes , it seems likely that the phosphorylating system may be at least partly retained in the synaptosome during homogenisation. Increased tyrosine hydroxylation rates have been found when cAMP is added to incubations of synaptosomes suspended in isotonic buffer. Boarder and Fillenz (1978) showed a 20-30% acceleration of 3H-DOPA formation in synaptosomes from hippocampus, hypothalamus and striatum (pH 6.1, tyrosine concentration 5 - 20 μM) when cAMP was added to a final concentration of 0.5mM. This activation was not dependent on release of NA, since removal of calcium from the incubation medium did not prevent the activation by cAMP. Similar results were earlier demonstrated for dopaminergic synaptosomes only (Patrick and Barchas, 1976; Goldstein,1976).

The two strands of evidence for a cAMP-mediated activation of TH, from studies of soluble enzyme and from synaptosomal work, though suggestive, do not amount to a demonstration that TH is regulated in vivo by the cAMP-dependent-protein kinase phosphorylating model described by Roth et al (1978, and see FIG. 3.1). But there is also evidence that cAMP can activate TH in preparations which retain neuronal integrity, such as the

FIG. 3.1 Model of a noradrenergic terminal.
(from Salzman and Roth, 1979)



Schematic model of a (central) noradrenergic neuron illustrating a hypothetical mechanism by which an increase in impulse flow may result in an activation of tyrosine hydroxylase. The abbreviations used are Ty = tyrosine, C = reduced pteridine cofactor, NE = norepinephrine, DA = dopamine, PP_i = inorganic phosphate, cAMP = 3′ : 5′-cyclic AMP, Ca^{2+} = calcium ion. Activation of tyrosine hydroxylase is characterized by increases in the apparent affinity of the enzyme for tyrosine and for pteridine cofactor and a decrease in the apparent affinity of the enzyme for the end-product inhibitor, norepinephrine. Details of a possible mechanism linking neuronal depolarization with tyrosine hydroxylase activation are presented in the text.

hypogastric-nerve-vas deferens preparation and hippocampal slices. Weiner (1979, using the vas deferens preparation) showed that addition of cAMP (0.5 mM) to the incubation medium resulted in increased TH activity in both control and electrically-stimulated vasa, but that the cAMP-induced increase was maximal in the unstimulated tissue. Use of calcium-free media did not prevent cAMP-activation. This suggested that the mechanism of activation of cAMP and electrical stimulation was , in some respects at least , similar.

Simon and Roth (1979) investigated the activation of soluble TH derived from hippocampal slices which had been incubated either with high potassium or with cAMP (2 mM). The enzyme was isolated from the slices after the stimulation period , and incubated at subsaturating concentrations of cofactor and substrate . The results of this study , in contrast with those of Weiner (1979) suggest that rather different mechanisms may underly activation of TH by cAMP and depolarization. In particular, the pH optimum of the cAMP-treated enzyme shifted from the non-stimulated optimum at pH 6.2 to a more physiological range based around pH 7.2. This corresponded with Weiner's findings for TH from electrically-stimulated vas deferens. But the K^+-stimulated hippocampus yielded a TH with the usual pH optimum at 6.2. The decay of activation after stimulation by cAMP or high-K^+ also differed .

Simon and Roth (1979) also discovered that calcium-dependence was a feature of TH for K^+-stimulation of striatal TH, but not for cAMP-stimulation. This finding reinforces the possibility that activation of TH can take different routes according to the nature of the stimulus . Given this much progress, it is still not clear how much influence differences in assay methods will have on whether enzyme activation will be perceptible in a given preparation. Certainly it seems that the kinetic changes found in the studies outlined above would change tyrosine hydroxylation rates only at subsaturating cofactor concentrations and/or under circumstances where NA concentration

is rate-limiting, since changes in Km and Ki are more reliable than changes in Vmax. However, it is clearly premature to extrapolate from these findings towards speculation about the mechanisms for in vivo changes in noradrenaline synthesis rate.

8. TYROSINE HYDROXYLATION IN NORADRENERGIC SYNAPTOSOMES.

Table E summarises some recent work in this laboratory using the synaptosomal preparation at the physiological pH, 7.4 (data taken from Nicholass and Fillenz, in press, and unpublished results). For the determination of the apparent Km for tyrosine, a range of concentrations of 5-40 µM labelled tyrosine was used; in other experiments labelled tyrosine was added in a final concentration of 20 µM. For details of the method, see Chapter 4.

TABLE E

SYNAPTOSOMAL TYROSINE HYDROXYLATION:

EFFECTS OF ALTERING COMPOSITION OF INCUBATION MEDIUM.

EXPERIMENT	Hippocampus	Cerebellum	Hypothal
Effect of tyrosine concentration; apparent Km for tyrosine	4.1 µM	12.4 µM	3.4 µM
Cofactor concentration; effect of adding 0.8 mM BH4	- 3 %	+ 5 %	+ 49 %
End product concentration; effect of adding 10^{-6}M NA	- 30 %	- 41 %	- 29 %
Removal of calcium ions; effect of replacement of Ca++ with Mg++, and addition of 1 mM EGTA	- 6 %	- 9 %	+ 24 %
Depolarization with 50 mM K^+	+ 56 %	+ 49 %	+ 97 %
Effect of cAMP addition of 2 mM 8-Br-cAMP	+ 36 %	+ 26 %	+ 67 %
Effect of depolarization and cAMP together	+ 105 %	+ 79 %	+ 115 %

Results are expressed as percentage deviations from control activity in Krebs-Ringer incubation medium without additives.
(data from Nicholass and Fillenz, in press; and unpublished data)

From these results it is clear that TH activity in synaptosomes from untreated rats can be altered by in vitro changes in the incubation medium. The synaptosomal preparation is susceptible to activation by addition of cAMP, and depolarization; activity is reduced in the presence of added NA. The hippocampus and cerebellum show similar patterns of response in all the experiments in Table A; but the hypothalamus is different from these regions in some respects. In addition to the above-mentioned effects, the hypothalamus is also activated by addition of cofactor, and removal of calcium ions from the incubation medium. These regional differences are not fully understood, but presumably they may reflect the different properties of noradrenergic and dopaminergic TH, since hypothalamic synaptosomal preparations contain both NA and DA terminals.

From the similarities of results obtained on soluble enzyme and in synaptosomes, as reviewed in this section, it appears that TH in noradrenergic neurons is subject to regulation by several mechanisms. There is evidence for regulation by end product inhibition ; and by availability of reduced pteridine cofactor ; and in addition, TH can be 'allosterically' activated by various means, often via a reduction of the Km for cofactor or an increase in the Ki for end product inhibition. In vitro activation in noradrenergic tissue can be achieved by increased calcium concentration, by addition of cAMP when phosphorylating conditions are present, and by incubating intact tissue with a raised K^+ concentration. Care is needed in interpreting results from brain regions with mixed noradrenergic and dopaminergic innervation, since there appear to be marked differences in the mechanism of activation of tyrosine hydroxylase from these two types of monoaminergic neuron.

9. TH REGULATION : IN VITRO EFFECTS OF PSYCHOTROPIC DRUGS ?

Direct effects of psychotropic drugs on TH enzyme can be assessed by adding the drug, in μM to M concentrations, to incubation mixtures containing solubilised enzyme. Alternatively the drug can be added to synaptosomal suspensions; but since synaptosomes are capable of active uptake and release of transmitter and intraneuronal storage and metabolism, drug effects on synaptosomal tyrosine hydroxylation may reflect these aspects of NA function. Also, without definite information as to the concentration of the drug in the synaptosomes (as opposed to the concentration in the incubation medium), the net effect on catalytic activity of TH will be impossible to assess.

There is a dearth of information about drug effects in both sorts of enzyme preparations with regard to noradrenergic neurons. Patrick (1977) reports that cocaine increases tyrosine hydroxylation in dopaminergic synaptosomes, and reserpine has been shown to inhibit tyrosine hydroxylation both in striatal dopaminergic synapses, and, in this laboratory (Boarder and Fillenz,1978), in hippocampal and hypothalamic as well as striatal synaptosomes. Since cocaine blocks transmitter uptake, and reserpine blocks transport of dopamine into storage vesicles, the effects reported on hydroxylation rate may well be due to alterations in intrasynaptosomal distribution of transmitter rather than to direct interaction of the drugs with tyrosine hydroxylase.

Even when drug interactions with TH in soluble form are reported, the concentrations of drug required are often rather higher than the levels which would occur in brain after in vivo administration , so it is difficult to assess their significance. For example, both parachlorophenylalanine (PCPA,discussed briefly in Section 6 of Chapter 5) and parachloroamphetamine

(PCA) both inhibited TH in vitro in very high concentrations (10^{-4}- 10^{-3}M). Morphine (see Section 3 of Chapter 5) had no effect on soluble TH in vitro at 10^{-3} M (Cicero et al, 1973).

But these negative results might also have been due to non-optimal assay conditions. Although Cicero et al used a range of substrate and cofactor (DMPH4) concentrations, the absence of catalase in the assay may have prevented the detection of drug-related changes. In the case of Dairman et al (1968), in which phenoxybenzamine and phentolamine were added to incubations of solubilised beef adrenal TH, no change in activity was found; but since the assay was performed only at saturating concentrations of cofactor and tyrosine, changes in affinity for substrate of cofactor were not , in fact, excluded. Reports of this nature should therefore be examined carefully before the stated conclusions are accepted. More exhaustive work is needed in order to elucidate the various ways in which psychotropic drugs affect noradrenergic neurones; but to eliminate a direct effect on synthesis of NA via interaction with tyrosine hydroxylase , for any drug which can accumulate in nerve endings, the soluble assay of TH should be performed at a range of drug , cofactor and substrate concentrations so that kinetic analysis can be undertaken.

I shall return to this aspect of neuropharmacological assessment with respect to individual drugs in Sections 3-6 of Chapter 5. At present, to paraphrase Lovenberg (1978), investigation of psychoactive drugs and catecholamine synthesis often amounts to an investigation of the effects of functional activity on synthesis, rather than the effects of changes in synthesis on the functional activity of neurons.

10. CHANGES IN TH ACTIVITY AFTER IN VIVO STIMULATION.

In this section the focus will remain on direct measurement of the rate of hydroxylation of tyrosine to DOPA in brain tissue; of necessity , changes in TH activity are measured post mortem, and therefore post-stimulation. In the next section, comparisons will be made with other measures of NA synthesis which can be performed in vivo.

With in vivo stimulation, the route by which the intended stimulus reaches the noradrenergic neuron may affect the outcome. For example , the uptake of the pterin cofactor, BH4, may be a limiting factor in its effect on TH activity. This jeopardises the attempt to find out whether endogenous cofactor concentrations are normally saturating. Large doses of BH4 were injected intraventricularly into rats (Kettler et al 1974), producing a 350% increase in cofactor concentrations in the brain; but the associated increase in tyrosine hydroxylation was only 2-fold; given a low-efficiency uptake system, the question of saturation remains unresolved.

Under the rubric of 'in vitro activation', I referred to several preparations in which TH was activated by treatment with different stimuli. It is not clear which of these in vitro stimuli have most relevance to in vivo changes in enzyme activity; but phosphorylation, depolarization, and calcium-related effects have been incorporated into theoretical models as mechanisms for in vivo activation in relation to normal neuronal function. Results obtained from isolated enzyme using these methods also seem to bear some resemblance to results of depolarizing stimulation in preparations containing whole neurons (hypogastric nerve-vas deferens preparation of Weiner , 1977; and the brain slices used by Roth et al, 1975). Further work by Roth and co-workers suggests similar modes of activation of TH by stimuli administered to intact animals (Roth et al,1974; Roth et al, 1975; Salzman and Roth, 1980a).

These studies involved electrical stimulation of noradrenergic neurons in the locus coeruleus of anaesthetized rats. The standard experiments used a 15-minute stimulation period (stimulation at 20 Hz, current 200-400 uA, pulse duration 2 msec). Unilateral stimulation was applied during a single session. The rats were killed at varying times after the end of the stimulation session, to examine the decay of activation in surviving animals. Biochemical measures of NA function used ipsilateral hippocampus and cortex , isolating soluble TH enzyme from homogenates and comparing with the enzyme from the corresponding tissue from the contralateral, unstimulated side,analyzed separately. The assay system involved a 5-minute preincubation period at 37°C before addition of the labelled tyrosine (10 µM final concentration, subsaturating) and a dopa-decarboxylase inhibitor was included in the incubation. (The reason for this addition is obscure since activity was measured in terms of tritiated water, not DOPA, separated by elution from a Dowex-50 column for scintillation counting.) Electrode placement in the locus coeruleus was confirmed histologically.

The first two experiments in this series (Roth et al 1974,1975) were concerned with establishing the brain-stimulation technique for comparison with the in vitro stimulation of hippocampal TH from untreated animals by addition of calcium ions and cAMP to the incubation medium containing soluble TH (Roth et al,1975) and by depolarizing slices with high potassium incubation media or electrical field stimulation (Bustos et al, 1978). A parallel effect was confirmed (see FIG.3.2). At subsaturating concentrations of cofactor and tyrosine , soluble TH activity from the stimulated hippocampus was raised to 440% of that on the contralateral, non-stimulated side. The kinetic changes elicited were of a similar pattern to those with the previous in vitro stimulation techniques; a reduction in Km for tyrosine and for cofactor, and an increase in Ki for inhibition by NA. These changes were even more

FIG. 3.2 Activation of soluble tyrosine hydroxylase by various methods, demonstrated by kinetic changes on Lineweaver-Burk plots. (from Roth et al, 1975)

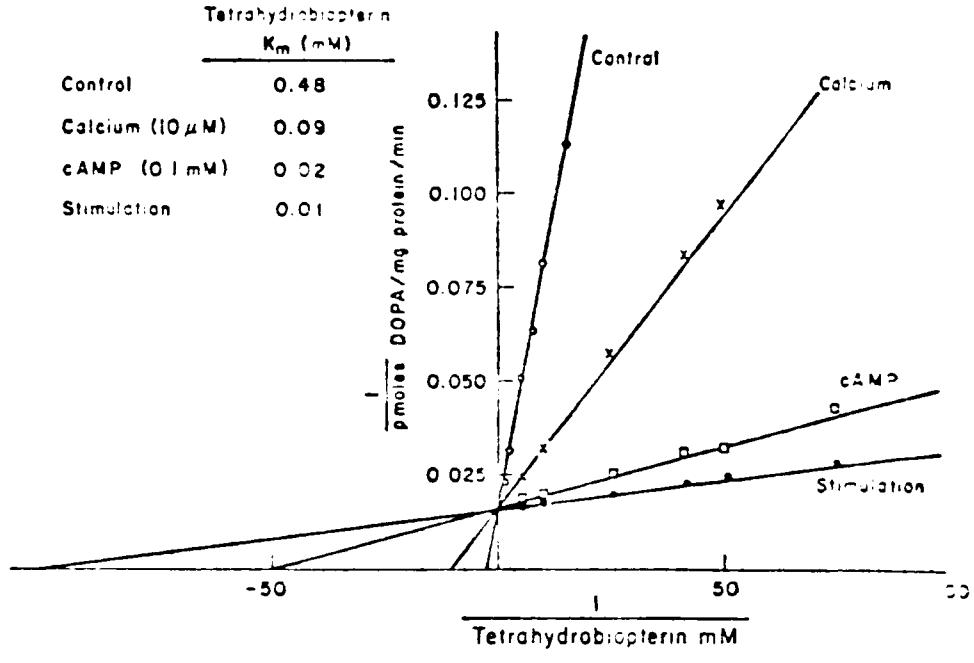
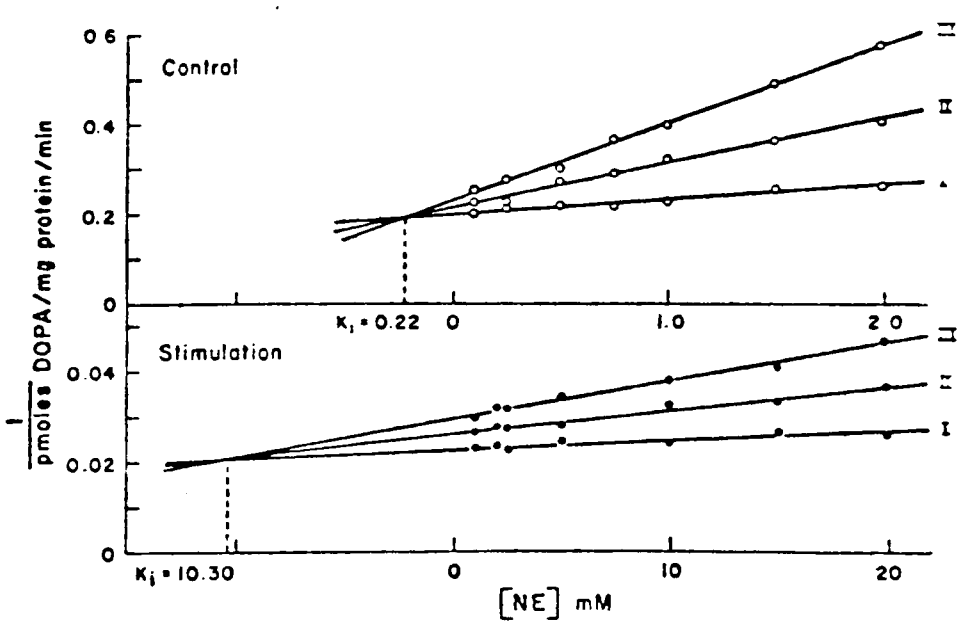


Fig. 3. Lineweaver-Burk plot demonstrating the effect of calcium, cAMP and electrical stimulation on the activity of soluble hippocampal tyrosine hydroxylase. The enzyme was prepared and the assay performed as indicated in the section on materials and methods. Each line is the best fit plot of the data obtained from the means of points generated from 6 separate assays. Assays were conducted in the presence of tyrosine (0.1 mM) and seven tetrahydrobiopterin concentrations ranging from 10 μM to 1 mM



Effect of electrical stimulation of the locus coeruleus on the K_i for norepinephrine of the tyrosine hydroxylase isolated from the hippocampus. The K_i was determined by the method of Dixon (1953) at three concentrations of tetrahydrobiopterin (10^{-4}, 10^{-5} and 10^{-6} M), a constant L-tyrosine concentration of 10^{-4} M and seven concentrations of norepinephrine. Each line is the best fit plot of the data obtained from the means of points generated from 6 separate assays. Stimulation parameters are the same as those indicated in Table 5

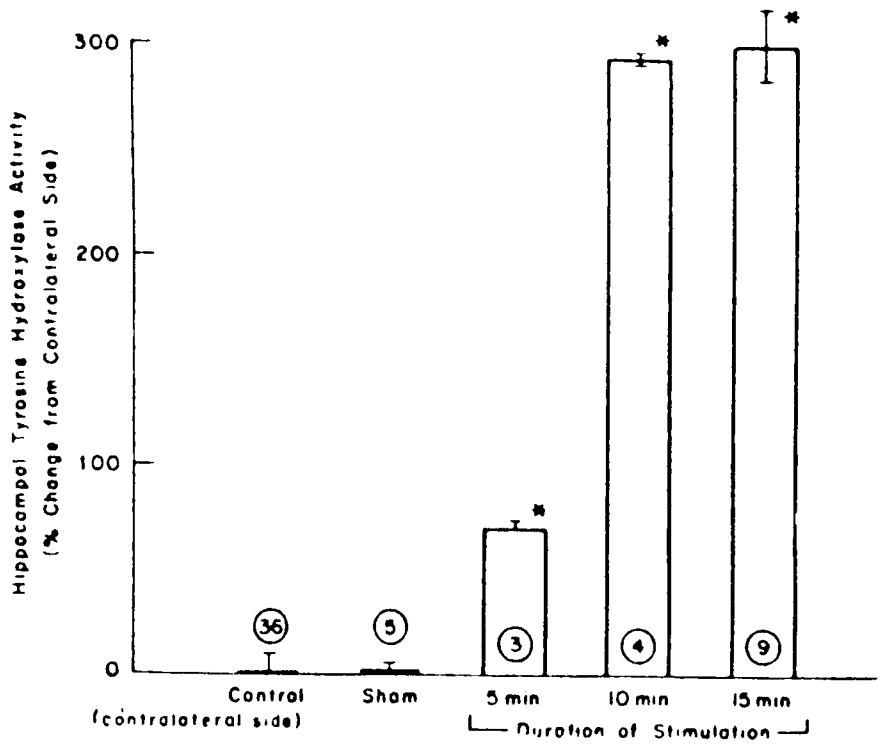
pronounced when the natural cofactor BH4 was used in place of DMPH4, as was the case in the 1975 experiment with in vitro activation of TH by calcium and cAMP. Changes in Vmax were not reliably found.

In another study (Salzman and Roth, 1980a), these results were re-established and extended.The elevation of subsaturating TH activity was shown to be present in rats killed up to 10 minutes after the end of the LC stimulation period; and the minimum stimulation period necessary to produce significant activation was defined as 5 minutes. Locus coeruleus stimulation for 10 minutes was sufficient to produce near-maximal activation (FIG.3.3). These results correspond with the in vitro results, although in the brain stimulation experiments the comparison reported is between the two hippocampi of each animal rather than between treated and untreated aliquots of enzyme from the same source. Salzman and Roth (1980a) indicate that the variation between animals is considerable (they do not report the range of enzyme activity in stimulated animals).

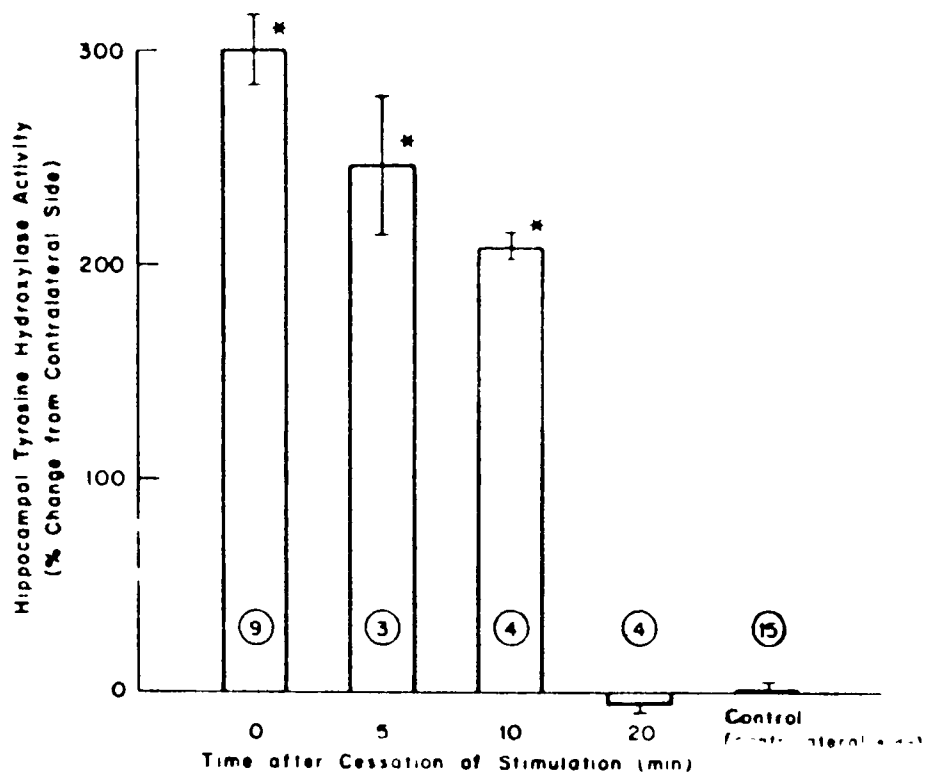
There are, of course disadvantages in this paradigm; particularly the possibility that , given the crossed anterior projection from at least part of the LC, the comparison between stimulated and 'non-stimulated' sides of the brain would not provide a true stimulation effect. However, this crossed projection is of minor importance in the rat, and a comparison with baseline hippocampal TH activity in anaesthetized but unstimulated animals in this study showed no significant difference from activity in the hippocampus contralateral to stimulation.

All the results reported by Roth and his coworkers and discussed here, with exception of Harris & Roth's (1971) experiment , have been concerned with changes in activity of the solubilised isolated enzyme from

FIG. 3.3 Post-stimulation activity of tyrosine hydroxylase after in vivo stimulation of the locus coeruleus.
(from Salzman and Roth, 1980a)



Time course of activation of hippocampal tyrosine hydroxylase by locus coeruleus stimulation. Chloral hydrate-anesthetized rats (400 mg/kg i.p.) were subjected to continuous, biphasic, unilateral LC stimulation (300 μA, 20 Hz) for 5 to 15 min. Sham-stimulated animals had an electrode lowered into one LC but current was not applied. Immediately upon termination of the stimulation, the hippocampi were removed, frozen and assayed for tyrosine hydroxylase activity. Control values represent the mean of the contralateral sides of all animals stimulated, regardless of electrode placement (=7.0 ± 0.3 pmol of dopa per min per mg of protein). The tyrosine hydroxylase activity in each stimulated hippocampus is expressed as the percent increase over the activity in the unstimulated, contralateral, control hippocampus. Enzyme activity in tissues of sham-stimulated animals was 7.0 ± 0.4 pmol of dopa per min per mg of protein on both electrode-implanted and contralateral sides. Values are the mean ± S.E.M. of the number of samples indicated in the circles. *Significantly greater than paired control, $P < .05$.



Time course of decay of hippocampal tyrosine hydroxylase activation following locus coeruleus stimulation. Chloral hydrate-anesthetized rats (400 mg/kg i.p.) were subjected to continuous, biphasic, unilateral LC stimulation (300 μA, 20 Hz) for 15 min. At the indicated times after the stimulation was terminated the rats were decapitated and the hippocampi were removed, frozen and assayed for tyrosine hydroxylase activity. Control values represent the mean of the contralateral sides of all animals stimulated, regardless of electrode placement (.0 ± 0.3 pmol of dopa per min per mg of protein). The tyrosine hydroxylase activity in each stimulated hippocampus is expressed as the percent increase over the activity in the unstimulated, contralateral control hippocampus. Values for 0 min (rats decapitated immediately upon termination of stimulation) are taken from figure 4 (15-min stimulation). Values are the mean ± S.E.M. of the number of samples indicated in the circles. *Significantly greater than paired control, $P < .05$.

neurones after homogenisation and high-speed centrifugation. The changes have been maintained for short periods after stimulation. Clearly these changes are not dependent on end-product feedback inhibition or on changes in cofactor concentration intraneuronally, any more than they are related to impulse traffic as such. The activity of the solubilised enzyme seems to reflect something about recent neural activity in the tissue. Given favourable (subsaturating) conditions for incubation, catalytic efficiency of TH is improved for some 10 minutes after the cessation of an activating (it is tempting to say 'galvanising') stimulus.

No other laboratory has shown allosteric activation of central noradrenergic TH after short bursts of stimulation of this nature. The dearth of evidence on activation of noradrenergic TH by drugs in vitro has been pointed out. There is little evidence for activation of noradrenergic soluble TH by drugs administered in vivo in single doses. (This contrasts with the evidence for effects of neuroleptic drugs on dopaminergic TH. Zivkovic et al (1974) have shown increased affinity for DMPH4 after in vivo administration of neuroleptics, though in vitro addition of the neuroleptics had no effect of TH in homogenates.) Masserano et al (1979) found a rapid activation of TH in caudate nucleus after a single electroconvulsive shock. Although this effect was also apparent in adrenal medulla, it was not found in central noradrenaline-containing brain regions such as hippocampus, hypothalamus , cortex or locus coeruleus.

It may be that the within-animal comparison , used in the brain-stimulation paradigm, is necessary for the detection of allosteric changes, especially if the inter-animal comparisons envisaged in drug studies are beset by large variations in baseline activity. Another problem is encountered in killing animals by stunning and decapitation, which may, as Weiner (1979) points out, result in a sympathetic discharge and may affect noradrenergic

activity in the brain, distorting pre-existing different states of enzyme activation in experimental and control animals. Dairman et al (1968) measured TH activity using an assay like that of Nagatsu (1964a) with DMPH4, mercaptoethanol and ferrous sulphate and tyrosine at saturating conditions after in vivo administration of phenoxybenzamine or phentolamine. No change in activity was found with these drugs, which are both alpha-adrenergic blocking agents; but this assay was, as pointed out above with reference to the in vitro use of these drugs, not ideal for demonstrating changes in kinetic properties of the anzyme.

Roth and his colleagues (1978) see the mechanism of kinetic activation by increased impulse traffic , as manifested in their experiments, as a mechanism of regulation which may be superimposed on a background of regulation by end-product inhibition. Regulation by end product inhibition alone would require a signal of 'substantial depletion' before the rate of hydroxylation of tyrosine would start to accelerate in response to ,say, increased firing rate and increased release of NA. If the neuron were also able to respond to a signal about increased firing rate (or prospective depletion) by rapidly increasing the affinity of TH for cofactor, or decreasing affinity for inhibition by NA, then synthesis rate could accelerate to keep up with requirements of NA for release. This capacity for increasing synthesis in order to maintain fairly constant levels of transmitter during periods of rapid neuronal firing and increased release has been mentioned as a characteristic of noradrenergic neurones. The hypothetical mechanism described above shows how this could come about.

What is missing so far in this discussion is a demonstration that synthesis of NA does in fact increase in vivo , during or after stimulation which is capable of eliciting kinetic changes in solubilised tyrosine hydroxlase. This will be discussed in the next section ; it is important to note that the changes demonstrated under subsaturating conditions are merely an indication of potential for acceleration of synthesis in neuronal tissue.

11. MEASUREMENT OF NORADRENALINE SYNTHESIS AND TYROSINE HYDROXYLASE ACTIVITY: EFFECTS OF STIMULATION AND STRESS.

Various methods have been used to measure noradrenaline synthesis and turnover in vivo (for review see Korf, 1980; and Salzman and Roth, 1979). The aim in developing these methods has been to obtain a sensitive and reliable index of NA function. NA levels seem to be maintained despite different levels of activity of both peripheral and central noradrenergic neurons, so that increased rates of firing and of release are not necessarily accompanied by reduction of transmitter levels.

In order to maintain stable levels of NA, synthesis rates increase in nerve terminals during increased activity. The methods outlined in Table F below have been used to reflect some aspects of the increased synthesis, release and metabolism of NA in the brain under conditions which activate the noradrenergic system, such as stress, cold environment,and certain drugs (see Stone,1975). All the methods involve making assumptions which may be violated in practice; but, as Korf (1980) has pointed out, the results from different methods agree at least in demonstrating increased synthesis or turnover under circumstances in which NA neuronal activity increases. Since NA metabolism as well as synthesis is occurring in nerve terminals , NA loss from terminals is due to intraneuronal metabolic breakdown by monoamine oxidase and by catechol-O-methyl transferase, as well as to release. So there is no simple relationship between NA release and NA turnover. Hence (Korf,1980), 'the turnover rate is a pharmacological tool, but its precise physiological significance remains obscure'. In what follows I have ignored 'turnover ' methods which do not depend upon synthesis rate, such as the rate of depletion of injected labelled NA.

TABLE F:

MEASUREMENT OF NA SYNTHESIS RATE IN VIVO: METHODOLOGY.

AMT-NA : measurement of NA depletion after synthesis inhibition by alpha-methyl tyrosine in large doses. Control animals are injected with vehicle instead of inhibitor .Carlsson et al (1972) have designated this as an index of utilization of NA; as a measure of synthesis, Korf (1980) and Salzman and Roth (1979) consider that it usually gives an under-estimate compared to other methods (see below). Korf (1973) and Brown et al (1974) used this method to show increased 'utilization' of NA immediately after a session of foot shock in rats. Gordon (1966) found increased NA depletion in brainstem after 6 hours in the cold,or after 3-5 hours exercise in a rotating drum.

DDCI-DOPA : measurement of DOPA accumulation after decarboxylase inhibition; a measure of the rate-limiting step in synthesis of NA and DA. The catecholamines are not distinguished from each other in this method, since the final steps of synthesis are blocked; some measure of specificity can be obtained by selecting regions for study which have either noradrenergic or dopaminergic input, but not both. The DOPA decarboxylase inhibitor is administered 30 minutes before killing, since accumulation of DOPA has been shown (Carlsson et al ,1972) to be linear over this period. High doses of inhibitors such as NSD 1055, NSD 1015, and Ro 4-4602 are used. These drugs also have monoamine oxidase inhibiting activity. This method gives a fair estimate of catecholamine synthesis rate, according to Salzman and Roth(1979) and Korf (1980), but drug interactions are possible and it is not ideal for pharmacological work; also the recommended set time of 30 minutes for DOPA accumulation ,during which synthesis rates are assumed to be constant, is a severe limitation for work involving different periods of stimulation or time courses of, say, drug effects.

3H-TYR-NA : measurement of 3H-NA newly synthesised from injected 3H-tyrosine. Usually the labelled tyrosine is injected as a bolus dose intravenously or intracerebrally , and at the end of a set period the animal is killed and labelled NA is isolated and measured by counting the radioactivity. Alternatively, a constant infusion of labelled precursor can be given, and synthesis rate is calculated from the difference between the specific activities of NA and tyrosine. The specific activity of tyrosine should remain the same in experimental and control animals at any given time, although it may vary during the period of accumulation of radioactivity. When a 'conversion index ' incorporating both labelled NA and specific activity of tyrosine is used, this method provides a good estimate of synthesis which is similar to that obtained after decarboxylase inhibition (Carlsson et al,1972; Costa, 1972). Unfortunately in many studies the specific activity of tyrosine was not measured. Stolk et al (1974) used this method to show an increase in NA synthesis in whole brain in rats made to swim in cold water as a form of stress. Gordon et al (1966) showed elevated NA synthesis by this method in rats after strenuous exercise and after cold, in the study mentioned above. Huszti (1976) found an increase in 3H-NA in brain after immobilization, at a time when total NA levels were unchanged.

FLA63-NA : measurement of NA depletion after inhibition of dopamine-beta-hydroxylase,and is another index of utilization of NA, but in the presence of dopamine accumulation. . Goldstein et al (1972) used this method to demonstrate an increase in NA synthesis during cold stress in rats. But the accumulation of dopamine may distort synthesis rates, as it is itself an inhibitor of TH.

TABLE F (continued)

MOPEG measurement : 3-methoxy-4-hydroxyphenylglycol and its conjugate, MOPEG-SO4, are major metabolites of NA in brain; see FIG 1.2 (Schanberg,1968; Bareggi et al, 1978) ; MOPEG accumulation can be measured, and its egress is blocked by probenecid in some studies. The best estimates of synthesis are obtained when total (free and conjugated) MOPEG , and also DOPEG, are measured (Nielsen et al, 1976). An alternative method ,which ensures measurement of synthesis-related changes, is to measure labelled MOPEG after injection of tritiated tyrosine (Nielsen and Braestrup,1977; Stone,1976). Bliss et al (1968) and Stone (1975) have shown increased brain NA synthesis after footshock using this method.

Table F shows some of the principal methods which have been used to measure synthesis of noradrenaline. They have been used to study drug actions as well as the effects of stress in the CNS, and the relevant pharmacological studies will be referred to in later sections of this thesis .

Let us return to the question of comparing changes in synthesis rate measured by in vivo 'accumulation' techniques and by enzyme assay in vitro. Electrical stimulation of the locus coeruleus has been shown to increase NA activity using biochemical measures (see Table G). Korf (1976) and Walter and Eccleston (1973) both measured MOPEG accumulated during stimulation by killing groups of control and stimulated animals immediately after the stimulation session. The recent study by Salzman and Roth (1980a) allows a comparison between TH activity and the in vivo measures of NA synthesis to be made. While the in vivo measures involve the measurement of accumulated products over a short period immediately following locus stimulation, the TH assay is performed on isolated enzyme from brain regions dissected post mortem and reflects the state of the enzyme at the time of death in terms of its capacity to hydroxylate exogenous labelled tyrosine. With Salzman and Roth's data (1980a) the simultaneous measurement of different aspects of NA turnover can be set against changes in the state of the enzyme which represent a potential for increased synthesis. As has been noted, the in vivo measures of synthesis reflect a submaximal rate of hydroxylation of tyrosine;

assay of TH under subsaturating conditions represents reaction rate at an arbitrary point on the concentration range of both cofactor and substrate; and assay at saturating concentrations represents maximal potential hydroxylation rate of the solubilised enzyme.

TABLE G:

EFFECTS OF STIMULATION OF THE LOCUS COERULEUS

With similar parameters of locus stimulation - 15 minutes of stimulation at 20 Hz, current intensity 200-400 uA, pulse duration 2 msec - the following results have been obtained for post-stimulation NA function in the hippocampus and cortex ipsilateral to the stimulated locus coeruleus . The figures are given as percentage change with respect to the contralateral, unstimulated side:

NA levels	40% depletion in cerebral cortex	(Korf,1973)
	33% depletion in cortex and hippocampus	(Salzman & Roth,1980a)
DDCI-DOPA	35% increase in DOPA in cortex + hippocampus	(Salzman & Roth,1980a)
3H-TYR-NA	63% increase in 3H-NA in cortex, 55% increase in hippocampus	(Salzman & Roth,1980a)
TH assay	(tyrosine 10 µM ,cofactor 100-170 µM)	
	340% increase	(Roth et al,1974)
	305% increase	(Roth et al,1975)
	310% increase in hippocampus	(Salzman & Roth,1980a)
With 45 minutes of stimulation at 2 Hz :		
MOPEG (conjugated)	61% increase,ipsilateral hemisphere	(Walter & Eccleston,1973)

Clearly, locus stimulation constitutes an extremely effective method of stimulating noradrenergic neurones. The normal rate of firing in anaesthetised rats is 1-2 Hz; unanaesthetised rats respond to noxious stimuli with a short burst of firing at about 10 Hz, (Segal, 1979) which is followed after a short interval (seconds) by inhibition of firing. In Salzman and Roth's study the neurons of the locus coeruleus were driven at 20 Hz for 15 minutes. Besides providing effective stimulation , therefore, this induces a highly

abnormal pattern of firing for these cells. The higher current intensities used in these experiments were found to cause lesions in the locus coeruleus (Salzman and Roth, 1980a). In Walter and Eccleston's study, maximum biochemical effects were obtained at a frequency of 2 Hz which is near the average rate for the locus coeruleus (although normal firing is not regular, consisting of bursts of firing interspersed with silences).

The fact that there is NA depletion immediately after the end of stimulation (cf Salzman and Roth, 1980a; see FIG. 3.4) means that at that point the assumptions of many of the methods cited above do not hold. By 30 minutes post-stimulation, NA levels were back to control values in most animals . The period used to measure accumulation of 3H-NA was the 30 minutes after the end of stimulation. Only the animals with cortical NA levels which had reached 90% of control by the end of this period were selected for calculation of NA synthesis rate by this method; and a large inter-subject variation was reported (range 350-7000 dpm/g tissue in control hippocampus). The period for measuring DDCI-DOPA, on the other hand, was the 30 minutes starting from the beginning of stimulation; and the region chosen for assay was the cortex combined with the hippocampus, not the hippocampus alone as in the other methods (see FIG.3.5).

These results from Roth's laboratory represent a breakthrough in demonstrating that stimulation of noradrenergic neurones in the brain resulted in increased synthesis, increased release (depletion of NA) and allosteric activation of TH in forebrain tissue from the stimulated side of the brain; and that these changes were in some respects similar in nature and time course to activation of noradrenergic TH in vitro by calcium, by potassium-induced depolarization, and by electrical stimulation. The largely unilateral response to unilateral stimulation was a fundamental component of the study, since the inter-animal variation was extremely large and significant results were

FIG. 3.4 Cortical norepinephrine levels at times following termination of locus coeruleus stimulation.
(from Salzman and Roth, 1980a)

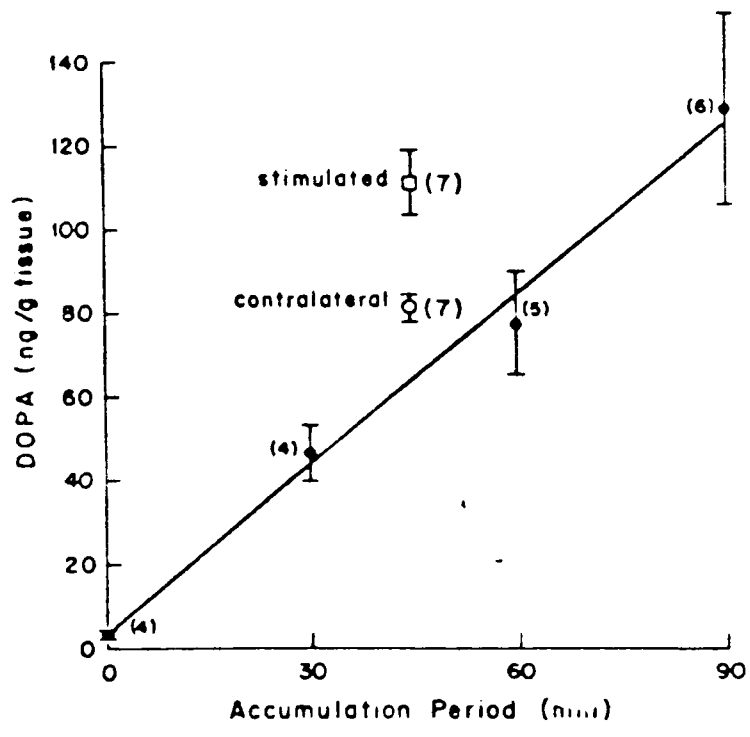
Cortical norepinephrine levels at times following termination of unilateral locus coeruleus stimulation

Rats were subjected to 15 min of unilateral LC stimulation and decapitated immediately, or 5 to 60 min after stimulation was terminated. Stimulated and contralateral hemicortices were removed and the NE content in stimulated tissues was compared to that of the contralateral control tissues. Sham-stimulated rats are those described in figure 4. Values for *n* refer to the number of stimulated hemicortices analyzed. Cortical NE content of unanesthetized rats was 254 ± 8 ng/g (*n* = 18). NE values are corrected for 73.5% recovery from alumina.

Recovery Period	NE		% Change from Contralateral Side	*n*
	Contralateral side	Stimulated side[a]		
min	*ng/g tissue*			
0[b]	275 ± 10	180 ± 10[c]	−34	5
5	303 ± 33	210 ± 31[c]	−31	5
10	299 ± 21	251 ± 22[c]	−16	4
20	304 ± 18	247 ± 17[c]	−18	5
30	289 ± 20	303 ± 14[d]	+7	5
45	345 ± 12	342 ± 32[d]	−1	9
60	333 ± 12	322 ± 22[d]	−3	4
Sham stimulation	324 ± 19	339 ± 20[d]	+5	3

[a] 20 Hz, 300 μA biphasic current, except sham stimulated and 0 min recovery groups.
[b] 20 Hz, 400 μA monophasic current.
[c] Significantly less than contralateral side, $P < .05$.
[d] Not significantly different from contralateral side.

FIG. 3.5 Accumulation of DOPA in cortex and hippocampus during in vivo stimulation of the locus coeruleus in the presence of a DOPA decarboxylase inhibitor.
(from Salzman and Roth, 1980a)



Accumulation of dopa in the combined cortex and hippocampus after Ro 4-4602. Chloral hydrate-anesthetized rats (400 mg/kg i.p.) were injected with the dopa decarboxylase inhibitor, Ro 4-4602 (800 mg/kg i.p.), and the cortex and hippocampus were removed 30 to 90 min later. Tissues from two animals were combined and assayed fluorometrically for dopa content. The zero time accumulation data represent dopa levels in tissue from rats which did not receive Ro 4-4602 but were decapitated 10 min after chloral hydrate injection. Open symbols represent data from rats receiving unilateral LC stimulation (20 Hz, 300 μA, 15 min beginning at 0 min) □, stimulated side; ○, contralateral side. Vertical bars indicate the S.E.M. and values in parentheses are the number of separate tissue samples analyzed.

obtained from matched-pair analysis of differences between stimulated and non stimulated sides of the same brain.

There is imperfect agreement between the results from entirely different methods for measuring NA function. The results representing in vivo synthesis rates show an increase ranging from 35% to 63% with different regions and time-periods, which might in themselves preclude closer agreement. Subsaturating TH activity shows increases of approximately 300% reported in three separate papers; it is not clear whether these are from entirely independent experiments, or whether the results are pooled with previous figures. The implications to be drawn from the large activation obtainable using this assay are : first, that in non-optimal hydroxylation conditions, the rate of hydroxylation of tyrosine would be responsive to changes in cofactor and substrate concentrations ; and second, that the enzyme in nerve terminals has some capacity to respond to nerve impulse traffic by increasing NA synthesis rate,even without demonstrable changes in substrate and cofactor availability.

Salzman and Roth (1980a) discuss whether end product inhibition could account for the changes in synthesis rate and enzyme activity, since NA levels were significantly depleted after LC stimulation . Taking the result of different methods together, Salzman and Roth say that this proposition seems unlikely, since the TH activity returns toward normal by 20 minutes after the end of stimulation, when NA levels are still depleted. They do not however give figures which show the relationship of synthesis to levels of NA in individual animals. NA depletion shows a threshold in that stimulation at lower frequencies and a slightly lower current intensity (5-10 Hz, 200 uA) does not produce depletion. Five minutes of stimulation at the higher frequency is sufficient to activate the enzyme. It therefore seems possible that TH activation does not require measurable depletion of NA levels; the kinetic

changes show that the affinity for NA as inhibitor is reduced by stimulation and affinity for cofactor is increased; so the competition between NA and cofactor for the binding site on the enzyme molecule may be reduced before there is any depletion of intraneuronal NA. This mechanism would , as discussed above, (p.76) endow the NA neurons with a more sensitive way of maintaining transmitter levels despite changing release rates, which is characteristic of the NA system.

More work is needed in experiments of this type comparing results from different methods, before the project of relating changes in kinetic parameters to in vivo synthesis can be realised. Extrapolation from isolated enzyme activity under arbitrary subsaturating conditions to the complexities of synthesis in vivo is as yet too great a leap to be profitable, especially when the stimulation concerned involves a firing rate which is outside the normal range for these neurones. A further complication is that the the simultaneous activation of non-noradrenergic neurones by the stimulation current may indirectly affect the noradrenergic neurones.

Measurement of tyrosine hydroxylation rate in synaptosomes is a method which has not yet been fully exploited. Some work has been done with synaptosomes in vitro (see section 8 of this Chapter, and section 7 of Chapter 2). As an index of capacity for NA synthesis in intact nerve endings, preserving some of the normal regulatory functions during post mortem incubation, synaptosomal tyrosine hydroxylation rate would seem to have many advantages. Some of the experiments described in this thesis were carried out in order to assess the usefulness of synaptosomal TH rate as an indicator of rapid short term changes in the activity of NA neurons, using mild stimuli without need for anaesthesia, operative procedures, or administration of precursors or inhibitors of synthesis.

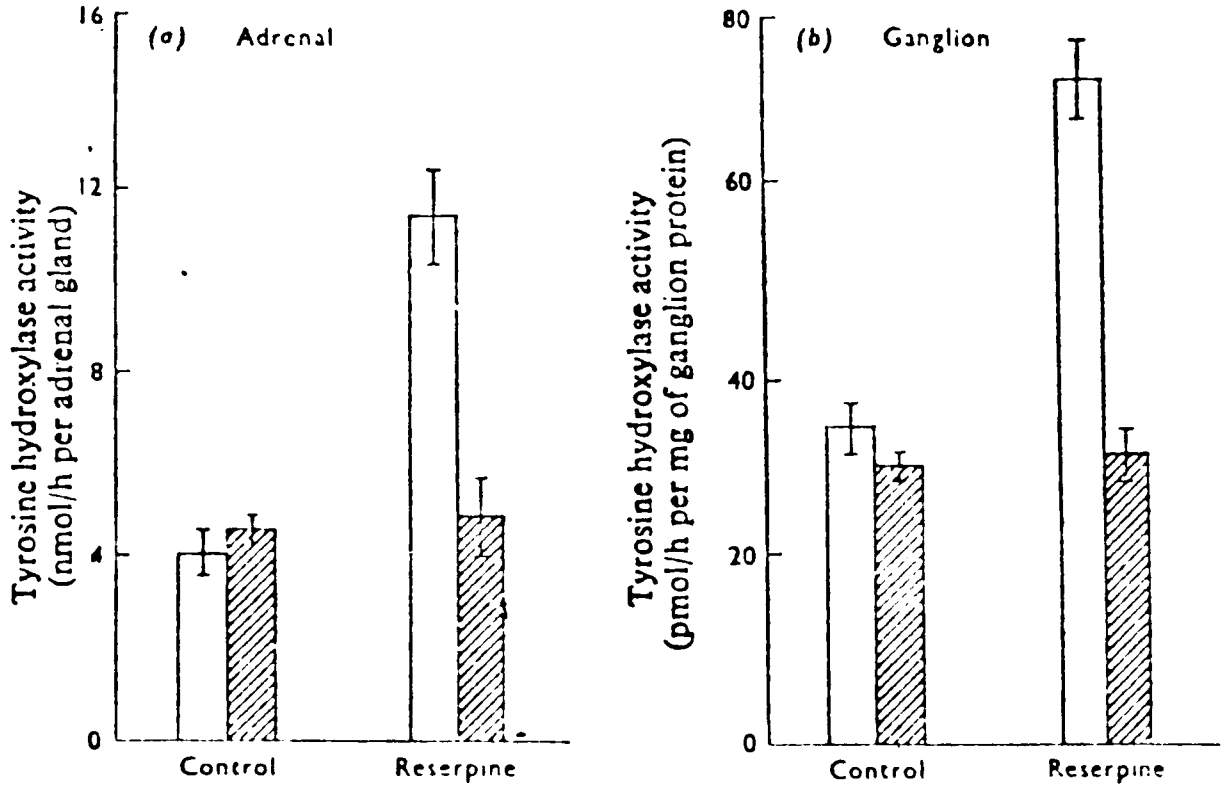
12. LONG TERM CHANGES IN TH ACTIVITY IN THE CNS.

The post-stimulus allosteric changes in tyrosine hydroxylase reported by Roth's group are short lasting; 20-30 minutes after in vitro or in vivo stimulation ceases, enzyme activity in the subsaturating assay is back at control levels. Noradrenergic neurones, in the brain and in the periphery , are also capable of long lasting changes in TH activity with potential for either increased or decreased synthesis of NA which can outlast the stimulus by weeks. Of the monoamine systems in the brain, noradrenergic neurones most commonly show these long lasting changes, whereas they are less marked in DA and 5HT neurones (Mandell, 1978).

PERIPHERAL NERVOUS SYSTEM AND ADRENAL MEDULLA:

Long lasting changes of this nature were first demonstrated by Mueller et al (1969) and Thoenen et al (1969), in adrenal gland(see FIG.3.6). Increases in TH activity were found 12 - 48 hours after administration of drugs which impaired synaptic transmission, and were thought to be linked to the reflex increase in sympathetic discharge which was associated with these drugs. Increases were found after 6OHDA, a neurotoxin specific to catecholaminergic neurones; repeated dosage with phenoxybenzamine , an alpha-adrenergic blocker (Dairman and Udenfriend, 1970); and reserpine, which depletes monoamine stores by blocking uptake into intraneuronal vesicles. Thoenen demonstrated that the increase in TH following NA depletion by reserpine was due to a trans-synaptic phenomenon mediated by nicotinic cholinergic receptors ; it can be prevented by treatment with cholinergic blockers , or by decentralization of the appropriate ganglia . The increase in activity can occur in the absence of impulse traffic, however, provided the cholinergic receptors remain intact. Stimulation of cholinergic

FIG. 3.6 Induction of tyrosine hydroxylase in adrenal medulla after reserpine administration.
(from Thoenen et al, 1969)



Effect of decentralization on the induction of tyrosine hydroxylase by reserpine (*Thoenen et al.*, 1969)

The preganglionic trunk of the right superior cervical ganglion was transected 2 days and the splanchnic fibres supplying the left adrenal 4 days before the beginning of the treatment with reserpine. The rats were injected with two 5 mg/kg body wt. doses of reserpine 24 and 48 h before the animals were killed. The total activity of tyrosine hydroxylase in adrenal glands (*a*) and its specific activity in the superior cervical ganglia (*b*) were then measured. □, Normal; ▨, denervated. The values given represent the means ± S.E.M. ($n = 6$).

receptors by physostigmine, pilocarpine, and nicotine in high doses also raised TH activity in the adrenal medulla (Lewander et al, 1977).

Joh et al(1973) and Reis et al (1974) showed , by the method of immunoprecipitation, that the amount of TH enzyme protein was increased during this process. Cycloheximide, a protein synthesis inhibitor , provided another means of preventing the change in total enzyme activity which was due to increased enzyme synthesis. The phenomenon of increased enzyme synthesis (related to activation of NA neurones over a long period) has been termed enzyme induction . In terms of NA synthesis, induction of TH clearly allows for a higher potential rate of synthesis of NA, but the mechanisms concerned in the short term regulation of synthesis (eg cofactor availability and affinity for end product inhibition) , will act to control NA synthesis however many enzyme molecules are present . An increase in amount of enzyme does not necessarily imply a higher NA synthesis rate; this , at subsaturating cofactor or substrate concentrations , will depend on the affinity of the enzyme for these substances. However, Dairman and Udenfriend (1970) showed that in vivo catecholamine synthesis from labelled tyrosine was elevated in adrenal medulla after repeated dosage with phenoxybenzamine.

Other manipulations which increase noradrenergic transmission have been shown to produce long-lasting increases in TH activity, although this is probably not always associated with induction of the enzyme . Cold stress for several hours, and immobilization (Kvetnansky,1971) were sufficient to increase TH activity in adrenal glands in one study, although Dairman and Udenfriend (1970) found no change in soluble TH activity in rats which had spent 23 hours in the cold. Dopamine-beta-hydroxylase activity is also increased in the adrenal by similar stimuli, but the same is not true of DOPA-decarboxylase. Pfeifer (1975) showed that handling rats in infancy resulted in adult rats with a lower basal adrenal TH activity than unhandled controls , and

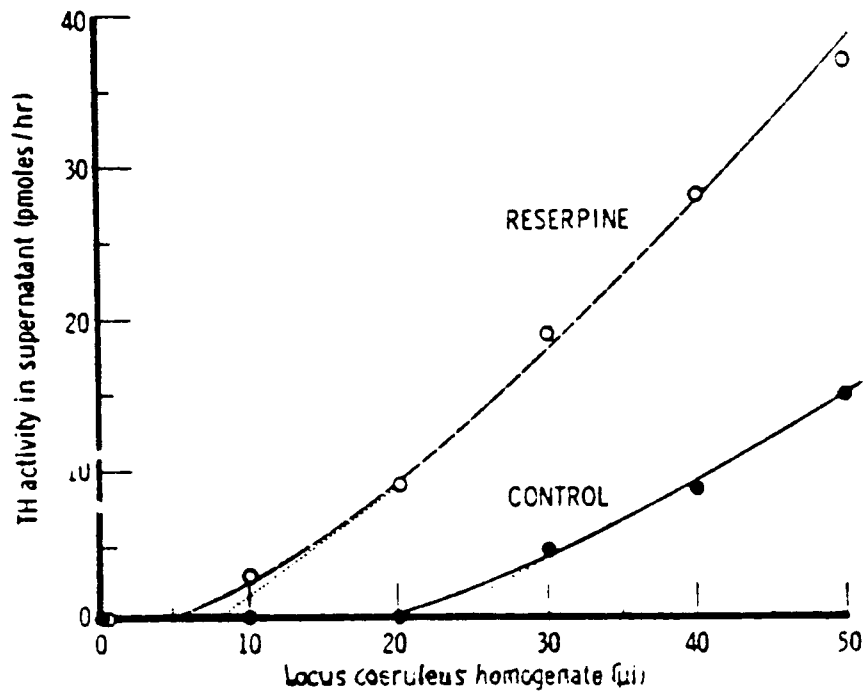
different responses to stress. The mechanism of this long-lasting effect is not known (see section 2 of Chapter 5 for a further discussion of the effects of handling).

CENTRAL NERVOUS SYSTEM :

Noradrenergic neurons in the brain also demonstrate increases in TH activity in response to similar stimuli; but the threshold dose for induction in brain is higher than in the adrenal, and the delay before increased amounts of enzyme are found is longer. Increases in TH enzyme protein (FIG.3.7) and total catalytic activity are found in the locus coeruleus 48 hours after a single injection of reserpine (Reis et al,1975; Black,1975; Zigmond et al, 1974;1979). The maximum rise is to 300% of control, and there is a slow decline over a period of several weeks. But in other regions such as frontal cortex, hippocampus and hypothalamus, which all receive projections from the locus, there is a delayed rise in enzyme activity after several days (see FIGS.3.8 & 3.9). Reis et al have termed this 'delayed augmentation'.The Vmax for tyrosine is increased ,but there is no change in Km for tyrosine or cofactor (DMPH4); see FIG 3.10 . Behavioural activation is a long-latency effect after reserpine administration, following the initial profound sedation of the first few days. Catecholamine levels are still reduced at this time.

The delay in the distal regions may be due to transport of the newly synthesised enzyme from the LC to the terminals. Lesions of the fibre bundles projecting from the LC interrupt the development of delayed activation in the distal regions ; but immunochemical demonstrations of increased enzyme protein in terminal regions (where TH concentration is low) are technically difficult and have not yet been reported. If the delay is due to transport of protein down the axon, the estimate of transport rate from the data of Black

FIG. 3.7 Immunoprecipitation of tyrosine hydroxylase in locus coeruleus from reserpine-treated and control rats. (from Reis et al, 1974)



Immunoprecipitation of TH in locus coeruleus from reserpine-treated (○——○) and control (●——●) rats. Six rats were treated with a single dose (10 mg/kg IP) of reserpine and killed four days later. Ten microliters of antibody was added to 10µl-50µl of homogenate of treated or control animals and final volume adjusted to 60µl with buffer. After the mixture was centrifuged, TH activity was assayed in the supernatant. Note that the equivalence point is shifted to the left in locus coeruleus from reserpine-treated animals, indicating accumulation of more specific enzyme protein.

(1975) and Zigmond (1979) suggests either that the speed of axonal transport for proteins is slower in the CNS (approximately 1.5 mm/day) than in the periphery; or that reserpine itself alters axoplasmic transport (Levin, 1980).

Because of the similarities between adrenal and locus coeruleus TH response to reserpine,and other stimuli, it is possible that the induction in the LC is mediated by a trans-synaptic mechanism, but this has yet to be clarified. Induction seems to be a characteristic exclusive to noradrenergic neurons; dopaminergic neurons, although depleted of DA by reserpine, do not show an increase of TH enzyme protein thereafter. Segal et al (1971) claim that there is an increase in TH activity after daily reserpine injections for 9 days in the striatum (following an earlier increase in midbrain TH activity) ; but this has not been confirmed as an increase in enzyme protein with immunoprecipitation studies.

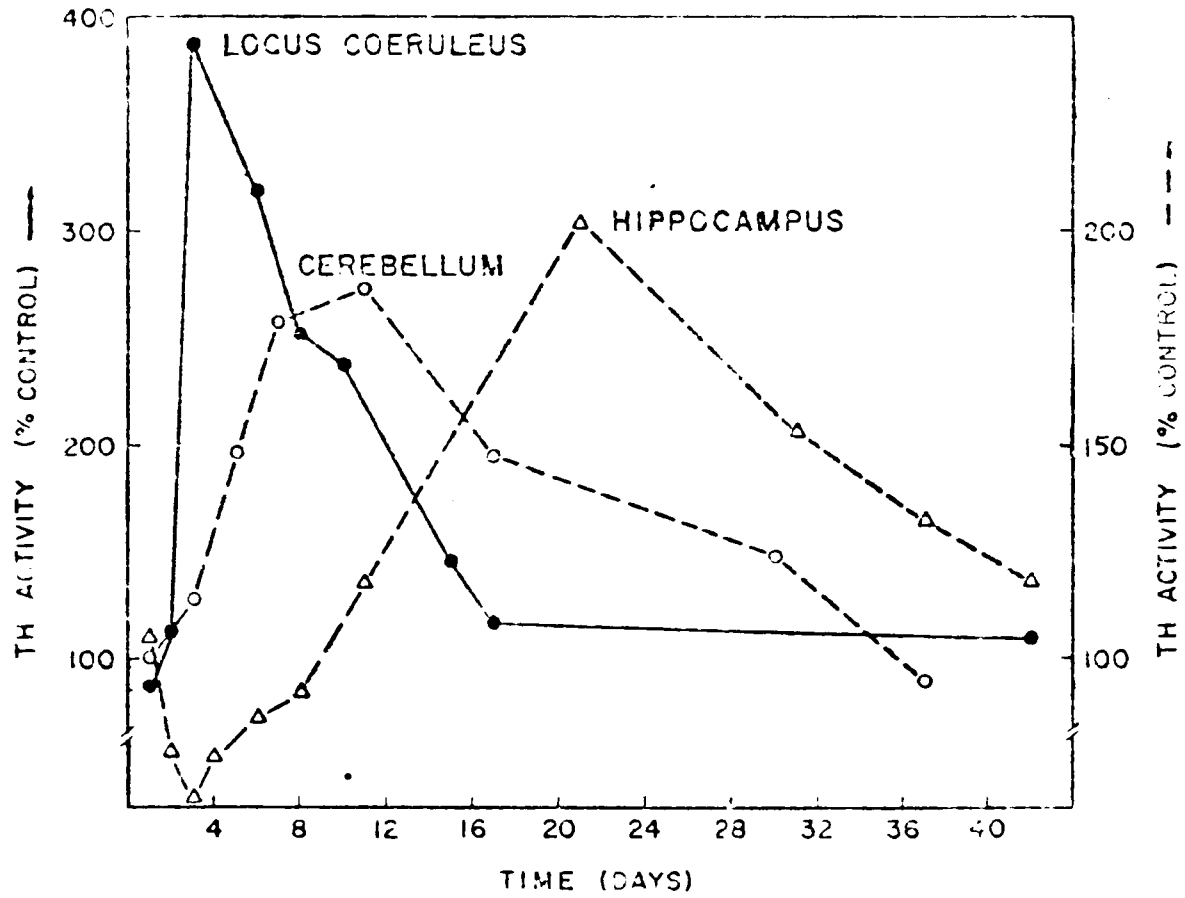
Other factors which emerge from the group of studies on reserpine which might help as pointers to the nature of induction are as follows:

1. TH induction in the LC is followed by a rise in the activity and total enzyme protein of dopamine-beta-hydroxylase. The increase in DBH total activity is not transmitted to noradrenergic terminal regions . DBH is generally transported rapidly in axons, since it is associated with the particulate vesicles; but it is released along with NA and therefore may not accumulate in terminal regions after induction.

2. Some workers have found critical periods for the initiation of the enzyme induction process after the onset of the triggering stimulus. The critical period is generally around 12 - 24 hours, after which induction occurs regardless of further interference (such as cholinergic blockade).

3. Noradrenaline levels are not reliably increased during or after TH induction. In a study by Carlsson et al (1975), NA levels rose from a minimum (shortly after reserpine administration) towards normal levels with a similar

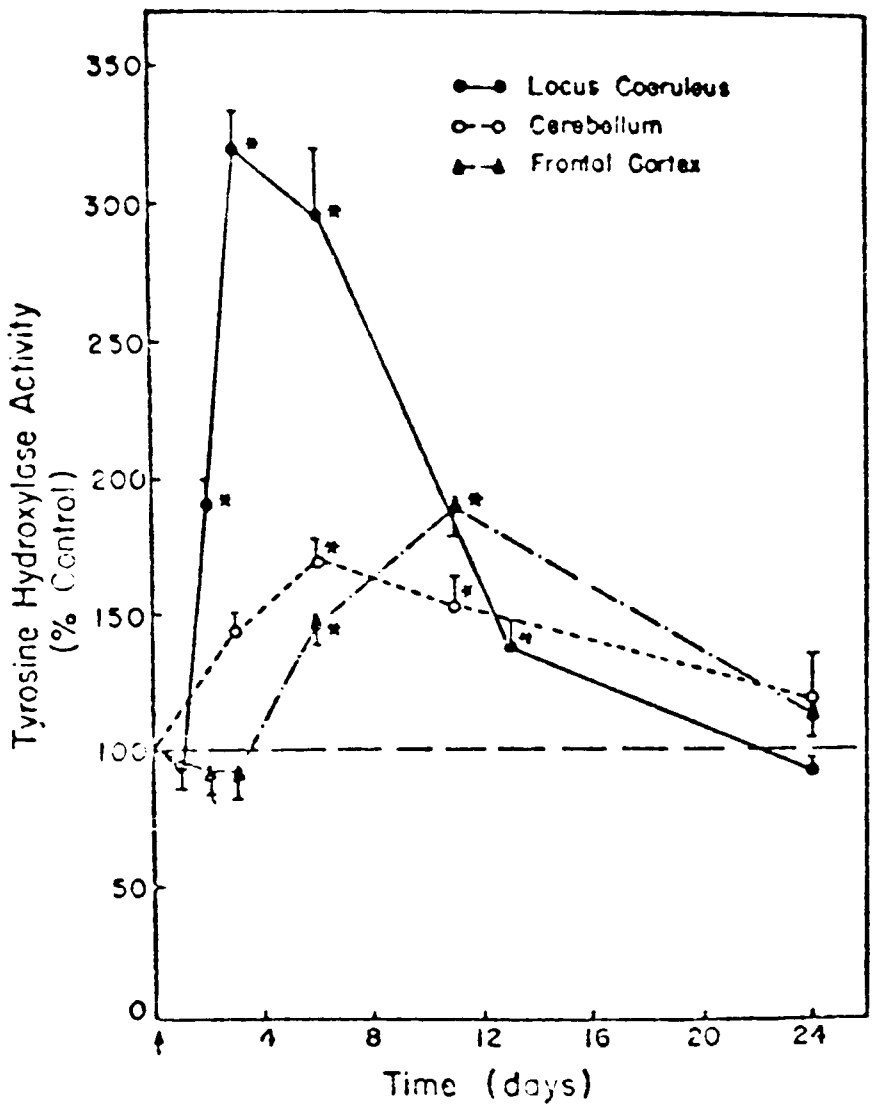
FIG. 3.8 A comparison of the time courses of the changes in tyrosine hydroxylase activity in different brain regions after a single injection of reserpine. (from Zigmond, 1979)



timecourse to the gradual decline in TH levels in the LC after induction. NA levels (and DBH activity and levels) will depend on release from terminals rather than on enzyme protein in cell bodies in the LC , and are therefore not a reliable indicator of the occurrence of induction. However, the work of Bacopoulos and Bhatnagar (1977) suggests that when brain regions are carefully selected and dissected according to the type of catecholamine innervation they receive, then total (saturated) TH activity is well correlated both with NA levels and with turnover (rate of depletion of NA after synthesis inhibition with alpha-methyl tyrosine), in terminal regions. In cell body regions of these untreated rats, TH levels are relatively high and NA turnover is low. Differences in NA turnover between regions, they suggest, are probably related to differences in the density of innervation and in steady state concentrations rather than to differences in firing rates. It would be useful to extend this work in brains of rats after induction of TH to see if these correlations still hold.

4. Most of the studies on induction have employed reserpine. But recent work from Pujol's laboratory (see McRae-Degueurce, 1980) has also shown evidence for increased enzyme quantity as well as increased activity. The precipitating stimulus in these studies is interference with the serotonergic innervation of the LC from the raphe nuclei. Induction of TH in the LC has been demonstrated after lesions of the serotoninergic raphe nuclei or after inhibition of the rate-limiting enzyme for 5HT synthesis with the inhibitor parachlorophenylalanine (PCPA). These studies will be discussed in section 9 of Chapter 5. The studies of Waymire (1977) on neuroblastoma cells in culture are also concerned with the mechanism of induction; but it is difficult to know whether the responses of these cells are related to their neoplastic properties rather than being normal responses, and they will not be further discussed here.

FIG. 3 9 A further comparison of changes in tyrosine hydroxylase activity in different brain regions after reserpine. (from Black, 1975)



5. If the amount of enzyme protein can be increased, it can also diminish. Reis and Joh (1978) refer to 'negative induction' in discussing the loss of TH protein found in the retrograde reaction after axonal lesions of NA fibres, and in the anterograde reaction in terminal regions. During the retrograde reaction an initial increase in activity and enzyme protein , due to build-up in the cell body because of failure of transport mechanisms, is followed by a decline to about 50% of control levels after 5 days. The activity and protein content remain at this low level for 2-3 weeks before slowly returning to normal. These changes in the cell body are reflected in remote terminals in regions not affected by lesions; this suggests that there is some control of enzyme protein in all projections of the neurone, via the cell body where enzyme protein is synthesised.

Other stimuli which cause a late activation of TH in brain are listed in Table H ; these do not all fulfil strict criteria for induction, since immunoprecipitation studies are lacking and since the TH activity has sometimes been measured under subsaturating conditions, rather than under saturating conditions . To draw a tentative conclusion, it seems that some of the stimuli which cause short term activation of NA neurons (see Table F) and are represented as 'stressors' can, after repeated administration, cause an increase in total TH activity in some brain regions, whether or not accompanied by an increase in NA levels. If no increase in Vmax is found after further investigation, these stimuli may be acting by increasing affinity of the enzyme for cofactors or substrate. In order to investigate the responses to these stimuli more thoroughly, the simultaneous assay of TH at saturating and subsaturating concentrations of tyrosine and cofactor, with kinetic analysis for both cell body and terminal brain regions, as well as immunoprecipitation studies, would be required, in addition to in vivo estimates of NA synthesis and release rate.

TABLE H
ACTIVATION OF TH (SOLUBLE) AFTER LONG TERM STRESS.

STRESS	TH ASSAY	REGION	RESULT (AUTHOR)
Electroconvulsive shock			
twice daily	100 µM tyrosine	whole brain	115% control
for 7 days	100 µM DMPH4	brainstem	124%
		cortex	120%
		hypothalamus	ns
		caudate	ns
			(Musacchio,1969)
once daily	100 µM tyrosine	LC	++
for 7 days	0.25 mM 6MPH4	hippocampus	ns
		hypothalamus	ns
		cortex	ns
			(Masserano,1979)
NA LEVELS after repeated electroconvulsive shock: increased (Kety,1967) but in unchanged in another study (Kato,1967).			
Immobilization			
3 hrs daily	200 µM tyrosine	hypothalamus	
for 5 days	6.4 mM DMPH4	(arcuate nucl)	130% control (Kobayashi,1975)
NA LEVELS after immobilization : increased according to Kvetnansky (1977) in individual hypothalamic nuclei, but not according to Lamprecht (1972).			
Isolation			
4 days	3 µM tyrosine	midbrain	++
16 days	or	midbrain	++
	100 µM tyrosine	striatum	+
	1.1 mM DMPH4		(Segal,1973)
NA LEVELS after chronic isolation: increased (Geller,1965).			
Cold			
4 days, 4°C	14.8 µM tyrosine	medulla obl	150% control
	0.72 mM DMPH4	hypothalamus	ns (Thoenen,1970)
15 days, 4°C	not reported	cortex	125% (Kennedy ,1979)
5 days	200 µM tyrosine	hypothalamus	ns
	6.4 mM DMPH4		(Kobayashi,1975)
NA LEVELS after chronic cold: increases found by Ingenito,(1968), Bhagat (1968) .			

FIG. 3.10 Kinetic analysis of tyrosine hydroxylase in the locus coeruleus from reserpine-induced, oxotremorine-activated, and control rat homogenates.
(from Reis et al, 1975)

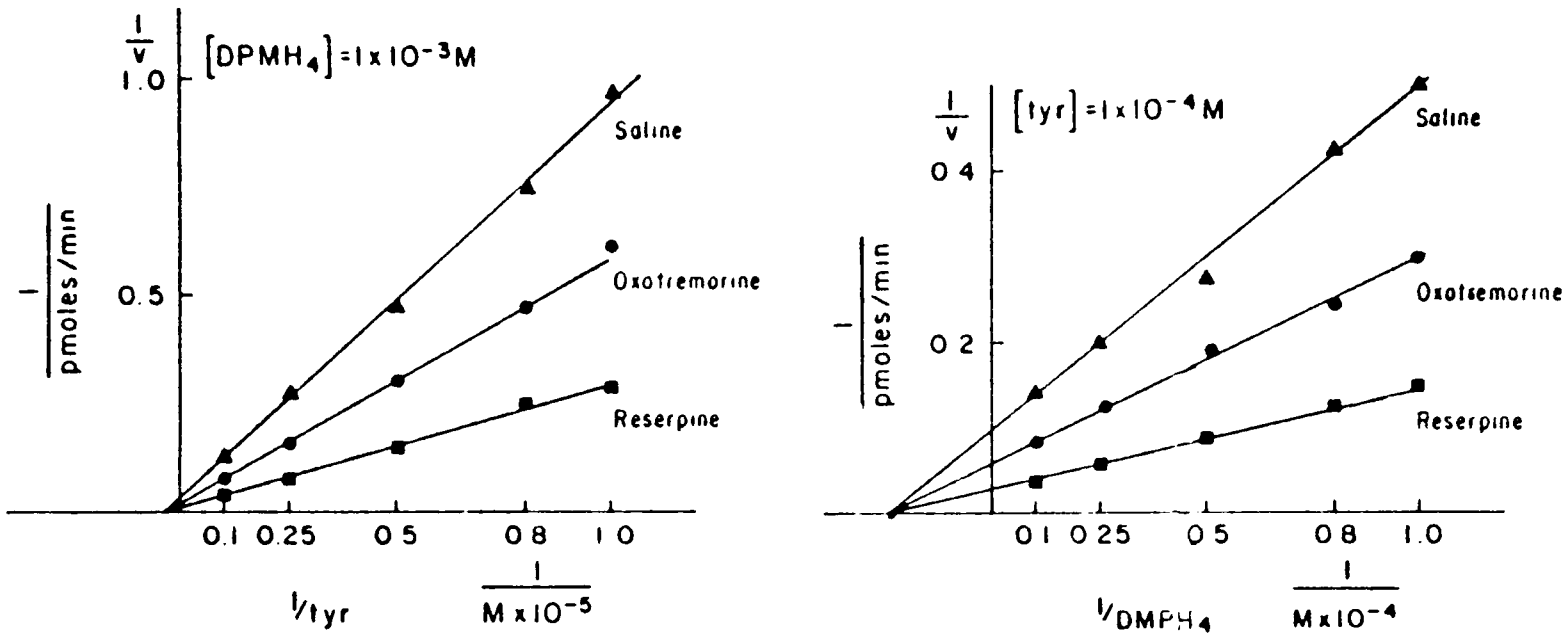
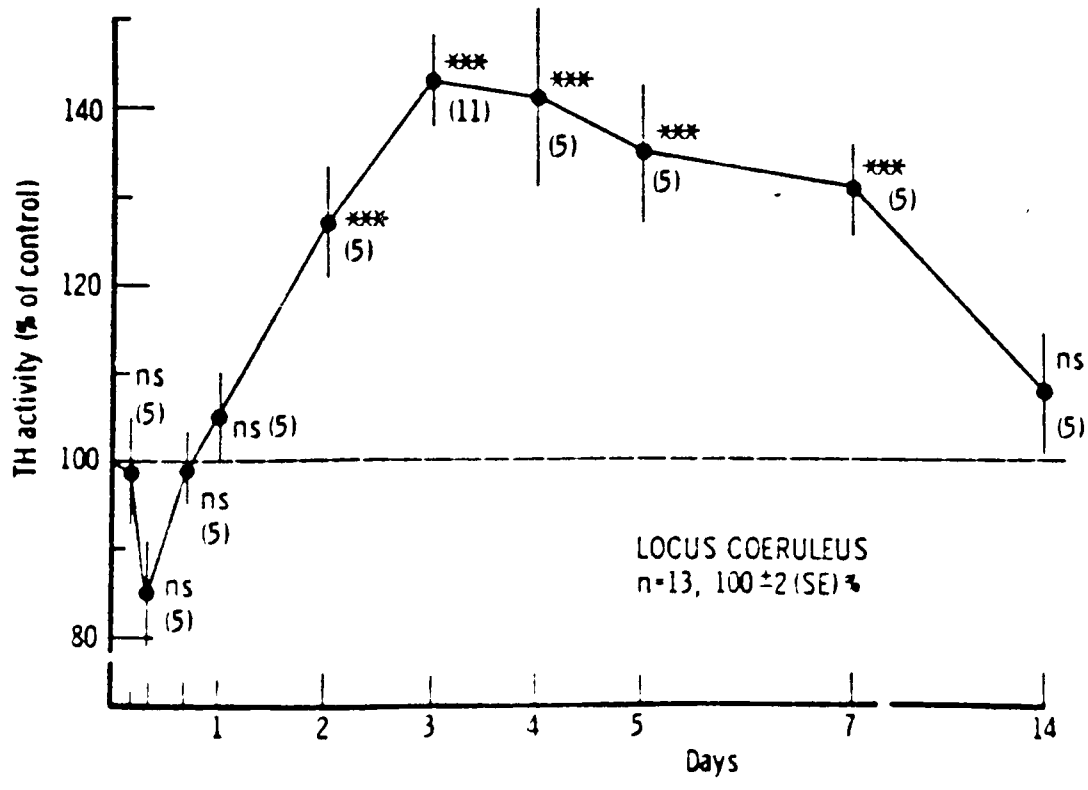


FIG. 3.11 Changes in activity of tyrosine hydroxylase in locus coeruleus at different times after administration of oxotremorine.
(from Lewander et al, 1975)

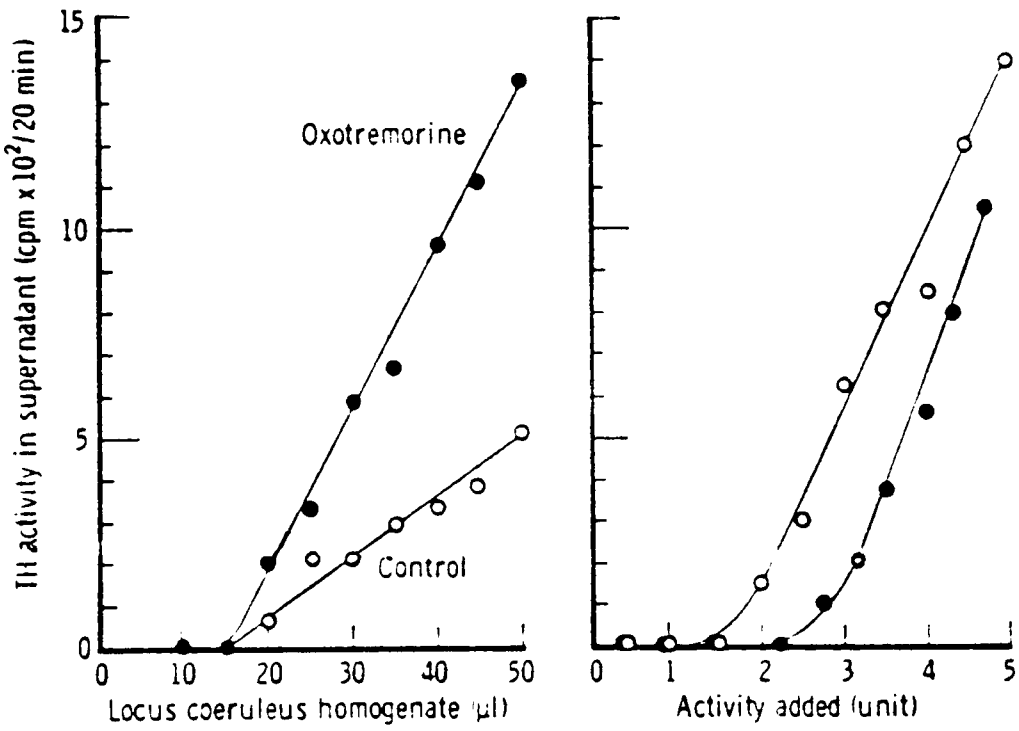


In the case of responses to oxotremorine , a muscarinic agonist (Reis et al,1975, 1977; Lewander et al, 1975, 1977) kinetic changes similar to those found with reserpine (increased Vmax, no change in Km) have been found; but there was no change in enzyme protein as measured by immunotitration in the LC (see FIGS.3.11 & 3.12). Nor was there an increase in DBH activity or quantityin the brain. This response has been termed 'delayed activation'. Since it may result from stimulation of cholinergic receptors on the LC cells or adjacent other cells, this may be a mechanism for trans-synaptic activation in vivo. Atropine administration soon after oxotremorine treatment can prevent the increase in TH activity. However, this mechanism clearly differs from that involving nicotinic receptors in the adrenal medulla, since the quantity of TH is not increased in the brain after oxotremorine.

There is no increase in TH activity in brain regions which contain noradrenergic terminals, such as hippocampus, hypothalamus and cortex after oxotremorine, even up to 12 days after treatment. The 'delayed activation' of oxotremorine is therefore confined to the noradrenergic cell bodies. Cholinergic stimulation is manifested behaviourally at 1 hour after oxotremorine injection; but the LC response is maximal at 72 hours after injection. The mechanism for enzyme activation is not clear.

Another index of function which may repay study in relation to long-term changes in noradrenergic function, and which has received little attention, is the hydroxylation rate of tyrosine in synaptosomes. These, as mentioned above (Chapter 2,section 7), retain many of the characteristics of intact NA neurons and can be conveniently studied in vitro. Do synaptosomes show increased synthesis of NA in terminal regions such as the hippocampus when induction of TH has taken place in cell bodies in the locus coeruleus?

FIG. 3.12 Immunotitration of tyrosine hydroxylase in locus coeruleus at 3 days after oxotremorine. (from Lewander et al, 1975)



Immunotitration of TH in LC at 3 days after oxotremorine. *Left panel*: Immunotitration with equal volumes of homogenate. Note overlapping equivalence points and different slopes. *Right panel*: Immunotitration with homogenate adjusted to have equal activity. Note curves are separate. These findings indicate activation and not accumulation of enzyme.

Boarder and Fillenz (1979) in this laboratory made a parallel study of soluble TH activity at saturating conditions (55 μM tyrosine, 1 mM BH4), and synaptosomal TH activity in tissues from the same brains , 7 days after a single injection of 10 mg/kg reserpine. Although there were significant increases in the solubilised TH activity in hypothalamus, cerebellum and hippocampus at this time, there was no increase in synaptosomal TH activity in cerebellum or hippocampus. By analogy with the data of Reis et al (1974,1975) it is likely that there was indeed an increase in enzyme protein at the terminals at this point; this result underlines the probability that transmitter synthesis is not wholly regulated by either end product inhibition , or by enzyme quantity. Among the other possible regulatory factors determining the expression of activity in synaptosomes , availability of reduced cofactor and the affinity of the enzyme for cofactor may be particularly important. Since the studies on reserpine have shown no change in affinity for cofactor (Km constant, Vmax increased) after induction, then if cofactor availability is limiting in synaptosomes ,even an increased amount of TH enzyme in the cytoplasm may not increase hydroxylation rate. Indeed the study of Besson et al (1973) showed a reduction of 3H-DA synthesis in cortex and striatum at the time of increased total TH activity, in slices, after reserpine.

Another study incorporating measures of TH activity both in synaptosomes and in soluble preparations was recently reported by Acheson et al (1980). These authors injected 6-hydroxydopamine (6OHDA) intraventricularly and measured recovery of function over 10 weeks subsequently. Initially and up to 5 days after the injection, NA content, uptake and TH activity in hippocampus were all markedly decreased owing to the neurotoxic effect of 6OHDA . By three weeks after the injection, TH activity at 75 μM tyrosine concentration showed some recovery (at first only in the LC,later in the hippocampus also), although affinity for cofactor was

unchanged. This was presumably due to synthesis of new enzyme in the few surviving cell bodies in the LC. Synaptosomal synthesis of NA also increased at this time, although content and uptake of NA were still reduced. Again, the interpretation was that the few surviving NA cells are responsible for the changes, and show compensatory functional activation once new TH is synthesised. However this activation is not brought about by changes in affinity of the enzyme for cofactor,as was the case for the short term activation by nerve stimulation in the experiments in Roth's group.

These two examples from a small literature on synaptosomal synthesis measurement in noradrenergic tissues serve to show that simultaneous measurement of different aspects of function is a productive means of investigating the complex changes taking place over long periods in noradrenergic neurones, as well as making for more satisfactory interpretations of short term changes. This is highlighted in studies which show a change in the immediate or short term response to a stimulus , after repeated presentation (or administration, in the case of a drug) . For example, Kennedy et al (1979), besides showing the increase in frontal cortex TH activity after 15 days of cold exposure, also found an increased elevation of DOPAC concentration after restraint for 15 minutes (29% increase before the chronic cold stress; 94% increase after cold stress) . It is not clear whether this increase is also found in noradrenergic metabolites; but if this were found to be associated with increased TH activity in synaptosomes , it would suggest that the increased enzyme which is available after induction can indeed be brought into play by short term activation during stress, although in the resting animal the presence of extra enzyme protein does not , per se, alter synthesis rate. Conversely, after reduction of total TH activity by 'negative induction', it might be possible to show a functional reduction in activity in response to acute stimuli even if resting transmitter levels were unchanged.

Thus the role of enzyme induction might be to increase the potential capacity for synthesis , to enable stores of NA to be maintained under stressful conditions which result in kinetic activation of the enzyme and increased synthesis and release of NA. In other words, reserpine, for example, amplifies the future capacity of the neurone to deal with similar stresses which tend to result in NA depletion (van Dongen, 1980). This formulation may require an expansion of the dual mechanism proposed by Fillenz (1977):

> 'Synthesis rate can either be adjusted in such a way that the releasable pool is kept constant in the face of fluctuating release rates; on the other hand changes in NA synthesis rate could produce changes in the size of the releasable pool and so changes in the amount of transmitter released per impulse. The first mechanism is an example of negative feedback designed to compensate for changes in release rate produced by some other mechanism;it is exemplified by the rapid regulation of TH activity. The second is a mechanism which does not compensate for changes in the rate of transmitter release but itself leads to changes in transmitter release. The new synthesis of TH would be such a mechanism.'

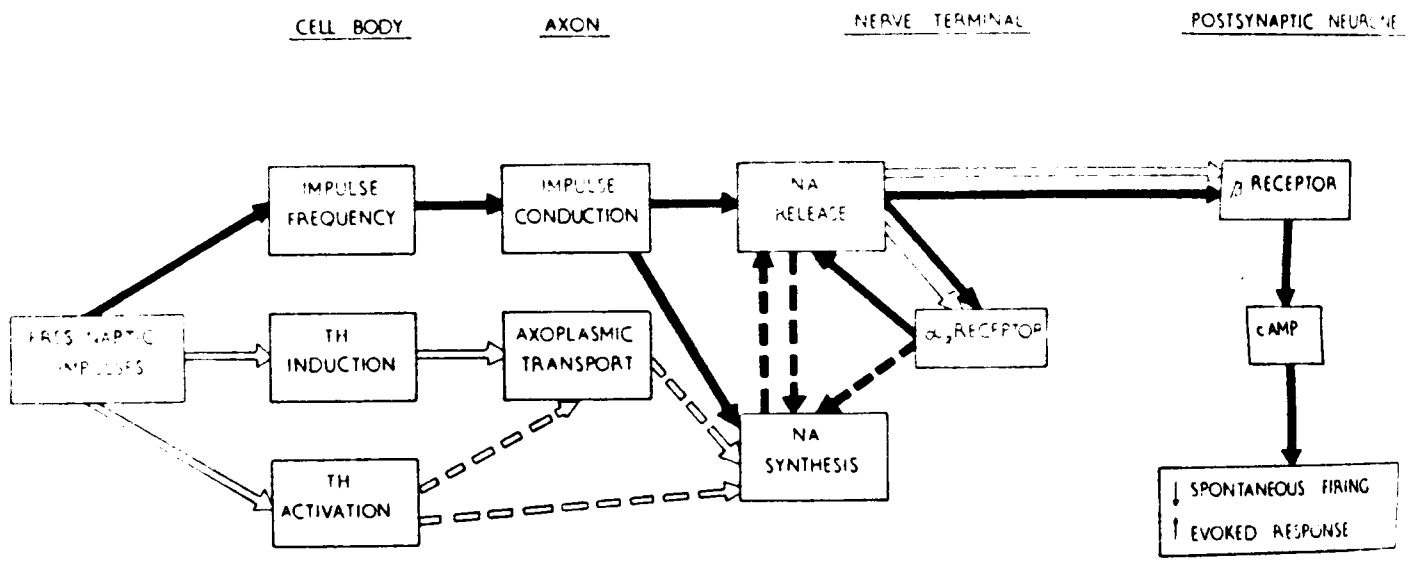
The experiments with repeated stimulation,exposure to behavioural training, and administration of drugs in this thesis are for the most part concerned with effects of long term stimulation on the resting synthesis rate and the total TH activity; further experiments using acute-on-chronic stimulation would be a valuable extension of this work.

13. REGULATION OF NORADRENALINE SYNTHESIS: MODELS AND MECHANISMS.

Noradrenaline neurons appear to be involved in many different processes and functions in the brain; but they are 'necessary and sufficient' to none of these functions. On the whole, the noradrenergic system is characterised by a great resilience to attack; despite near-depletion of noradrenaline levels and extreme sedation after a dose of reserpine, behavioural hyperactivity later ensues, followed by a return to normal catecholamine levels. Lesions of the NA neurons cause sprouting of residual axons and activation of synthesis in the residual neurons within a week or so. And, on the other hand, stimulation of LC neurons at 10 times their normal rate of firing results in activation of synthesis , but levels and synthesis rate are back to normal within half an hour of the end of the stimulation period.

After about 50 years of study, we still have very little idea of the role of NA neurons in the brain. Their adaptability is puzzling. It has been suggested that they may in fact impart adaptability to the animal; but this is a vague notion as yet. I want to summarise the adaptations that we know of with regard to the synthesis of noradrenaline, to look at the evidence from this chapter in terms of modes of regulation of tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of noradrenaline. It will become clear that there are many overlapping mechanisms at work: overlapping both in time , and in the extent to which the activity of the enzyme is constrained by the 'absolute requirements' discussed in Chapter 2 and the earlier sections of the present Chapter. Mandell (1978) has pointed out the 'redundancy' inherent in the control of NA synthesis , and some of the implied differences between the noradrenergic system and other monoamine systems. FIG.3.13 illustrates the interdependence of impulse traffic, noradrenaline synthesis and noradrenaline release and shows that we are far from a complete understanding of these relationships.

FIG. 3.13 The relationship of impulse traffic,noradrenaline synthesis and release, and post-synaptic receptor activity.
(from Fillenz, 1980)



Summary of regulatory mechanisms for noradrenaline synthesis

1. NA synthesis , or measures of TH in vivo or in slices, synaptosomes or homogenates , always represents a limited expression of the total catalytic activity of the enzyme after isolation and purification . There is always, as it were, a 'brake' on the activity of the enzyme.About 5% of the total activity is expressed in vivo in the resting animal. In order to explain this discrepancy between potential and observed activity in the organism, it has been postulated that the intraneuronal environment contains barriers , or compartments, which limit access to the necessary activating substances. Various models have been put forward : 'strategic pools' of transmitter effectively inhibiting synthesis were proposed by Weiner (1970); 'inhibitors'of the enzyme in vivo were proposed by Nagatsu (1964); Kuzcenski & Mandell (1972) postulated differences in enzyme activity dependent upon the physical state of the enzyme. Alternatively, the enzyme itself can be seen as incorporating its own inhibitory mechanism in a structure which has a catalytic sub-unit which can be closed off from activity by a regulatory sub-unit (Weiner, 1979). The kinetic studies performed with the isolated enzyme suggest that there is competition between cofactor and catecholamine end product for a binding site on the catalytic part of the molecule , even when the regulatory sub-unit is not blocking the site. As yet we do not know which of these models is likely to provide any further insight into the normal constraints on enzyme activity.

2. Rapid changes in activity of TH occur in enzyme preparations in vitro ; similar changes in vivo may be reflected in post-stimulus changes in response to sudden and drastic depolarizing stimuli. They occur at the same time as changes in NA synthesis rate in vivo,and are of short duration. Kinetic studies show that the activation usually takes the form of increased affinity for cofactor and reduced affinity for the inhibitory catecholamine end

product. There is no change in Vmax ; more of the existing potential synthetic activity is expressed in the standard conditions of the incubation. Other stimuli which produce these kinetic changes are calcium ions , and phosphorylating conditions, incorporating cAMP, ATP and magnesium ions. The model designed to account for these findings by Salzman and Roth (1979,1980b) specifies two stages, to account for their findings with calcium in vitro. First, an activator is formed as a result of the precipitating stimulus; this requires interaction with calcium ions intraneuronally. Then the activator combines with the enzyme to bring about the allosteric change. This process is independent of calcium and can therefore occur without an increase in release of transmitter. The model incorporates the finding of activation by phosphorylation by suggesting that the second stage involves phosphorylation of a protein, either TH itself or an 'activator protein ', by a cAMP-dependent protein kinase.

Weiner's data (Weiner et al,1977; Weiner, 1979) suggest that the enzyme exists in two forms; the difference lies in a ten-fold difference in affinity for cofactor. Changes in expressed activity of the enzyme are attributed to differences in the proportion of the total which is in the active form at any given time. Loss of activity during lengthy preincubations is explained by a tendency to revert to the inactive form in the absence of any stimulus.

Clearly there is considerable agreement between these models; each simply incorporates extra features to explain additional data. There has been little attempt to test the models independently in different laboratories, and there is still much unexplained data; such as the differences between NA and DA tyrosine hydroxylase in reaction to changes in calcium concentration . However, these models serve as the basis of a description of the capacity of NA neurons to maintain transmitter levels in the face of sudden ,mild

stimulation. When 'exhaustive stimulation' is used (Stone, 1973) and depletion of levels does occur, it seems that some feedback mechanism has been overtaxed; but it is not clear where the failure occurs.

3. Slow changes resulting in increased synthesis of TH itself have been described in the noradrenergic neurones in the periphery and in the CNS, but not in dopaminergic neurones. In the periphery there is evidence that the mechanism is trans-synaptic and dependent on cholinergic input; in the brain muscarinic cholinergic stimulation does not result in induction, and the source of the initiating stimulus for enzyme synthesis is unknown. Triggers such as neurotoxic lesions of the LC, lesions of the contralateral LC, reserpine, and lesions or synthesis inhibition in the serotoninergic neurons of the raphe nuclei have been found to cause the increase in enzyme protein which is the hallmark of induction. Here too there is a suggestion of a two-phase process, since induction in peripheral tissues can be interrupted by protein synthesis inhibitors or de-afferentation within 24 hours of the onset of the process, but subsequently proceeds regardless of these interfering stimuli . Costa and Guidotti (1974,1978) have produced a model for this process which describes the sequence of events necessary for new protein synthesis ,and this too involves interaction of enzyme protein during a critical period with a c-AMP-dependent protein kinase before transcription of the RNA. There is scope for incorporation of genetic differences in TH in this model, as discussed by Mandell (1978). Natali et al (1980) describe diurnal variations in protein content and TH activity in mice which may be under genetic control.

Induction of extra enzyme protein does not necessarily imply increased activity of the enzyme or increased NA synthesis; it does signify increased potential for synthesis. It may be thought of as contributing a protective back-up to the operation of the rapid adjustments to NA levels and neuronal firing rate; since the cell in which induction has occurred will be able

to respond to stimulation with a larger increase in synthesis rate of NA until cofactor or substrates become limiting. But often the precipitating stimulus and the increased requirements for synthesis will disappear before the extra enzyme is manufactured. The induction process, once triggered, has an inexorable quality and cannot be switched off. Negative induction of TH has only been discussed in relation to lesion-induced changes,so far (see section 12 of this Chapter) .

5. 'Delayed augmentation' is presumably brought about by the transport of increased quantities of newly synthesised TH to terminal regions as a result of TH induction in cell bodies. This mechanism will therefore increase the total TH activity in terminals, without necessarily affecting expressed activity, which will be under moment-to-moment regulation in the terminals. In vivo synthesis rate in terminals several days after reserpine needs to be measured; it would be interesting to know whether it is raised when behavioural hyperactivity is seen.

6. Long-lasting activation of TH without a change in protein quantity has also been demonstrated ; the response to oxotremorine ('delayed activation') comes into this category. Further kinetic studies are needed with the stresses which have been applied on a chronic basis, such as repeated cold stress, electroconvulsive shock and immobilization, to distinguish between kinetic activation with changes in affinity for cofactor,and changes in Vmax without changes in Km. Equally, in these stress experiments immunoprecipitation studies are needed to exclude the possibility of induction.

Long-lasting activation probably occurs in both noradrenergic and dopaminergic neurons; (Tissari, 1979; Kennedy et al, 1979) but may utilise different mechanisms. Some possibilities are: removal of an inhibitor; change in the enzyme molecule; or, possibly, an increase in the amount of reduced

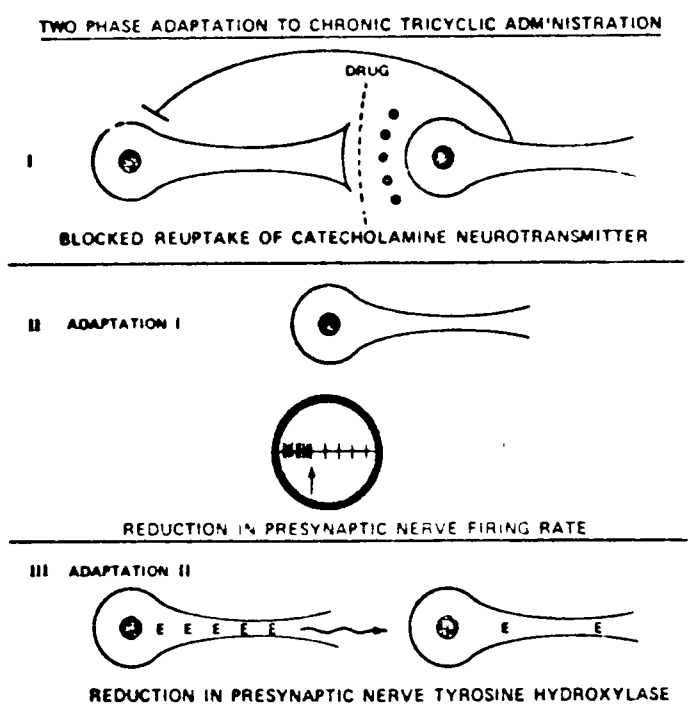
pterin cofactor. There is no definitive evidence to favour any of these possibilities. This type of activation may, in some circumstances, occur in addition to induction; for example, McRae-Degueurce (1980) shows that certain lesions of raphe nuclei in the midbrain result in activation of TH in the region of the LC which is not entirely accounted for by the induction mechanism. Long-lasting activation may be brought about by continued bombardment of the neurones, under conditions of stress ; but studies to exclude a 'critical period' mechanism (i.e. an early phase of latent activation, followed by a later phase of consolidation of the change) would be needed to prove this.

6. Besides the kinetic changes in the terminals which may provide a rapid adjustment of the activity of TH in response to neuronal events linked with depolarization and calcium entry to the terminals, there are other possible sources of in vivo regulation of TH to be considered, especially in the light of recent developments in our understanding of the regulation of NA release. Presynaptic alpha-2 adrenergic receptors on NA terminals in many regions innervated by the LC are capable of inhibiting both spontaneous and stimulus-evoked release of NA from in vitro and in vivo preparations (Langer, 1980). Similar receptors, known as autoreceptors, situated on the cell bodies of the locus coeruleus, have an inhibitory effect on firing rate in the LC, and are probably part of a recurrent collateral autoregulatory system for these neurons. Since these receptors influence both the NA content of terminals and the firing rate of the neurons, they may, directly or indirectly, influence synthesis rate (see Chapter 5,sections 3,4 and 5). There is an analogy here with dopaminergic neurons, which also have presynaptic receptors which appear to have an inhibitory effect on dopamine synthesis (Lovenberg,1978).

Recently it has been shown that alterations in the numbers or binding affinity of receptors on NA neurones can be brought about by various

treatments in vivo, and this allows for speculation as to possible long-term regulation of release, firing and synthesis rates under the influence of slow changes in receptor populations. Mandell et al (1972, 1975) have put forward a model for long-term regulation of TH and NA synthesis and the effects of receptor agonists and antagonists , based on the concept that the long term effects of drugs could be seen as adaptations of neuronal function in response to the early,acute effects of the drugs (FIG.3.14). This model incorporates changes in synthesis capacity, activation and receptor populations. It is relevant to the discussion above (p.95) of the role of 'adaptation ' in noradrenergic neuronal function, and whether this serves a homeostatic or protective , anticipatory role, or sets up a new steady-state. Like the other models described above, Mandell's is essentially a descriptive model ; but because it makes these links with other aspects of function besides synthesis of transmitter, it may have some value in generating predictions about drug action which can be tested. This is the subject of further discussion in Chapter 5.

FIG. 3.14 Metabolic adaptation to antidepressant drugs. (from Mandell et al, 1975)



CHAPTER 4

METHODS

1. RATS

2. PURIFICATION OF TYROSINE

3. TYROSINE HYDROXYLASE ASSAY

preparation of samples

preparation of the p_2 synaptosomal pellet

preparation of homogenates

incubation stage : synaptosomal assay

incubation stage : soluble assay

Dowex columns

scintillation counting

protein assay

calculation of results

range of blank and control values

4. NORADRENALINE ASSAYS

A.fluorimetric

B.radioenzyme method

5. STATISTICAL ANALYSIS OF RESULTS.

1. RATS.

All experiments were performed using male Sprague-Dawley rats from the Oxford Laboratory Animals Colonies (OLAC). The rats were usually kept 4-6 to a cage , with food pellets and water available ad libitum unless otherwise indicated. A 12 hours light - 12 hours dark cycle was maintained in the animal house. Rats were used in experiments when they weighed between 150 and 300 g, unless otherwise indicated.

2. PURIFICATION OF TYROSINE.

Tritiated tyrosine was purchased from the Radiochemical Centre at Amersham as L(3,5,^{3}H) tyrosine with specific activity 40-52 Ci/mmol according to batch. For use in the tyrosine hydroxylase assays described later in this Chapter, this radiolabelled tyrosine was diluted with 'cold' (unlabelled) tyrosine (Sigma) and purified by alumina and Dowex resin chromatography to eliminate catechols and other impurities, as described by Coyle (1972).

To prepare a batch of purified tyrosine for several assays, 1 ml of the Amersham solution containing 1 mCi of ^{3}H-tyrosine of high specific activity was mixed with 0.24 mg of 'cold' L-tyrosine and 4 ml sodium acetate buffer, 0.2 M, pH 8.6. After counting the radioactivity in an aliquot of this diluted mixture, it was run through a 3 cm column of activated alumina (BDH) . The effluent was titrated to pH 3-3.5 with 1 M HCl and run onto a 3 cm column of Dowex-50 resin (200-400 mesh, H+ form, Sigma) which had previously been prepared by washing with HCl and distilled water. With the tyrosine on the Dowex column, more water was run onto the column to wash away impurities (20-50 ml). The tyrosine was then eluted into a conical flask with 10 ml of 2 M HCl.

The acid eluate was evaporated to dryness under a stream of nitrogen gas, or freeze-dried. It was then taken up in ethanol and an aliquot was counted to determine recovery. After evaporating again, it was taken up in a volume of ethanol determined by the recovery, to yield a stock solution of labelled tyrosine with concentration approximately 100 μCi/ml. This was stored at 4°C. For each assay, an appropriate amount of stock solution was evaporated to dryness and dissolved in water for addition to incubation media.

Purification of the commercial 3H-tyrosine was found to be critical for improving the sensitivity of the method (personal communication, M.R.Boarder); this was an essential step for the measurement of TH activity in noradrenergic regions of the brain, which contain less enzyme activity than the adrenal medulla and the striatum, which were used in earlier studies of this enzyme.

3. TYROSINE HYDROXYLASE ASSAY METHODS :

The methods described in this section owe much to the work of Mick Boarder and Janet Stewart in this laboratory. The many different methods for assaying tyrosine hydroxylase activity have been reviewed in Chapter 2. It is clear that the enzyme activity recorded depends on many different features of the preparation and incubation, and different aspects have been treated as of paramount importance in different laboratories. In the studies reported here, the natural cofactor tetrahydrobiopterin has been used where possible; however, this substance is not commercially available, and DMPH4 was used as a substitute in many experiments.The concentration of tyrosine chosen was 20 μM (Km x 2) for the synaptosomal assay; for the solubilized enzyme, saturating tyrosine concentrations of 110-150 μM were used to estimate maximal activity. 'Subsaturating' concentrations of 40 μM were employed in addition, in some experiments (see APPENDIX). The time course of the

reaction, and the confirmation of Michaelis-Menten kinetics, were carried out with the solubilized enzyme from striatal tissue (see FIG.4.1, personal communication, Boarder and Stewart) in this laboratory. The choice of mercaptoethanol and ascorbate as anti-oxidants, and catalase for peroxidase activity, were made on the basis of experimental work with several different assay preparations. Ferrous sulphate was not found to enhance activity in this preparation (experiments using DMPH4) and was therefore not included. The reaction was linear for about 30 minutes in the synaptosomal assay, and 20 minutes in the solubilized assay.

PREPARATION OF SAMPLES

Rats were killed within 30 seconds of removal from cages by a blow to the head and immediate decapitation. The brain was removed rapidly (within 1-2 minutes) and brain regions were dissected on an ice-cooled glass plate. Starting with the dorsal surface of the brain uppermost, the corpus callosum was divided and the hemispheres reflected outwards to expose the hippocampus on either side. The hippocampi were separated from fornix and septum and again reflected outwards,dividing remaining fibres where necessary with fine forceps. The corpus striatum was easily visualised on the inner surface of the hemisphere and could be removed by fine dissection. The locus coeruleus was dissected after removal of the entire cerebellum by division of the peduncles; when the brainstem region caudal to the inferior colliculi was well illuminated, the small elevations either side of the midline overlying the locus coeruleus could be dissected out by forceps after cuts had been made both rostral and caudal to the locus itself. In order to dissect the hypothalamus, the brain was turned over,again reflecting the hemispheres outwards. The hypothalamus was clearly visible ; cuts at its rostral and caudal margins gave a slice which was laid caudal side downwards. Three further

FIG. 4.1 Plot of tyrosine hydroxylase activity (in cpm) in partially solubilised preparation of rat striatum with increasing concentration of substrate. (Boarder and Stewart, unpublished data)

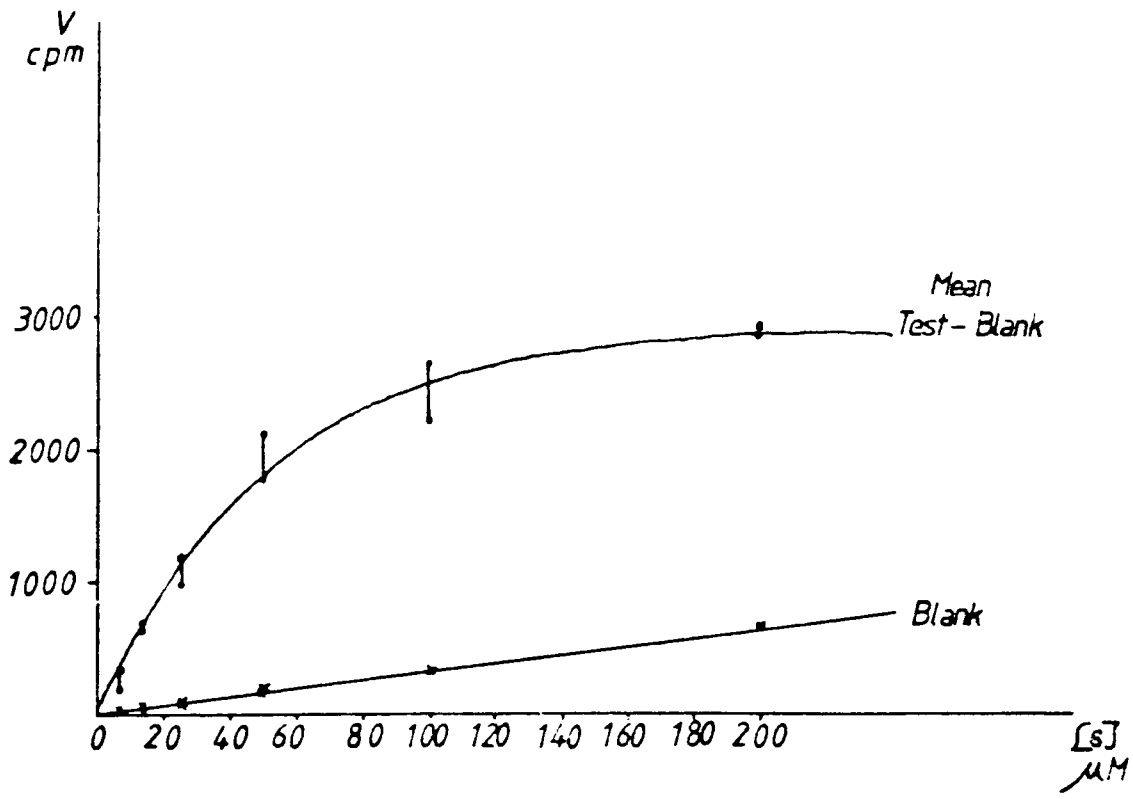
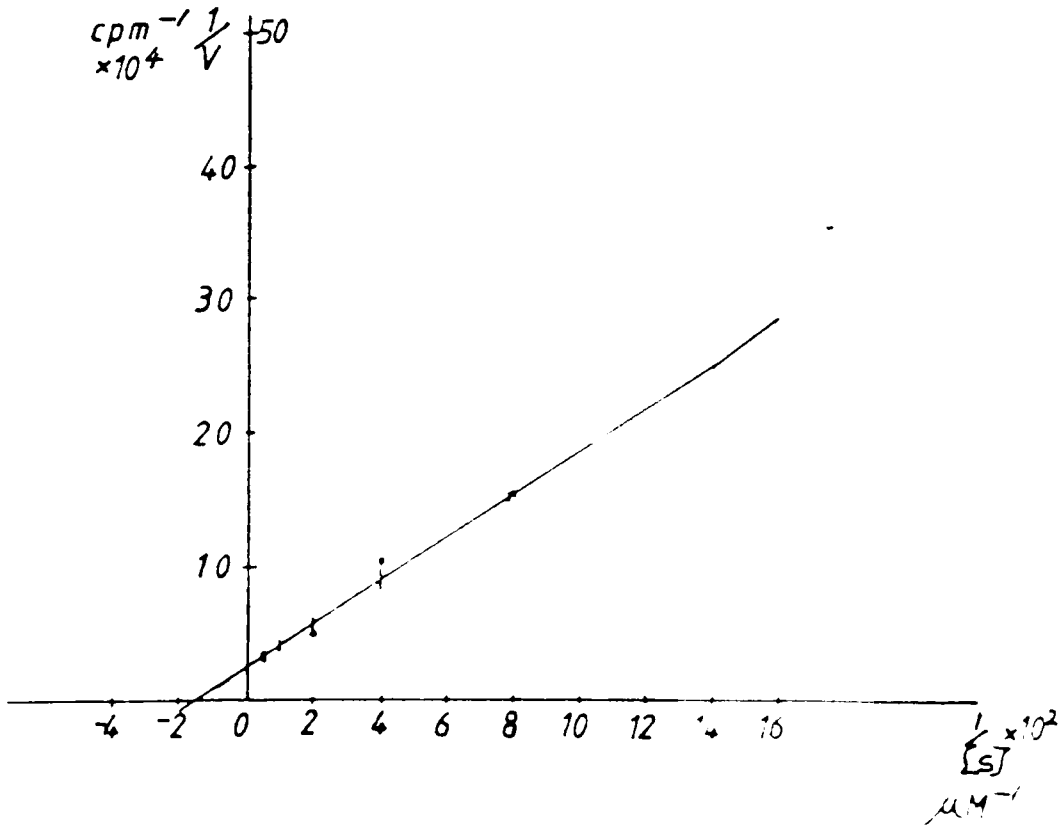


FIG 4.2 Lineweaver-Burk double reciprocal plot of data from FIG. 4.1.
(Boarder and Stewart, unpublished data)



cuts separated the lateral margins and the dorsal border,defined by the white matter of the bilateral medial forebrain bundles.

The locus coeruleus samples were put in a 1-ml glass homogenizer with (usually) 150 µl $NaPO_4$ buffer , 5mM, pH 6.5, with 0.2% Triton X-100 (w/v).

Other regions were placed in 15 ml centrifuge tubes containing 2 ml 0.32M sucrose. All samples were kept at 4^oC throughout preparation and assay except during incubation.

PREPARATION OF THE P_2 SYNAPTOSOMAL PELLET

All regions except the locus coeruleus (LC) were individually homogenised in a 20 ml glass homogeniser with a teflon pestle at low speed ; approximately 12 strokes were required to obtain a uniform milky suspension. Soon after homogenising, the samples were centrifuged at $4^{o}C$ at 1900 rpm (1000 x g) in a Mistral centrifuge for 10 minutes to sediment the cell debris and myelin. The supernatant from each sample was decanted into a plastic ultracentrifuge tube and spun at $4^{o}C$ at 15,000 rpm (16,000 x g) for 20 minutes in an MSE Superspeed 65 ultracentrifuge. The supernatant S_2 was discarded ; the pellet P_2 was the crude synaptosomal fraction containing synaptosomes,ie pinched-off nerve terminals,and mitochondria. The P_2 was resuspended in 100-200 µl of incubation medium by vortexing,and was kept on ice until incubation took place (see Karobath, 1971; and Boarder and Fillenz, 1978).

PREPARATION OF HOMOGENATES

This was the method used to prepare LC samples; in some experiments it was also used for other brain regions. The buffer used was 5 mM $NaPO_4$, pH6.5, plus 0.2% Triton X-100 . Brain regions were dissected on ice and either put in a appropriate volume of buffer in a homogeniser immediately, or frozen on dry ice and stored, wrapped in foil, until homogenised at a later date. Homogenising was carried out in glass homogenisers with ground glass pestles . Homogenising with Triton X-100 , a surface-acting detergent, has the effect of breaking down nerve cell membranes and thus ensuring that tyrosine hydroxylase (TH) is brought into solution.

INCUBATION STAGE : SYNAPTOSOMAL TYROSINE HYDROXYLASE

After resuspending the synaptosomal pellet in incubation medium (A or B, see below), 45 µl duplicate aliquots were taken into small glass test tubes on ice. A quantity of the purified tritiated tyrosine solution in alcohol (100 µCi/ml) was dried off under nitrogen and resuspended in distilled water in two-thirds of the original volume of alcohol; the volume had to be adequate to allow 5 µl for each assay tube, including blanks, and 40 µl extra (for standards, and wastage during pipetting). Addition of a 5 µl aliquot of this solution to a 45 µl aliquot of P_2 resuspension gave a final concentration of 20 µM tyrosine in the incubation ; this is approximately twice the Km of tyrosine hydroxylase with respect to substrate in this preparation.

Blanks were prepared in two ways. Tissue-free blanks, used in all experiments, consisted of 45 µl incubation medium + 5 µl 3H-tyrosine. Tissue blanks consisted of an aliquot of pooled remaining tissue resuspension to which 100 µl trichloroacetic acid (TCA) 5% w/v was added before the 5 µl aliquot of 3H-tyrosine.

Sample and blank tubes were incubated simultaneously in a water-bath at 37°C for 20 minutes. The reaction was stopped by plunging the tubes into ice and adding 100 µl TCA 5% to all tubes except tissue blanks. This produced a protein precipitate which was left to settle for 20-30 minutes on ice.

INCUBATION MEDIUM A : for synaptosomal TH assay at pH 6.2. (Boarder and Fillenz,1978). This was made up as follows: 50 ml $NaPO_4$ buffer 30mM, pH 6.2 containing NaCl 250 mM, KCl 10 mM, $CaCl_2$ 2 mM and $MgCl_2$ 2 mM ; mixed with 50 ml glucose (20mM) in distilled water and 18 mg ascorbate (1 mM ascorbate in incubation medium). The incubation medium was freshly prepared from the component solutions for each experiment, and saturated with oxygen , which was bubbled through the solution for 20 minutes immediately before resuspension of the synaptosomal pellets.

INCUBATION MEDIUM B : for synaptosomal TH assay at pH 7.4 (Fillenz, Graham-Jones and Gray,1979). This incubation medium was made up in 50 mM Tris-HCl buffer at pH 7.4 , instead of phosphate buffer. 50 ml of the Tris-HCl buffer containing NaCl, KCl, $CaCl_2$ and $MgCl_2$ (in the same concentrations as in A) were mixed with 50 ml glucose (20 mM) and 18 mg ascorbate ; the solution was oxygenated for 20 minutes before pellet resuspension.

INCUBATION STAGE : SOLUBLE TYROSINE HYDROXYLASE.

SAMPLE PREPARATION: this assay requires solubilization of the enzyme TH as a preliminary step. In some experiments the brain regions were homogenised in a buffer containing Triton X-100 (Segal and Kuczenski, 1974; Lewander, 1977), and in these the homogenate could be used for assay either fresh or after freezing. The LC was always prepared in this way. However, we were also able to use the synaptosomal preparation to obtain solubilized synaptosomal TH. Usually the resuspended P_2 remaining from a synaptosomal assay was stored in plastic tubes at -18°C and assayed on a subsequent occasion. This freezing process will effectively lyse membranes; and in order to solubilize the enzyme still further, equal volumes (approx 50 µl) of resuspension and buffer ($NaPO_4$, 5 mM, pH 6.5 or pH 7.3 , plus Triton X-100, 0.4%) were vortexed together. Duplicate aliquots of 35 µl were used in the soluble TH assay.

INCUBATION MIXTURES : the solubilized enzyme from brain regions has an absolute requirement for pteridine cofactor ; in these assays, mercaptoethanol and catalase were also used to optimise the conditions for the assay (Nagatsu, 1964b; Lloyd and Kaufman, 1974; Boarder and Fillenz, 1979). The pH was also varied in some experiments,as was the 3H-tyrosine concentration. The various resulting 'cocktails' are detailed below. The total

incubation volume for each sample was kept constant at 50 ul, which was composed of 35 µl sample, 5 µl of a catalase-and-buffer solution, 5 µl of a solution of cofactor and mercapto-ethanol, and 5 µl of 3H-tyrosine suspended in distilled water (DW). Blanks were prepared from buffer plus additives as in the relevant mixture (tissue-free), or from pooled sample with TCA 5% (100 µl) added before 3H-tyrosine, with other additives as in the relevant mixture (tissue blanks).

SATURATING MIXTURE WITH TETRAHYDROBIOPTERIN:

110 µM tyrosine; BH4 1.27 mM; pH6.2

35 µl sample

plus 5 µl from solution containing 50 µl catalase (Boehringer-Mannheim) + 150 µl DW + 200 µl NaAcetate buffer 1M,pH 5.8.

plus 5 µl from solution containing 1.2 mg tetrahydrobiopterin in 250 µl of 2 mM mercapto-ethanol.

plus 5 µl 3H-tyrosine solution ; (if W µl is total volume required for pipetting, 8 x W µl from stock solution of ^{3}H-tyrosine is dried off and resuspended in W µl of DW to give final concentration of 110 µM tyrosine in incubation).

SATURATING MIXTURE WITH DMPH4 :

150 µM tyrosine ; 2 mM DMPH4; pH6.2

35 µl sample

plus 5 µl from solution containing 50 µl catalase + 150 µl DW + 200 µl Na Acetate buffer 1 M, pH 5.8.

plus 5 µl from solution containing 1.92 mg DMPH4 (Sigma)per 400 µl DW + 10 µl mercaptoethanol (2 mM DMPH4 in final incubation.)

plus 5 µl 3H-tyrosine (if W is volume required for pipetting, 11.17 x W is taken from stock solution and dried off; gives 150 µM tyrosine in final incubation.)

SATURATING MIXTURE AT HIGH pH :

150 µM tyrosine; 2 mM DMPH4 ; pH6.5

35 µl sample

plus 5 µl from solution containing catalase 50 µl + DW 223 µl + 127 µl Na Acetate buffer 1 M, pH 5.8

plus other solutions as in previous mixture.

SUBSATURATING MIXTURE AT HIGH pH:

40 µM tyrosine; 0.5 mM DMPH4; pH7.2

35 µl sample prepared with $NaPO_4$ 5mM, pH 7.3 + 0.4% Triton X 100.

plus 5 µl from solution containing 50 µl catalase + 350 µl $NaPO_4$ buffer 0.5 M, pH 7.0.

plus 5 µl from solution containing DMPH4 1.2 mg per 975 µl DW + 25 µl mercaptoethanol (DMPH4 0.5 mM in final incubation).

plus 5 µl from 3H-tyrosine suspension (if Y µl is required volume for pipetting, take $\frac{1}{3}$ x 3 µl from stock tyrosine solution, dry off and resuspend in Y µl of distilled water;this gives a final concentration of 40 µM tyrosine.)

Incubation of the soluble enzyme preparation was for 10 minutes at 37°C. The reaction was stopped by plunging the tubes into ice and adding TCA 5% (100 µl) to all tubes except tissue blanks.

Following the end of the incubation stage, the remaining steps of the assay were identical for synaptosomal and all solubilized preparations of the enzyme TH.

DOWEX COLUMNS FOR TRAPPING OF TYROSINE AND METABOLITES.

Columns were prepared using Pasteur pipettes shortened at both ends. Glass beads were inserted as stoppers and the pipettes were inserted into specially-constructed racks, thin end downwards. A double column of Dowex resin (Sigma) was used to trap both tyrosine and acid metabolites in the reaction mixture (Boarder and Fillenz, 1979). The lower layer was of Dowex-1 x 8 (200-400 mesh, Cl^- form converted to OH^- form before use) and measured approximately 150 µl. The upper layer was Dowex 50 x 4 (200-400 mesh); approximately 300 µl was used, to give a total column volume of 450 µl.

The columns were left to run dry of excess water for 30 mins; meanwhile the acid precipitates from the reaction were centrifuged at 1000 rpm for 10 minutes to spin down the precipitated protein . The tubes were put back on ice and 100 µl aliquots of supernatant were carefully run onto the columns. After the columns had run 'dry' again (20-30 minutes), scintillation vials were placed beneath the columns and the tips of the columns were wiped to remove excess moisture. The next step was to elute the 3H-H_2O formed (molecule for molecule with 3H-DOPA) in the reaction. This was done by running 500-600 µl of DW onto the column and collecting the effluent (1-2 hours).

SCINTILLATION COUNTING

Radioactivity in the effluents collected from samples and blanks was measured in an ISOCAP Nuclear Chicago scintillation counter. Ethanol (absolute,4 ml) and Toluene-PBD scintillation fluid 8 ml (2500ml for 10 g PBD) were added to the eluates in the scintillation vials,and the samples were

counted overnight for 10 minutes each,measuring tritium in dpm (efficiency approximately 30%).

PROTEIN ASSAY

Protein assays were performed on all samples,whether or not the samples were weighed at the time of dissection. After taking off the supernatant from the reaction mixture, the protein precipitates left in the tubes were dissolved overnight in 0.5-2.0 ml NaOH, 0.5 M. Protein assay was performed by the Lowry method (Lowry et al,1951) on 200 µl aliquots of these solutions.

CALCULATION OF RESULTS FOR TH ASSAY

Tyrosine hydroxylase activity was measured as fmol 3H-H_2O produced per minute of incubation and per mg of protein in the assay sample.

This was calculated for each sample as follows:

TH = (sample cpm - blank cpm) x k/protein(mg) fmol/min/mg protein

where k is a constant determined by counter efficiency, specific radioactivity of 3H-tyrosine supplied by Amersham Radiochemicals,incubation time, volume of reaction mixture and column aliquot; k was generally approximately 0.304 for synaptosomal assays, and 0.608 for solubilized assays. Protein varied according to region used and dilution of the P_2 pellet on resuspension.

RANGE OF BLANK AND CONTROL VALUES.

There was considerable variation in control TH activity in all brain regions between experiments. This is illustrated in the following examples from hippocampal results.

SYNAPTOSOMAL TH.

In a series of 19 experiments with 3-4 tissue-free blanks in each experiment, the mean blank value was 222 counts per minute (cpm). One way analysis of variance showed that there was significant variation between experiments; the main effect of REPLICATIONS ($F=55.1$, df 18/50, $p < 0.001$) was highly significant.

The control values for hippocampal TH in this series of experiments gave a mean of 150 fmol/min/mg P_2 protein. These values also gave a significant REPLICATIONS effect on one way ANOVA ($F=4.53$, df 18/80, $p < 0.001$).Mean blank and mean control TH activity were not significantly correlated (Pearson's $r= 0.16$, df 17).

SATURATING TH.

a. When homogenates were used,BH4 was used as the cofactor and the results were expressed in terms of fmol/min/mg wet weight. Tissue-free blanks ranged between 700 and 1500 cpm, with a mean of 1033 cpm; the mean control TH activity in a series of 9 experiments was 138 fmol/min/mg wet weight. No correlation between mean blank and mean control activity was found ($r=0.002$, df 7).

b. When the P_2 pellet was assayed with BH4 as cofactor, the mean blank in a series of 7 experiments fell in the range 140-600. The mean control TH activity for this series was 1686 fmol/min/mg P_2 protein, and there was no

correlation between mean blank and mean control TH activity for these experiments (r=0.001, df 5).

c. When the saturated TH assay was performed as in b. above but using DMPH4 as cofactor, the mean blank from a series of 13 experiments was 936 cpm, with a range of 360 - 1875 cpm. Control TH mean for this series was 1095 fmol/min/mg P_2 protein, and again there was no significant correlation between mean blank and mean control TH activity (r=0.3, df 11).

SUBSATURATING TH ASSAY

When solubilised enzyme from the P_2 pellet was assayed at subsaturating concentrations of cofactor(DMPH4) and tyrosine, the range for mean blank values in a series of 7 experiments was 320-924cpm, with a mean of 622 cpm. The control TH activity in this series was 634 fmol/min/mg P_2 protein,and there was no significant correlation between mean blanks and mean control TH activity (r=-0.176).

Thus although both blanks and control TH activities vary between replications in all the assays used, the lack of correlation demonstrates that the variation in control values is not attributable to the blank variation. Other factors which might have contributed to the shifting baseline of control TH activity were the following:

factors associated with subjects, e.g. age of rats, diurnal variation in TH activity, strain differences;

factors associated with storage of samples and reagents, e.g. storage time of frozen tissues in buffer or triton; batch life of purified labelled tyrosine, cofactors, catalase suspension;

differences in technique between assays , eg length of Dowex columns, time taken to kill different numbers of rats and prepare synaptosomes;

lastly, seasonal variations could have affected the rats themselves, or other aspects of the assays (Natali et al, 1980).

However, none of these factors has been found to account satisfactorily for the variation in either blank or control means between experiments. The techniques and conditions for animal housing and assay were kept as constant as possible and no regular variation associated with known changes in the above factors was demonstrated in the results despite periodic review.

4. NORADRENALINE ASSAYS

In some experiments , described in Chapter 5, sections 7 and 8, noradrenaline assays were performed in addition to tyrosine hydroxylase assays. Two methods were used for the determination of noradrenaline in homogenates of brain regions, or in the P_2 resuspension; a fluorimetric method and a radioenzymic method.

A. FLUORIMETRIC METHOD FOR ASSAY OF NORADRENALINE .

This method was based on that of Holman et al (1977). Brain regions were homogenised after weighing in 1 ml of sodium phosphate buffer, 5 mM, pH 6.5, with 0.2 % triton, as for tyrosine hydroxylase assays. Approximately 0.8 ml was used for the noradrenaline assay. Samples were vortexed with 0.5 ml perchloric acid 1 M, and 35 µl of 10 % EDTA solution, and then centrifuged. The supernatant was adjusted to pH 5.5-6.0 with 2M potassium hydroxide.

Biorex 70 resin, prepared by the method of Barchas et al(1972), was pipetted into glass columns to give 3 cm x 0.5 cm diameter columns. These were washed with 0.1 % EDTA before running on the noradrenaline-containing supernatants. Further columns were used as 'internal standards' prepared from stock solutions of noradrenaline (2.5 - 250 ng/ml) adjusted to pH 5.5-6.0 as above. The columns were then washed with 1 ml sodium phosphate buffer ,20 mM, pH 6.5, containing EDTA 0.2 %, and then with distilled water. 1 M HCl (300 µl) was passed over the column and this effluent was discarded; the noradrenaline was then eluted with a further 400 µl of HCl, 1 M.

The 400 µl eluates were then assayed by the trihydroxy-indole method . Each sample was divided into two duplicates of 200 µl each. Sodium phosphate buffer, 0.5 M, pH 7.0 was added (200 µl) and pH adjusted to 6.5-7.0 with potassium carbonate 1 M. 20 µl potassium ferricyanide 0.25 % was added to each sample to start the reaction, and 4 minutes later 200 µl of alkaline ascorbate in sodium hydroxide (10 M) was added. The samples were then diluted with 600 µl distilled water, vortexed, and readings were taken on a Minko Bowman fluorimeter within 15 minutes at excitation wavelength 410, emission 510. 'External standards' were used in the assay for estimation of recovery from the columns.

Results were expressed as ng noradrenaline per g of tissue wet weight.

B. RADIOENZYMIC METHOD FOR ASSAY OF NORADRENALINE.

This method was used in some experiments as an alternative to the fluorimetric method. Homogenates in sodium phosphate buffer were prepared as in A. above. Samples of 70-90 µl were vortexed with 5 µl of 2M perchloric acid , and centrifuged. The supernatants were used for noradrenaline assay. Duplicate aliquots of 10 µl were assayed, along with standards made up from stock solutions of noradrenaline (0.2 - 1.0 ng/10 µl); in addition to these standards, another set of samples was assayed after the addition of known amounts of NA. The incubation mix was made up as in the method of Coyle and Henry (1973), and contained Tris base (final concentration 40 nmoles/tube), EGTA, pargyline, dithiothreitol, COMT enzyme prepared according to Coyle and Henry (1973 b), and ^{3}H-S-adenosyl-methionine.

15 µl of incubation mix was added to all samples , standards, and perchloric acid blanks to give a final incubation volume of 35 µl, and the tubes were incubated at 37°C for 45 minutes. The reaction was then stopped , on ice, by the addition of 100 µl of a saturated solution of borate containing normetanephrine as carrier.

Extraction of NA from the resulting mixture was carried out in 3 stages; toluene-isoamyl alcohol (T-IAA, 3:2 mixture) was added to each tube and extraction of the O-methylated products was performed by vortexing and centrifugation; the organic layer was removed by automatic pipette and extracted again into acid. Extraction was performed a third time with T-IAA and acid, to wash the acid layer. The acid layer was then used to perform the cleavage reaction with sodium peri-iodate and sodium hydroxide. This reaction was terminated by adding sodium metabisulphite and HCl, and extracting into toluene. The toluene phase was then pipetted into a scintillation vial and counted with PBD in toluene (0.4 %).

Results were expressed as ng NA per g wet weight of tissue.

4. STATISTICAL ANALYSIS OF RESULTS.

Each of the experiments described in Chapter 5 involved the assay of brain regions from 10 - 30 rats, all of which were killed on the same day. In each experiment there was a control group and at least one experimental group. In some experiments rats from control and experimental groups were matched by weight, and killed in pairs.

Results of TH assays were expressed in fmol of tritiated water formed , per minute of incubation, per mg of protein in the P_2 resuspension. Group means are given in the results tables in Chapter 5 along with the standard error of the mean (mean $\pm$ S.E.M.) and the number of subjects in each group (e.g., n=5). Analysis of results was performed by analysis of variance (ANOVA) by treatment condition, whenever a single experiment contained more than two groups; and by two way ANOVA, when additional data were obtained either from additional brain regions of the same rats, and/or by replicating the experiment according to the same design in a different set of rats. Variance ratios (F) are reported as follows: ($F=10.82$, df 1/16, $p < 0.01$) for a variance ration of 10.82 with one degree of freedom in the numerator and 16 degrees of freedom in the denominator.

Two way ANOVA was performed using the GENSTAT programme on an ICL 2980 computer. When the data showed a marked deviation from the normal distribution, as shown by widely differing arithmetic mean and median in any experiment, a $\log_{10}$ transform of the data was generally found to achieve normality.

Further analysis of results was carried out by t tests when ANOVA demonstrated significant main effects or interactions in the data; these t tests utilised the error variance in the ANOVA, and are reported as two tailed tests

in all cases. Probability levels of less than 5% are reported as statistically significant.

In experiments using matched pairs of animals, paired comparisons were made by two-tailed t tests for matched pairs. These are reported as 'paired' t tests and again two tailed probabilities of less than 5% are reported as statistically significant.

Relationships between TH activity in different brain regions, and between different measures in the same animal were explored using Pearson's product-moment linear correlation coefficient and/or analysis of covariance where appropriate.

CHAPTER 5

EXPERIMENTS

The experiments described in this thesis fall into 9 groups, each of which is concerned with a different experimental paradigm in which rats were exposed to some stimulus which was expected to affect noradrenergic activity in the brain. In each group the basic measure has been tyrosine hydroxylase activity as measured by one or more of the methods outlined in Chapter 4. TH activity in synaptosomes was used as an index of noradrenergic activity immediately ante-mortem. Solubilised TH activity under saturating conditions provided an index of total enzyme activity, and subsaturating TH assay was used for the detection of changes in affinity for cofactor or substrate. Different brain regions were assayed: noradrenergic terminal regions, such as hippocampus, hypothalamus , cerebellum, and cortex, as well as the locus coeruleus which contains noradrenergic cell bodies.

An attempt was made, in each paradigm, to predict the direction of change for TH assays in accordance with current hypotheses about noradrenergic function , with particular regard to NA synthesis , as discussed in the earlier chapters of this thesis.

On the next page is a list of experimental paradigms. In subsequent sections (1 - 9) of this chapter, each paradigm is separately introduced and the experiments performed are reported . The results are discussed briefly at the end of each section.

CHAPTER 5

LIST OF EXPERIMENTS.

SECTION 1. SINGLE FOOTSHOCK.

To determine whether the synaptosomal TH method was sensitive enough to register the activation produced by a mild but noxious stimulus, TH activity in synaptosomes of NA terminal regions was measured after a single exposure to electrical footshock. Saturated TH activity was measured in the same animals, for assessment of the proportion of total enzyme activity expressed in the control and shocked animals.

SECTION 2. REPEATED FOOTSHOCK.

The effects of daily repetition of mild footshock,and the effects of handling naive adult male rats, were assessed by measurement of both synaptosomal and saturated TH activity in NA terminal regions.

SECTION 3. SINGLE DOSES OF CLONIDINE, MORPHINE, DESIPRAMINE, AND CHLORDIAZEPOXIDE

These drugs,which have all been found to inhibit firing in the locus coeruleus,were administered in single doses to rats. Synaptosomal and subsaturating TH assays were performed .

SECTION 4. SINGLE DOSES OF YOHIMBINE AND 2-DEOXYGLUCOSE.

Synaptosomal and subsaturating assays were performed after single doses of these drugs, which have been found to increase the firing rate of NA neurones.

SECTION 5. LONG TERM DRUG ADMINISTRATION.

The effects of repeated administration of clonidine, desipramine, morphine and 2-deoxyglucose ,and the effects of subsequent withdrawal from some of these drugs was investigated with all three methods for measuring TH activity. Both terminal and cell body regions were assayed.

SECTION 6. SINGLE DOSE OF PARACHLOROAMPHETAMINE.

TH activity was measured in synaptosomes at different time intervals after a single dose of this drug, which first releases 5HT and subsequently blocks 5HT synthesis.

SECTION 7. PARTIAL REINFORCEMENT ACQUISITION TRAINING.

TH activity and NA levels were measured in a series of experiments in which rats underwent acquisition training in different reinforcement schedules (continuous vs partial reinforcement) , for food reward, with different inter-trial intervals.

SECTION 8. PARTIAL PUNISHMENT AQUISITION AND CONFLICT.

TH activity and NA levels were measured during aquisition and conflict phases of partial punishment experiments using food reward and footshock simultaneously.

SECTION 9. ELECTRICAL STIMULATION OF THE SEPTAL AREA.

The long term effects of 10 sessions of electrical stimulation of the septal area , resulting in hippocampal theta driving at 7.7 Hz, were investigated by measuring TH activity (synaptosomal and saturated) at various intervals after theta driving and operant behaviour-shaping schedules.

SECTION 1 :

EFFECT OF A SINGLE FOOTSHOCK ON TYROSINE HYDROXYLASE ACTIVITY IN SYNAPTOSOMES AND SOLUBILISED PREPARATIONS.

Rationale :

The activation of NA neurones in the brain during stress has not been easy to demonstrate under physiological conditions. Arduous regimes of electric shock have been shown to deplete NA levels in brain, as shown by Maynert and Levi (1964), Bliss et al (1966), Taylor et al (1969a), and Campos and Romero (1977), who managed to obtain up to 35% depletion. To do so, they had to administer footshock of high current intensity - 1 to 5 mA - with sessions of intermittent shocks lasting at least 15 minutes; and even then, levels of noradrenaline were restored to normal in rats which survived this procedure within 1-2 hours. Less intense stimulation did not result in depletion , presumably because under these circumstances synthesis is able to increase sufficiently to compensate for increased release (see Chapter 3). The in vivo measures of turnover , synthesis and utilization, as in a study by Korf et al (1973b) , showing increased cortical NA depletion after alpha-methyl tyrosine in rats subjected to electrical footshock , have been cited as more sensitive to stress-induced activation of NA neurones (see Stone,1975, for review); some of the difficulties with these methods have been discussed in Chapter 3.

More recently, methods other than severe footshock-stress have been used to demonstrate the increase of firing rate in NA neurones in response to milder noxious stimuli. Segal (1979) used electrophysiological recording to show that the locus coeruleus of anaesthetized rats responded to a mechanical pinch-stimulus to the leg with a burst of firing followed by inhibition of firing. Bodnar et al (1978) also implicated noradrenergic mechanisms in the response to aversive stimulation by showing that lesions of the locus coeruleus elevated

the nociceptive threshold to footshock in rats ; see also Redmond, 1979. In the light of the work by Roth et al described in Chapter 3 , showing kinetic activation of tyrosine hydroxylase isolated from the brain and assayed in vitro after relatively short periods of stimulation of the locus coeruleus, it seemed possible that mild noxious stimulation might result in activation of TH.

In the series of experiments described in this section, a single mild footshock was used as the experimental 'stress', and TH activity was measured in synaptosomes and soluble enzyme preparations from brain regions dissected immediately after the footshock. Some of these results have been described in a preliminary communication (Fillenz et al, 1979).

The brain regions used were the hippocampus,cerebellum and hypothalamus. The hippocampus and cerebellum receive NA projections from the ipsilateral locus coeruleus via the dorsal aascending NA bundle. These regions contain relatively little dopamine. The hypothalamus, on the other hand, has NA projections from the medullary NA nuclei in addition to the input from the LC; and it also receives a dopaminergic input from the brainstem ; there are also DA neurons in the median eminence which send fibres to terminate in various hypothalamic nuclei. Thus the TH in the hypothalamus will be a mixture of dopaminergic and noradrenergic types (see Chapter 2).

Cells of the locus coeruleus possess bifurcating or branching axons. The wide distribution of efferent axons over the forebrain was assumed by various workers (e.g. Olson and Fuxe,1971) to allow a single LC cell to project to various different terminal regions . If this is the case, then stimuli which activate neurons of the LC (Segal,1979) might be expected to generate somewhat similar responses in different NA terminal areas. The present experiments constituted an investigation of the response to mild noxious stimulation in different terminal regions.

Method:

Male Sprague-Dawley rats weighing between 150 and 300 g were housed in groups of 4-6 with food and water freely available. They were left undisturbed in home cages until the day of the acute experiment. The shock-delivery apparatus consisted of a Campden Instruments shocker and scrambler connected to deliver electric shock across the 12-bar grid floor of an aluminium bow measuring 8 x 8 x 13 inches. The shock intensity was 0.3 mA (root mean square) and duration was set at 0.5 sec.

In each experiment, rats were taken one at a time out of the home cage; alternate rats were then put in the Skinner box and footshock was delivered 5 seconds later. The rats usually reacted with a squeak and flinch response; threshold current for this response was about 0.15 mA. These levels of shock current are mild compared with up to 5.0 mA used in other laboratories. Shocked rats were removed from the Skinner box within 10 sec of the shock and immediately killed by a blow to the head ,followed by cervical dislocation and decapitation. Control rats were removed alternately from the home cage and killed in the same way without placement in the Skinner box. There were 4-7 animals in each group and they were usually treated in pairs, a control and a shocked rat in each pair. All the animals in any given experiment were killed on the same day and the brains assayed together, as there was considerable variation in results between groups of controls assayed on different occasions.

Brains were dissected on ice; synaptosomal preparations were made as described in Chapter 4, using hippocampal , cerebellar and hypothalamic samples. Synaptosomal TH activity was assayed at 20 µM tyrosine and pH 7.4, in 45 ul aliquots of synaptosomal resuspension containing approximately 0.3 - 0.7 mg protein.

The remaining synaptosomal suspension from each sample was stored at -17°C for subsequent solubilisation and assay of saturated TH activity, using the methods outlined in Chapter 4 (tyrosine at 110 µM, BH4 at 1.27 mM). Jane Nicholass helped with the assays in some of the experiments in this series.

Results :

EFFECT OF A SINGLE FOOTSHOCK ON SYNAPTOSOMAL TYROSINE HYDROXYLATION IN THE HIPPOCAMPUS.

The results from a series of 10 experiments are set out in Table 1.1. The first 7 experiments (series A, Experiments i - vii) had 5 rats in each control group, and another 5 rats in each shocked group. These experiments were reported in a preliminary communication (Fillenz, Graham-Jones,and Gray, 1979). Two way analysis of variance was performed with SHOCK and REPLICATION as factors. The main effect of SHOCK was significant ($F=4.54$, df 1/56, $p < 0.05$); the overall group means show a 12% increase in synaptosomal tyrosine hydroxylation rate in shocked rats as compared to controls.

TABLE 1.1

SYNAPTOSOMAL TH IN HIPPOCAMPUS

AFTER A SINGLE FOOTSHOCK

Experiment	CONTROL (n=5)	SHOCK (n=5)	SHOCK / CONTROL %
Series A:			
i	141 ± 9	168 ± 10	119
ii	100 ± 11	132 ± 22	132
iii	185 ± 14	205 ± 8	111
iv	120 ± 12	148 ± 14	123
v	163 ± 22	168 ± 23	103
vi	176 ± 12	165 ± 20	94
vii	144 ± 10	161 ± 9	112
Series A group means	(n=35) 147 ± 7	(n=35) 164 ± 7*	112%
Series B:			
viii	190 ± 15 **	165 ± 9	87
ix	128 ± 5	145 ± 7	113
x	144 ± 12	153 ± 15	106
Series B group means	(n=17) 158 ± 10	(n=17) 155 ± 6	99%
Combined group means	(n=52) 151 ± 6	(n=52) 161 ± 5	107%

results expressed as fmol/min/mg P_2 protein

* $p < 0.05$; see text

**(n=7 for control and shock groups in experiment viii)

There was also a highly significant effect of REPLICATIONS (F=6.02. df 6/56, $p < 0.001$). The SHOCK x REPLICATION interaction term was not significant (F=0.52, df 6/56). None of the individual experiments with 5 animals in each group showed significant between-group differences when t tests for differences between 2 means were performed using the error variance from the two way ANOVA described above. .

When paired comparisons were made between the control and the shocked rat in each pair, a mean difference of 16.9 fmol/min/mg P_2 protein was calculated for the 35 pairs. This difference was significant on a t test for matched pairs (paired; t=2.43, df 34, $p < 0.05$). Matched-pair tests and overall ANOVA gave similar results in this series, suggesting that there was no contamination by order effects in the assay procedure .

A further three experiments (series B) were later performed; these results are shown in the lower part of Table 1.1. These experiments failed to yield any significant difference between controls and shocked rats, although there was no change in the conditions used for the experiment.

The results from all 10 experiments were pooled for another two way ANOVA. In this analysis, the main effect of SHOCK did not reach significance at the 5% criterion level (F=2.89, df 1/84, $p < 0.1$) although REPLICATION was again significant as a main effect (F=5.6, df 9/84, $p < 0.001$). The interaction term was not significant. The overall group means, with 52 rats in each group, showed an increase of only 7% in the shocked group and the mean intra-pair difference was not significant on a paired t test (paired; t=1.81, df 51, $p < 0.1$,two tailed).

Thus the mean synaptosomal hydroxylation rate for shocked groups was higher than that for controls in 8 of the 10 experiments reported here;

but, as further data were accumulated and the results analysed with larger numbers of subjects, the size and significance of the apparent shock-induced rise diminished. In an attempt to explore this phenomenon, further analysis of the results was undertaken.

One way ANOVA's were run to examine the variation between experiments in controls and shocked rats separately. Neither set of results deviated significantly from a normal distribution; the means and medians did not differ by more than 8%. Amongst the controls, there was a significant main effect of REPLICATIONS ($F=5.06$, df 9/42, $p < 0.001$). Amongst the shocked groups, however, the main effect of REPLICATIONS did not reach significance ($F=1.76$, df 9/42, $p < 0.2$) . A Kolgorov-Smirnov 2-sample test was performed, the results of which showed no significant difference between the distributions of the control and shocked group data ($D=0.19$, $p < 0.2$).

When the data from all 10 experiments were ranked in ascending order of control synaptosomal hydroxylation rates, it became apparent that the shock-induced rise was not seen in experiments with higher control mean rates. Between-group differences in mean values were significantly but inversely correlated with control means ,across the 10 experiments ($r= -0.76$, df 50, $p < 0.015$). Similarly it was shown that the results of the 52 individual intra-pair comparisons were significantly inversely correlated with values of the control rats of each pair ($r= -0.63$, df 50, $p < 0.001$). These correlations are illustrated in Fig.1a and 1b.

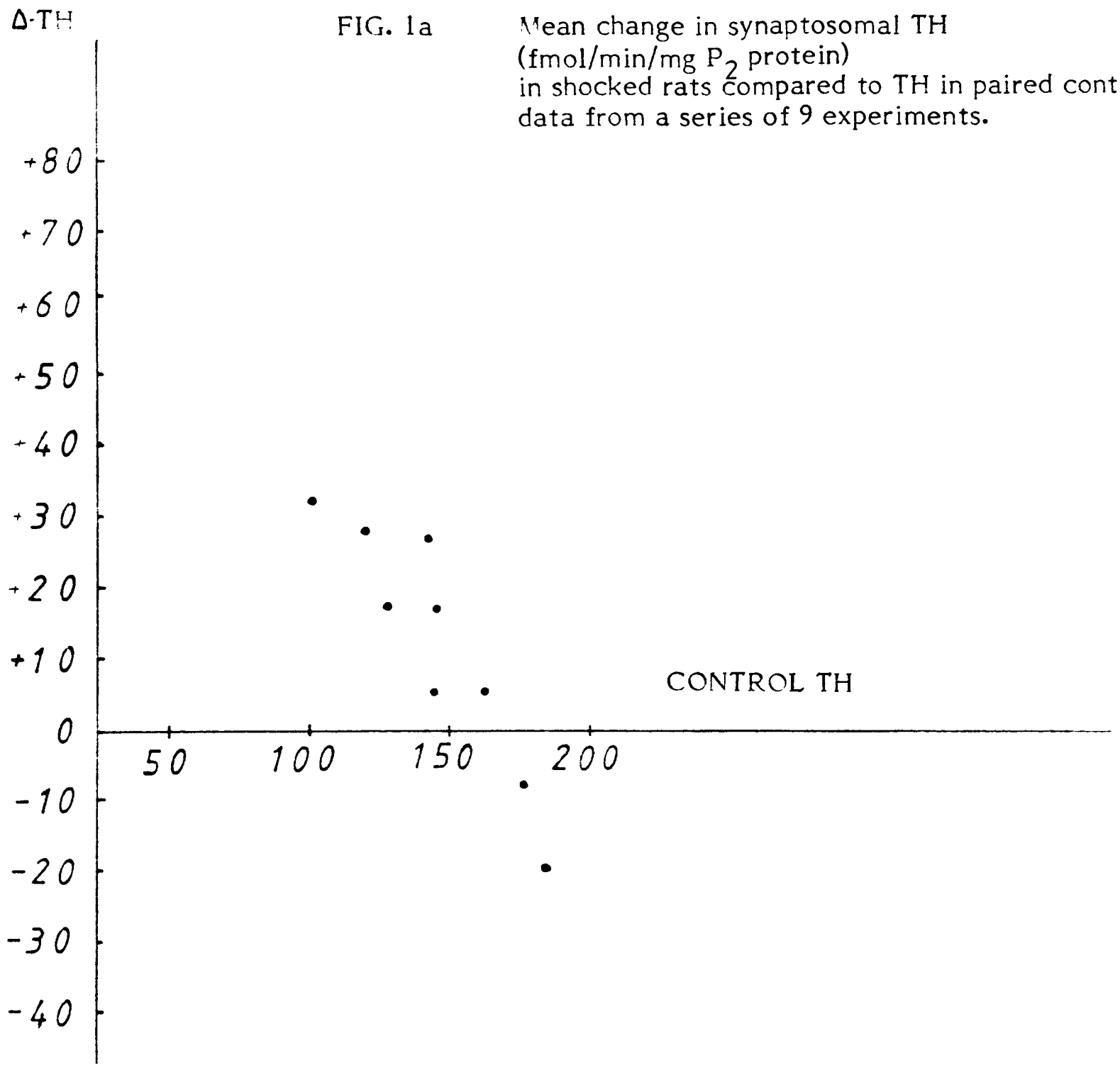
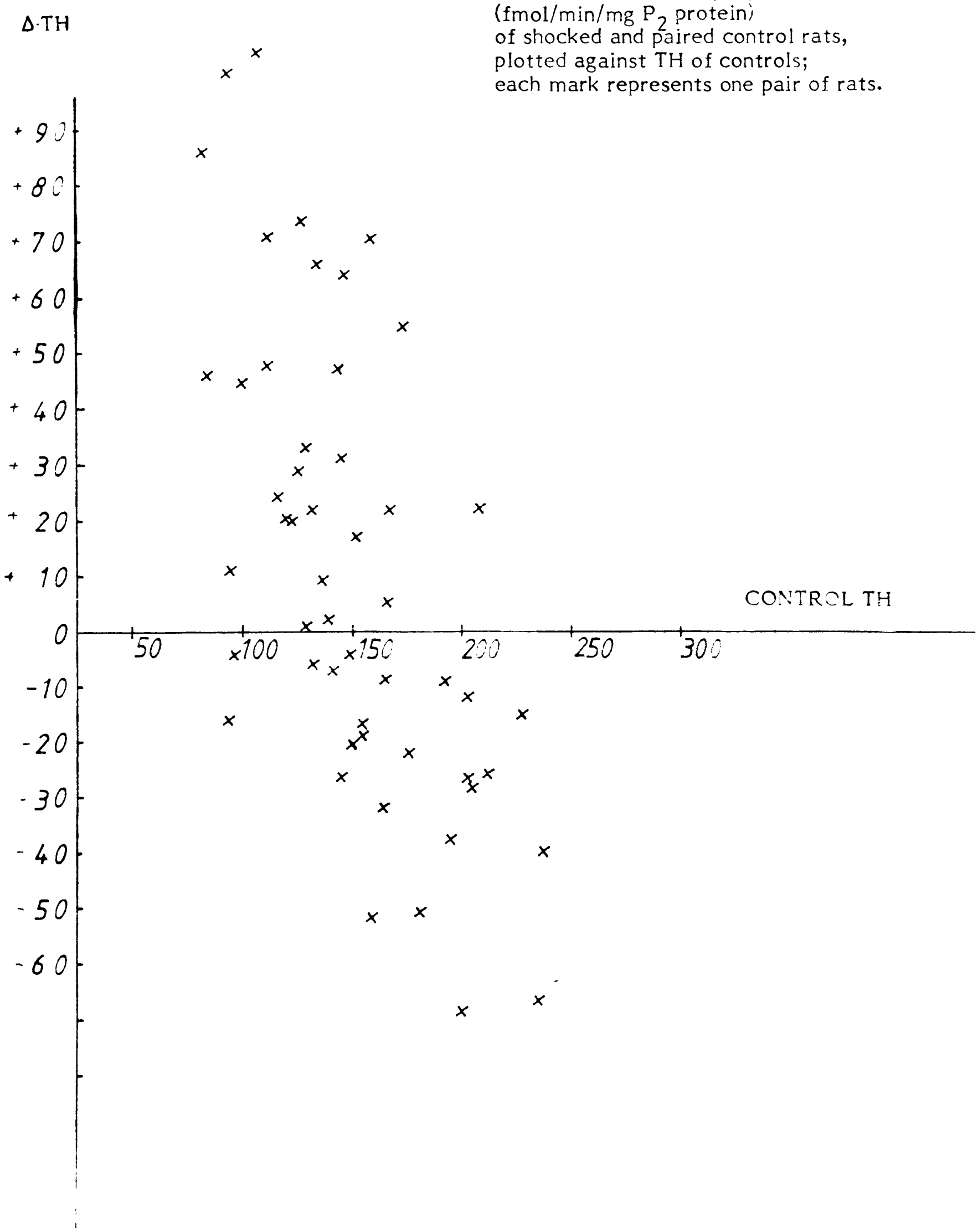


FIG. 1a Mean change in synaptosomal TH (fmol/min/mg P_2 protein) in shocked rats compared to TH in paired controls; data from a series of 9 experiments.

FIG. 1b Difference between synaptosomal TH (fmol/min/mg P_2 protein) of shocked and paired control rats, plotted against TH of controls; each mark represents one pair of rats.



SYNAPTOSOMAL TYROSINE HYDROXYLATION IN THE HYPOTHALAMUS.

Hypothalamic samples were prepared in 5 of the 10 experiments involving a single footshock (Experiments i,ii,iii,vi and ix). The results are shown in Table 1.2. In the first three experiments, increases in the shock groups similar to those in the hippocampus were seen. Later experiments did not confirm this finding. Two way analysis of variance over all five experiments did not yield a significant main effect of SHOCK ($F=2.66$,df 4/40, $p < 0.2$). The main effect of REPLICATION was significant ($F=27.99$,df 4/40,$p < 0.001$). The interaction term was not significant.

Matched-pair t tests were performed , as for the hippocampal results. Tests restricted to data from the first three experiments showed a significant intra-pair effect of shock ($t=2.95$, df = 14, $p < 0.02$); but when all 25 pairs from the five experiments were included, the hypothalamic synaptosomes did not show significant intra-pair differences between shocked rats and controls (all 25 pairs; $t= 1.73$, df= 24, $p < 0.1$).

One way ANOVA of control and shocked groups separately showed that there was significant between-replication variation in hydroxylation rate in controls ($F=12.97$,df 4/20, $p < 0.001$) , but not in in shocked rats ($F=0.29$,df 4/20). But control values for hypothalamic samples were not significantly correlated with the intra-pair difference scores as was the case in the hippocampus (means: $r=0.33$, df 3; individual pairs: $r=-0.19$, df 23).

Overall, therefore, there was little definite evidence of activation of synaptosomal TH in the hypothalamus after a single footshock. It remains to explain the reduction in between-experiment variation among shocked rats, compared to controls. This seems to be a statistically robust consequence of some aspect of the shock experiment in both hypothalamus and hippocampus.

TABLE 1.2

HYPOTHALAMIC SYNAPTOSOMAL TH

AFTER A SINGLE FOOTSHOCK

Experiment	CONTROL (n=5)	SHOCK (n=5)	SHOCK / CONTROL %
Series A			
i	245 ± 20	276 ± 22	113 %
ii	368 ± 29	439 ± 21	119 %
iii	461 ± 11	489 ± 27	106 %
vi	278 ± 17	255 ± 29	92 %
Series A	(n=20)	(n=20)	
group means	338 ± 21	365 ± 26	108 %
Series B	(n=5)	(n=5)	
ix	384 ± 36	405 ± 30	105 %
Combined group	(n=25)	(n=25)	
means	347 ± 19	373 ± 22	107 %

results expressed in fmol/min/mg P_2 protein

SYNAPTOSOMAL TYROSINE HYDROXYLATION IN THE CEREBELLUM.

Cerebellar synaptosomes were prepared in three of the single-shock experiments and the results are shown in Table 1.3. There is no significant difference between shocked and control rats . A t test for matched pairs over all 15 pairs did not reach significance (paired t=0.59, df 14), though the mean value for the shocked rats showed an overall increase of 7% in hydroxylation rate compared to controls. This was identical to the increase in the hippocampus and hypothalamus, although the figures were obtained on different numbers of animals for each region.

As in the hippocampus, there was a significant inverse correlation between the absolute value for synaptosomal hydroxylation rates in control rats, and the difference between the rates in synaptosomes from the shocked and control rats in each pair (r= -0.52, df 13, $p < 0.05$).

TABLE 1.3

CEREBELLAR SYNAPTOSOMAL TH

AFTER A SINGLE FOOTSHOCK

Experiment	CONTROL (n=5)	SHOCK (n=5)	SHOCK / CONTROL %
Series A			
iii	194 ± 39	186 ± 26	96 %
vi	253 ± 48	293 ± 39	116 %
vii	235 ± 15	245 ± 40	104 %
group means combined	(n=15) 227 ± 21	(n=15) 241 ± 22	106 %

fmol/min/mg P_2 protein

SYNAPTOSOMAL TYROSINE HYDROXYLATION 30 MINUTES
AFTER A SINGLE FOOTSHOCK.

The experiment was repeated with a 30-minute interval between shock and decapitation. 5 control rats and 5 experimental rate were used. Controls were left in the home cage whilst experimental rats were removed one at a time and put in the shock-box. Shock was delivered (0.3 mA, duration 5 seconds) as in the series described above; but the rat was then removed from the shock-box and put in a small individual holding box (10 x 6 x 6 inches). 30 minutes later the shocked rat and the paired control were removed and killed by stunning and decapitation. There was no difference in synaptosomal hydroxylation rate between these two groups, in hippocampal or hypothalamic samples,as shown in Table 1.4. The results shown were taken from a single experiment without replication. Note, however, that control values for hydroxylation rates were at the lower range of normal (see Table 1.1, and the discussion following).

TABLE 1.4

SYNAPTOSOMAL TH IN HIPPOCAMPUS AND HYPOTHALAMUS
30 MINUTES AFTER A SINGLE FOOTSHOCK.

	CONTROL (n=5)	SHOCK (n=5)	SHOCK / CONTROL %
HIPPOCAMPUS	136 ± 15	129 ± 12	95 %
HYPOTHALAMUS	287 ± 12	263 ± 11	92 %

fmols/min/mg P_2 protein

DISCUSSION: Synaptosomal TH after a single footshock.

The results from the earlier experiments in this section suggested that a single low-intensity footshock could elevate synaptosomal tyrosine hydroxylation rate in hippocampus. Considering the arduous and long lasting shock sessions required to obtain NA depletion, metabolite accumulation, or 'utilization' in the studies of Maynert and Levy (1964) and others already cited, it seemed that synaptosomal TH activity might be a sensitive index of rapid changes in NA synthesis. By analogy with Segal's (1979) results on the pattern of firing in the locus coeruleus after a mild noxious stimulus, it was tempting to suggest that the noradrenergic response to such stimuli consists of both increased firing rate and increased synthesis of NA , both responses being of short duration. Attempts to confirm the early findings and to replicate them in cerebellar synaptosomes, however, have led to a different picture.

The evidence for 'activation' of TH in these experiments is tenuous; although mean synaptosomal tyrosine hydroxylation rates in all three regions are raised by 7 % in shocked compared to control groups, the main difference between shocked and control results is the reduction in the variation between experiments in shocked groups, as shown in one way analysis of variance by replications. Synaptosomal TH in both hypothalamus and hippocampus is subject to considerable variation between experiments, although at first sight the mean control values for several series of experiments (series A and B, above, and other experiments to be described later) appeared not to differ greatly. However, the hippocampal hydroxylation rates calculated for shocked rats clustered closely and with less between-experiment variation around a mean of 161 $\pm$ 5 fmol/min/mg P_2 protein. When the control mean fell below this figure, the mean effect of shock was seen as a 'rise' (ranging from 10 to 30 %). Where there was a high control mean, as in Experiments iii,vi and viii, the effect of shock appeared to be a 'fall' in hydroxylation rate (6 to 14%).

In the hippocampus and cerebellum the magnitude of the difference between control and shocked values was inversely correlated with the absolute values for control animals. In the hypothalamus, this correlation was not found, although the reduction in between-experiment variation was found in both hippocampus and hypothalamus after shock. After a delay of 30 minutes between shock and killing, there was no difference between shocked and control synaptosomal hydroxylation rates (see Table 1.4) although since the control values in both hippocampus and hypothalamus fell in the lower range of normal in this experiment, a between-group difference should have been perceptible at least in the hippocampus.

These results may be interpreted as follows. Possibly there is a rate of hydroxylation which is normal in aroused, but unharmed animals. The mild footshock used in these experiments may perhaps act as a stimulus which is sufficient to encourage hydroxylation at this rate, but is not capable of raising synthesis rate to supranormal levels. Another possibility is that the reduction in variance after shock is analogous to the relative inhibition which follows a burst of firing in the locus coeruleus after noxious or high-intensity sensory stimulation. In other words, the post-firing state may be coded in a particular range of hydroxylation rates; shock will have the effect of inducing this state in the experimental rats, but, owing to the normal variation in arousal state (to use this term loosely), some of the control rats will also be in this state and there will not be a clear dividing line between shocked and control rats. Between-experiment variation due to other factors in the environment will affect control groups more than shocked groups, on this model, since shocked animals will all be in the 'post-firing' state.

This analogy suggests a parallel with Weiner's (1979) model which postulates that TH exists in noradrenergic terminals in two states distinguished by the Km for cofactor. In 'activated' tissue,according to Weiner,

the TH is mainly converted to the high-affinity state; this can be achieved by electrical stimulation. In resting tissue, however, the enzyme reverts to the low-affinity form . Pre-incubation of enzyme preparations (or, to stretch the analogy, establishment of a baseline state of low arousal in vivo) may encourage this process, and therefore activation is most effective in transforming the enzyme to the active form when tissue is first preincubated.

A full 'dose-response' analysis of the effects of altering shock intensity and duration would have gone beyond the scope of the present enquiry; as will be seen later, the shock parameters used in the present series of experiments were selected for comparison with the results of behavioural work using the same shock levels. However, results from 3 pilot experiments gave no indication that a more reliable 'activation' effect of shock would be obtained by using either a higher intensity of shock (0.5 mA), or two shocks spaced 30 seconds apart.

On the assumption that NA projections from the LC to different terminal regions carry similar impulse traffic , because the regional projections are collaterals of the same set of neurons, we might expect the synaptosomal hydroxylation rates in different regions to be significantly correlated. Table 1.5 shows the Pearson's 'r' coefficients calculated for different combinations of brain regions. Control and shocked groups were treated separately.

TABLE 1.5

CORRELATION (Pearson's 'r') BETWEEN SYNAPTOSOMAL TH ACTIVITY IN DIFFERENT BRAIN REGIONS.

REGIONS	n	CONTROLS	n	SHOCKED
Hippocampus-hypothalamus	20	0.11	20	0.05
Hippocampus-cerebellum	15	0.37	15	0.37
Hypothalamus-cerebellum	10	-0.42	10	-0.68 *

* $p < 0.02$

Control rats in Table 1.5 show no significant inter-regional correlations; in shocked rats , there is a significant (negative) correlation between hypothalamus and cerebellum, but not between any other pair of regions. This does not support the notion of parallel regulation of synaptosomal TH activity in terminal regions via the firing rate of LC cells , since the TH in the hypothalamus and the cerebellum are derived partly from different sources, as discussed at the beginning of this section. On the contrary, these results suggest two other possibilities: either there are additional regulatory mechanisms in the terminals which can over-ride the centrifugal influence from the LC; or the LC output is not uniform, but is differentiated with respect to the different terminal regions.

The first of these propositions will be further discussed in later sections of this thesis; and recent studies by Mason and Fibiger (1979) support the second proposition. When horse radish peroxidase was used for retrograde tracing of axons from terminal regions back to the LC, injection of tracer material into different projection areas resulted in heterogeneous patchy labelling of the LC. Thus there appears to be some anatomical specificity of projections from the LC , and the ascending noradrenergic dorsal bundle should no longer be visualised as a parallel output from a particular set of LC cells to all terminal areas in the forebrain, at any rate as regards the regulation of

EFFECT OF A SINGLE SHOCK

ON SATURATED SOLUBLE TYROSINE HYDROXYLASE ACTIVITY.

Hippocampus :

Solubilized TH from hippocampus of control and shocked rats was measured at saturating levels of cofactor and tyrosine in Experiments i,ii,iii and iv. The results are shown in Table 1.6. Two way ANOVA showed a highly significant effect of REPLICATION ($F=28.83$, df 3/32, $p < 0.001$). SHOCK was not significant as a main effect ($F=0.08$, df 1/32). The interaction term was not significant. It can be seen from Table 1.6 that control values differ between experiments; one way ANOVA was undertaken on the control and shocked results separately to determine whether the distribution of the variance due to REPLICATION differed with respect to the two groups. The main effect of REPLICATION was significant in both (controls: $F=20.66$, df 3/16, $p < 0.001$; shocked group: $F=10.42$,df 3/16, $p < 0.001$).

Hypothalamus:

Hypothalamic soluble TH was assayed in Experiments i,ii, and iii (see Table 1.6). Here too the changes between replications were more significant than any between-group differences. Two way ANOVA of soluble TH showed a significant effect of REPLICATION ($F=52.11$,df 2/24, $p < 0.001$) but no significant effect of SHOCK ($F= 0.024$, df 1/24) . The interaction term was not significant. The hypothalamic values appear to have doubled in Experiment iii compared to previous experiments, and this is the case in both control and shocked groups.

Cerebellum :

Cerebellar soluble TH was assayed in Experiment iii only; results shown in Table 1.6 show no significant difference between shocked and control groups.

TABLE 1.6

SATURATED SOLUBLE TH AFTER A SINGLE FOOTSHOCK.

EXPERIMENT	CONTROL	SHOCK	SHOCK% CONTROL
HIPPOCAMPUS	(n=5)	(n=5)	
i	1308 ± 109	1423 ± 232	109 ± 18
ii	1718 ± 145	1591 ± 121	93 ± 7
iii	2342 ± 145	2312 ± 156	99 ± 7
iv	856 ± 152	1021 ± 138	119 ± 16
	(n=20)	(n=20)	
combined	1555 ± 141	1587 ± 132	102 ± 8
	(n=5)	(n=5)	
shock + 30 min delay	1572 ± 80	1643 ± 145	105 ± 10
	fmol/min/mg P_2 protein		
HYPOTHALAMUS	(n=5)	(n=5)	
i	12.98 ± 0.43	11.71 ± 0.54	90 ± 4
ii	11.15 ± 0.68	10.61 ± 0.47	95 ± 4
iii	27.29 ± 2.94	27.68 ± 2.67	101 ± 10
	(n=15)	(n=15)	
combined	17.14 ± 2.15	16.67 ± 2.25	97 ± 13
	(n=5)	(n=5)	
shock + 30 min delay	18.35 ± 2.42	17.32 ± 1.64	94 ± 9
	pmol/min/mg P_2 protein		
CEREBELLUM	(n=5)	(n=5)	
iii	3908 ± 503	4279 ± 555	109
	fmol/min/mg P_2 protein		

DISCUSSION : Relationship between synaptosomal and soluble TH activity.

The use of synaptosomal TH measurement as a measure of 'expressed activity' in NA neurones has been discussed in Chapters 3 and 4 , as an alternative to the measurement of total TH activity in solubilised preparations; this latter measurement is related to total potential enzyme activity. However, the present experiments show that the baseline from which changes in TH activity are to be measured, namely the activity in brain regions of untreated control rats, shows considerable shifts between experiments, whether measured in synaptosomes or in preparations of solubilised enzyme. In synaptosomes, the effect of shock appeared at first as a small rise in hydroxylation rate; later re-evaluation of the shock effect suggested some sort of 'normalization' process. In the solubilised preparation, however, shock had no impact on total TH activity, as seen in the very small variance ratios for shock effects.

Clearly the shifting baseline encountered in these experiments,especially the major changes between experiments in control soluble TH activity, may render meaningless attempts to express synaptosomal TH activity in relation to soluble activity, at least when several different experiments are under consideration. To clarify this issue, some aspects of the relationship of synaptosomal and soluble TH activity will now be considered, using the data from the 4 experiments in which both assays were performed.

Hippocampus

Two way ANOVA of hippocampal synaptosomal TH for these 4 experiments showed a significant effect of SHOCK ($F=8.41$, df 1/32, $p < 0.01$). There was an overall increase of 19 % in the shocked groups relative to controls (mean control 137 $\pm$ 9 fmol/min/mg P_2 protein, mean shocked 163 $\pm$ 9, with 20 rats in each group). The main effect of REPLICATION was also

significant (F=13.82, df 3/32, $p < 0.001$) . Since it was assumed that the expression of synaptosomal TH might be related to the total quantity of enzyme as measured by soluble TH, analysis of covariance was performed with soluble TH as covariate. The effect of covariation was not significant (F=3.67, df 1/31, $p < 0.1$). After adjustment, synaptosomal figures again showed a significant effect of SHOCK (F=8.51,df 1/31, $p < 0.01$) and of REPLICATION (F=8.19, df 3/31, $p < 0.01$) . Thus the only change after covariance was a reduction in the degree of significance of the REPLICATION effect.

The correlation coefficient is another measure of relatedness between two measures; the coefficient for synaptosomal vs soluble TH activity in the animals in these experiments was calculated ($r= 0.52$, $p < 0.02$ for the 20 controls; $r= 0.58$, $p < 0.01$ for the 20 shocked rats). Plots of the regression of soluble upon synaptosomal TH (not shown) indicate that the relationship is not linear ; although the correlation is significantly different from zero, the linear coefficient is not high enough to denote a close relationship between the expressed activity of TH in synaptosomes and the maximal potential activity in saturated enzyme from the same tissue. Only 27-33% of the total variance in either sample would be accounted for by the correlation.

The highly significant replications effects in the ANOVA's of both soluble and synaptosomal data certainly seem to obstruct the investigation of the relationship between synaptosomal and soluble TH activity. Within experiments, it may nevertheless be possible to see whether 'activation' of TH in synaptosomes after shock is manifested as the postulated increase in expressed activity relative to total activity. Table 1.7 shows the mean values derived when synaptosomal TH is expressed as a percentage of the soluble TH from the same animal; the linear correlation coefficients for synaptosomal vs

saturating activity are also shown. Data are shown for all experiments separately (n=5 in each experimental group). The table shows that, in spite of the significant rise in synaptosomal TH after shock in the experiments analysed here, this is barely apparent in terms of the relative activity in the two preparations. Hippocampal synaptosomes hydroxylate tyrosine at about 10% of the saturating rate for soluble enzyme. There is some suggestion of an increase in this ratio after a single footshock (Experiments i-iii) , but it is very slight in these data, since the mean ratios of synaptosomal to soluble TH activity differ by less than 1% between shocked and control groups overall.

High and significant correlations between synaptosomal and soluble TH activity are apparent in two individual groups in this series of experiments, one shocked group and one control group, as shown in Table 1.7. However, this may be due to chance.

TABLE 1.7

RELATIONSHIP BETWEEN SYNAPTOSOMAL AND SATURATED TH ACTIVITY IN THE HIPPOCAMPUS .

EXPERIMENT	CONTROL 'r'	syn/sol %	SHOCKED 'r'	syn/sol %
i	0.75	10.9	0.96**	12.6
ii	0.17	5.9	0.45	8.4
iii	0.95*	7.9	0.22	9.0
iv	-0.73	16.7	-0.033	15.3
mean (n=20)		10.4%		11.3%
overall correlation (n=20)	0.52*		0.58**	

* $p < 0.02$; ** $p < 0.01$

Hypothalamus

Table 1.6 also shows the hypothalamic saturated soluble TH results for the three experiments in which both synaptosomal and soluble TH were

measured. As in the hippocampus, the difference in synaptosomal TH between these shocked and control groups was significant ($F=5.71$, df 1/24, $p < 0.01$), and analysis of covariance with soluble TH as covariate served mainly to reduce the variance ratio for the REPLICATIONS effect in synaptosomal results slightly ($F=48.07$, df 2/24, $p < 0.001$, before covariance; $F= 27.25$, df 2/23 , $p < 0.001$, after covariance) . The significance of the SHOCK effect was very slightly reduced after covariance of soluble TH ($F= 5.48$, df 1/23, $p < 0.025$). The effect of covariance was not significant ($F=0.01$, df 1/23). From Table 1.8, it can be seen that hypothalamic synaptosomes express only about 2% of the maximal activity, again with only the slightest hint of an increased relative activity after shock.

TABLE 1.8

RELATIONSHIP BETWEEN SYNAPTOSOMAL AND SATURATED TH ACTIVITY IN THE HYPOTHALAMUS AND CEREBELLUM.

EXPERIMENT	CONTROL	syn/sol	SHOCKED	syn/sol
	'r'	%	'r'	%
HYPOTHALAMUS				
i	0.04	1.9%	0.01	2.4%
ii	-0.22	3.4	0.86	4.2
iii	-0.1	1.0	0.16	0.9
overall (n=15)	0.61*	2.1%	0.56**	2.4%
CEREBELLUM				
iii	0.80	4.8%	0.63	4.4%

* $p < 0.02$ **$p < 0.05$

Cerebellum

Tables 1.6 and 1.8 also show the results from one experiment in which cerebellar sample were taken. Synaptosomal activity was 4% of the total saturated activity, and the relationship between the two measures did not alter after shock.

It is interesting to note that the hypothalamic rise of 6 - 19 % in synaptosomal TH activity after shock in this set of experiments was of a similar order of magnitude to the hippocampal changes (and see the overall 7% synaptosomal activation in all regions, discussed earlier). Thus despite different constraints operating on the expression of TH activity in the two regions, a similar increase in synaptosomal activity can be evoked by a stimulus which arouses the animal but does not cause debilitation.

Variation of the baseline for solubilized TH activity has been discussed briefly in Chapter 4. This has remained problematic , as none of the efforts to isolate and eliminate particular sources of variation has succeeded in stabilising the control levels over more than a few experiments. Particular factors of concern in the experiments discussed here included a long batch-life for the cofactor BH4, which might have contriubuted to the low control values in Experiment iv ; but the abrupt rise in hypothalamic values (and hippocampal values though the rise was less pronounced in this region) in Experiment iii remains unexplained.

SECTION 2 :

EFFECT OF REPEATED FOOTSHOCK ON SYNAPTOSOMAL AND SOLUBILISED TYROSINE HYDROXYLASE ACTIVITY.

Rationale :

Animals respond to repetition of an aversive stimulus in a number of ways. These include habituation or 'tolerance', when the repeated stimulus fails to evoke the same response as it did when originally presented; on the other hand, increased reactivity, or 'sensitization' , may also occur. Stone (1975) has reviewed the effects of chronic stress on noradrenaline levels and 'turnover' in the brain . The stress stimuli used in many cases were severe and exhaustive regimes which led to acute depletion of NA . There was great variation in the reported results after repetition of such stresses as footshock and exercise in cold water, but Stone was able to conclude that NA levels did not generally fall after repetition of stress, but were maintained at or even increased beyond the control level. The increased 'turnover' (measured in a variety of ways) did not appear generally to habituate to stress, but stayed at an elevated level during and sometimes beyond the period of repetition of the stress stimulus. This elevation of turnover was thought to represent adaptation to stress , in that neurons were rendered capable of increasing synthesis and release of NA during subsequent exposure to stress (see Chapter 3 on induction and related changes).

Further work on the characteristics of regimes that produce depletion of NA has been reviewed by Gray et al (in press); there is some evidence, though it is by no means conclusive, that the greatest increases in NA release and depletion occur when shock stress is uncontrollable and/or unpredictable (Weiss et al,1975; Schutz et al , 1979).

In the present experiments rats were subjected to a mild aversive stimulus for seven successive days, and the activity of TH incubated with saturating concentrations of tyrosine in synaptosomes and solubilised preparations was measured in hippocampus , hypothalamus and cerebellum. Synaptosomal TH was regarded as an indicator of ongoing noradrenergic activity at the time of death, whereas the saturating soluble assay was used to discover whether changes in total enzyme activity had occurred.

The 'stress' was electrical footshock with the same parameters as in the experiments described in section 1. This level of shock , rather than a more severe stress, was chosen precisely because it was 'tolerable'. This study was intended as part of an investigation of the role of NA function in the development of tolerance to stress (see section 8 on the partial punishment effect).

Method :

Because the repeated shock procedure involved a certain amount of handling, in contrast to control animals which were left in home cages until they were killed, a handled-only condition (HC) was incorporated into the design of this series of experiments. In Experiments I,II and III, all groups except naive controls (C) were handled for 10 minutes daily during the week before the shock procedure started.

The shock delivery apparatus was as described in section 1. Shocked rats were taken to the apparatus and subjected to one shock (0.3 mA , duration 0.5 seconds, as in acute experiments) each day at the same time for 7 days. Some rats (shock-and-kill group, SK) were killed within 10 seconds of the last shock; others (shock-and-wait group, SW) were put back in the home cage and killed 24 hours after the last shock.

A series of 5 experiments is described below. In Experiments I and II, there were 2 groups of 5 rats each; handled controls (HC) and rats shocked daily and killed after a 24 hour delay (SW). In Experiments III and IV, there were four groups; C, HC, SW and SK. In Experiment V, there were two groups; C and SK.

All the rats in any given experiment were killed in one morning, in pairs or squads of 4 animals containing one rat from each group. Brain regions were dissected and assayed as described in Chapter 4. Soluble TH was assayed at 110 μM tyrosine and 1.27 mM BH4. Synaptosomal TH was assayed at 20 μM tyrosine, at pH 7.4.

Results :

EFFECT OF REPEATED FOOTSHOCK ON
SYNAPTOSOMAL TH ACTIVITY IN THE HIPPOCAMPUS

The results are shown in Table 2.1 . Two way ANOVA of data from Experiments III and IV, which incorporated all four conditions, showed a significant effect of TREATMENT ($F=3.45$, df 3/32, $p < 0.05$). Neither the REPLICATIONS effect ($F=0.71$, df 7/32) nor the interaction term ($F=0.84$, df 3,32) was significant . A t test showed that neither of the repeated-shock groups differed significantly from naive controls; but the handled animals had higher synaptosomal TH rates than controls (increased by 37%, $p < 0.02$, df 32) or rats shocked 24 hours before death (increased by 42%, $p < 0.01$,df 32).

The effect of handling on synaptosomal TH rates was confirmed in Experiments I and II. Two way ANOVA of all the HC and SW data (four experiments) by TREATMENT and REPLICATIONS showed a highly significant

effect (F=13.45,df 1,32, p < 0.001) for TREATMENT. Handled animals show a rise of 40% over SW hydroxylation rates. Neither the REPLICATION effect (F=1.26, df 3,32) nor the interaction term (F=1.73, df 3,32) were significant.

Two way ANOVA of the results of the control and 'shock-and-kill' groups to assess the 'acute-on-chronic' shock effect did not show a significant effect of TREATMENT (F=2.0, df 2,23, p < 0.2); nor was there a significant effect of REPLICATION (F=1.33, df 2,23). No significant elevation of synaptosomal TH above control level was found in rats given repeated footshock, whether they were killed immediately after the last shock or 24 hours later. Handled controls , however, show a highly significant elevation of TH in hippocampal synaptosomes.

One way ANOVA of the HC results from all the 4 relevant experiments shows no significant effect of REPLICATIONS (F=0.70, df 3,16). The controls (C) in this series (experiments III,IV and V) show slightly more between-experiment variation, but this did not reach significance (one way ANOVA, F=2.16,df 2,11, p < 0.2).

TABLE 2.1

EFFECT OF REPEATED FOOTSHOCK ON

SYNAPTOSOMAL TH IN HIPPOCAMPUS .

	CONTROL	HANDLED	SHOCK(SW)	SHOCK(SK)
	(n=5)	(n=5)	(n=5)	(n=5)
Experiment				
I	-	182 ± 10	166 ± 2	-
II	-	223 ± 28	158 ± 16	-
III	141 ± 13	176 ± 31	143 ± 7	167 ± 30
IV	107 ± 4	163 ± 25	97 ± 14	116 ± 17
V	104 ± 4*	-	-	123 ± 17
Combined	(n=14)	(n=20)	(n=20)	(n=15)
I - IV	-	186 ± 13	141 ± 9	-
III-V	118 ± 9	-	-	146 ± 17

results expressed in fmol//min//mg P_2 protein ± S.E.M.

* (n=4)

HYPOTHALAMIC SYNAPTOSOMAL TH

The results are shown in Table 2.2. No rise in TH rate was seen in the handled animals compared to controls (Experiment III) or to SW groups (Experiments I,II,and III). Nor was there a rise attributable to acute effects of shock in SK rats killed immediately after the last shock (Experiments III and V).

CEREBELLAR SYNAPTOSOMAL TH

Handled rats had a higher mean TH rate than SW rats in the one experiment in which cerebellum was assayed (see Table 2.2), but this difference did not achieve significance at the 5% level (unpaired t test, t=1.80, df 8).

TABLE 2.2

EFFECT OF REPEATED FOOTSHOCK ON SYNAPTOSOMAL TH IN HYPOTHALAMUS AND CEREBELLUM.

	CONTROL	HANDLED	SHOCK(SW)	SHOCK(SK)
HYPOTHALAMUS	(n=5)	(n=5)	(n=5)	(n=5)
Experiment				
I	-	282 ± 17	295 ± 31	-
II	-	411 ± 15	401 ± 16	-
III	437 ± 24*	454 ± 44	442 ± 36	453 ± 23
V	365 ± 73	-	-	312 ± 27
CEREBELLUM				
II	-	183 ± 18	140 ± 16	-

results expressed in fmol//min//mg P_2 protein ± S.E.M.

* (n=4)

CORRELATIONS BETWEEN REGIONS : SYNAPTOSOMAL TH ACTIVITY AFTER REPEATED SHOCK OR HANDLING.

Hippocampus-hypothalamus correlations were not significant in either the SW group (r=0.09,n=15) nor in the handled group (HC: r=0.23, n=15). Since the cerebellum was only assayed in one experiment, the numbers of animals for other inter-regional comparisons were small and the correlation coefficients were not significant (hippocampus-cerebellum, SW: r=0.54,n=5; HC: r=0.78,n=5; hypothalamus-cerebellum, SW: r=0.59, n=5; HC: r=0.26, n=5).

EFFECT OF REPEATED SHOCK ON SOLUBLE
SATURATED TYROSINE HYDROXYLASE ACTIVITY.

The results of solubilized TH assay under saturating conditions are shown in Tables 2.3 and 2.4. Hippocampus was assayed in Experiments I-IV, hypothalamus in Experiments I and II, and cerebellum in Experiment II only.

The four experimental groups do not differ in soluble TH activity in the hippocampus. In each experiment , the mean TH rates are clustered together, with no effect of shock or of handling . However, there is a large variation in the overall mean activity between the different experiments, which suggests variation in some aspect of the assay.

TABLE 2.3.
SOLUBLE TH ACTIVITY AFTER REPEATED SHOCK.

	CONTROL	HANDLED	SHOCK(SW)	SHOCK(SK)
HIPPOCAMPUS	(n=5)	(n=5)	(n=5)	(n=5)
Experiment				
I	-	1872 ± 141	1858 ± 197	-
II	-	2076 ± 204	2136 ± 161	-
III	572 ± 256	486 ± 62	421 ± 47	335 ± 116
IV	347 ± 151	470 ± 105	438 ± 67	464 ± 84
Combined		(n=20)	(n=20)	
I - IV	-	1226 ± 184	1255 ± 197	-
	(n=10)	(n=10)	(n=10)	(n=10)
III-IV	459 ± 145	438 ± 70	386 ± 53	399 ± 71

results expressed as fmol/min/mg P_2 protein.

Two way ANOVA of the hippocampal soluble TH activity in the HC and SW groups in Experiments I-IV gave no significant main effect of treatment ($F=0.02$, df 1/31), but the main effect of replications was highly significant ($F=83.55$, df 3/31, $p < 0.001$).

Cerebellar soluble TH (see Table 2.4) showed no significant difference between HC and SW groups. In the hypothalamus, there was a significant difference between groups in soluble TH activity, with higher activity in the handled group than in the SW condition in Experiment II ($t=5.01$, df 4, $p < 0.01$, paired t test). This was not so in Experiment I, which had a lower TH rate in both groups. This , as in the hippocampal results, probably denotes some variation in the assay between days, since each region was assayed for soluble TH on different days. Possible contaminating factors in these assays which might account for these shifts are long storage times for the tissue and for purified tyrosine.

TABLE 2.4.

SOLUBLE TH ACTIVITY AFTER REPEATED SHOCK.

	CONTROL	HANDLED	SHOCK(SW)	SHOCK(SK)
CEREBELLUM	(n=5)	(n=5)	(n=5)	(n=5)
Experiment III	-	3274 ± 388	3064 ± 52	-
HYPOTHALAMUS				
Experiment I	-	14447 ± 127	14468 ± 131	-
II	-	28794 ± 1213	21954* ± 829	-

results expressed in fmols/min/mg P_2 protein.

* HC > SW, paired t test, $p < 0.01$.

Because of the unreliability of soluble TH control values in these experiments, covering an even wider range than that encountered in section 1, it is unlikely that the ratio of synaptosomal TH to soluble TH can provide a valid estimate of enzyme activation in relation to total potential activity in this series of results. However,since all significant between-group changes were in synaptosomal TH, apart from one hypothalamic assay, the synaptosomal-to-soluble ratio would be elevated in handled controls to a degree varying with the control soluble activity. Thus the mean ratio of synaptosomal to soluble TH in the HC group was 16% as compared to 11.3% in the SW group. In the hypothalamus the corresponding ratios were 1.6 and 1.9 % respectively.

The correlation between hippocampal synaptosomal and soluble activity did not reach significance in the HC group, possibly reflecting the increase in the synaptosomal but not soluble activity ($r= 0.28$, $df=18$); whereas this correlation in the SW group reached the same value as for the control rats in Section 1 ($r=0.52$, $p < 0.02$, $df=17$). In the hypothalamus, the synaptosomal-soluble correlation was significant and high in the HC group ($r=0.85$, $p < 0.01$, $df=8$) and lower, though still significantly different from zero, in the SW group ($r=0.67$, $df=8$, $p < 0.05$).

DISCUSSION

Repetition of a mild, aversive footshock stimulus for seven days was not associated with any significant alteration in saturated soluble or synaptosomal TH activity in hippocampus, hypothalamus or cerebellum, when shocked animals were compared with naive controls. A small, non-significant 'acute shock ' increase was perceptible in the hippocampal and hypothalamic synaptosomes of the SK group; but since the statistical reliability of the single shock effect (section 1) is somewhat doubtful, it is not possible to interpret this unambiguously as physiological tolerance , or habituation , to stress after repeated presentation of the stimulus. Also, there was no long-lasting elevation of synaptosomal TH after repeated daily shock . In fact no evidence to suggest activation of NA synthesis after repeated mild footshock was found in these experiments.

Experiments on repeated severe footshock stress show behavioural 'helplessness' after exposure to intense and inescapable or unpredictable shock. Gray (in press) argues that the kind of helplessness which ensues after severe stress and is accompanied by NA depletion, especially in the hypothalamus, may be a model for human 'endogenous depression'. Clearly the present experiments do not constitute severe stress . (Nor do they involve enough shocks to produce 'helplessness', according to Anisman and Sklar,1979; or long enough shock trains, according to Childress and Thomas,1979, and Glazer and Weiss, 1976). But they do involve repeated presentation of an aversive stimulus, and the rats seemed capable of anticipating shock, as on several occasions attempts were made to jump out of the Skinner box before, as well as during , delivery of shock. Thus the paradigm used here is probably sufficient to induce fear when the rats were exposed to stimuli (the Skinner box) associated with shock. Gray (in press) suggests that fear and anxiety are related to increased noradrenergic activity in the forebrain. The present

experiments , although not intended as a critical test of this hypothesis, do not show any activation of NA synthesis after aversive footshock, and therefore do not support this formulation.

Hippocampal synaptosomes showed a significant and unexpected rise in TH activity after 1 or 2 weeks handling without shock. This was replicated in four experiments and was not accompanied by any change in saturated TH activity. Hypothalamic synaptosomes showed no change in TH activity, but in one experiment there was an increase in solubilised soluble TH activity. Experiments III and IV , which included all 4 conditions, showed that the handling effect in the hippocampus was due to elevation of TH rates above control levels. The similarity of the values for controls and repeated-shock groups , despite the handling undergone by all shocked animals, suggested an inhibitory role for the repeated shock, suppressing a rise in NA synthesis due to handling. It may be that this mechanism , which seems to be confined to the hippocampus in the present experiments, is also involved in 'habituation' or tolerance to shock.

One possible explanation is that , whereas handling and shock both cause an increase in noradrenergic activity, the impact of repetition of the shock stimulus is sufficient to activate an inhibitory input to the locus coeruleus, or to hippocampal terminals. In the former case, firing rate of NA neurons may be reduced; in the latter case, release of NA from terminals or NA synthesis could be inhibited. Handling alone (ex hypothesi) does not activate this input. Conceivably, some system such as the enkephalinergic input to the LC could mediate such a mechanism in response to repetitive aversive stimulation (cf Fanselow (1979) and Fanselow and Bolles (1979); work on naloxone ; and Goldstein, 1978).

As for the effects of handling per se, Levine and Mullins (1966) showed long-lasting effects of handling of infant rats. These were similar to the effects of mild footshock. When adult, these rats showed a rapid, but short-lasting, corticosterone response when placed in an open field; they were termed less 'fearful' than unhandled rats. Coscina et al (1975) found that handling in adulthood produced long-lasting effects on rat emotional behaviour and on neurochemical measures. Handled rats showed no changes in NA,DA or 5HT levels, but 5-hydroxyindoleacetic acid (5HIAA) was increased in whole brain, suggesting activation of 5HT neurons. Rats which were handled for 6 days before NA depletion with the neurotoxin 6-hydoxydopamine (6OHDA) showed less DA and NA depletion after the neurotoxin than unhandled rats; and handling after the 6OHDA injection reduced emotional reactivity (startle responses, irritability, and weight loss) . Coscina emphasised that whilst no direct relationship emerged between the crude neurochemical and behavioural measures employed, handling and other 'mild, brief somatosensory stimuli' should be regarded as capable of influencing the outcome of experiments on the neurochemical basis of behavioural change. The results discussed in this section support this view; moreover, it seems likely that the noradrenergic response to mild aversive stimuli is part of a complex interplay with other neurochemical systems which may well include serotonergic and enkephalinergic mechanisms.

EXPERIMENTS : SECTION 3

TYROSINE HYDROXYLATION RATE AFTER SINGLE DOSES OF DRUGS WHICH DECREASE FIRING RATE IN NORADRENERGIC NEURONS.

Rationale:

These experiments were designed to test the hypothesis that synaptosomal tyrosine hydroxylation rate would tend to reflect the rate of firing in noradrenergic neurons in the brain. The work of Roth and his co-workers (see Chapter 3, section 10) has shown that stimulation of NA neurons in vivo can produce changes in TH activity which persist in postmortem tissue. Stimulation of the locus coeruleus at higher-than-normal rates of firing resulted in changes in the kinetic properties of TH isolated from the hippocampus and increased accumulation of MOPEG in the cortex. The experiments reported here have used less invasive stimulation , in the form of parenteral administration of relatively non-toxic drugs to unanaesthetized rats; and measurements have been made of synaptosomal TH activity in all cases.

The drugs used in the experiments described in this section were chosen because there is evidence from electrophysiological experiments in various laboratories that the firing rates of single units recorded in the locus coeruleus of rats are decreased after single doses of these drugs, administered by intraperitoneal or intravenous routes as well as by iontophoresis (see Table 3.1). These electrophysiological experiments are performed on anaesthetized rats and therefore show changes from a baseline rate which is lower than the firing rate in the awake animal; nevertheless it has been assumed that the reductions in firing rate also occur when the drug is administered to the unanaesthetized animal.

TABLE 3.1

DRUGS WHICH REDUCE FIRING RATE IN CELLS OF THE LOCUS COERULEUS

DRUG	DOSE & ROUTE	PROBABLE MODE OF ACTION	REFERENCE
CLONIDINE	6 μg/kg,i.v.	agonist at inhibitory autoreceptors	Svensson,Bunney & Aghajanian,1975
"	iontophoresis		Aghajanian,1978
CHLORDIAZEPOXIDE	0.5-4.0 mg/kg i.v.	?benzodiazepine receptors, GABA mediated inhibition	Grant,Huang & Redmond, (pers. comm.)
DESIPRAMINE	0.05mg/kg/min, i.v.	NAuptake inhibition causes stimulation at autoreceptors	Dresse & Scuvee-Moreau 1980 Nyback et al,1975
MORPHINE	iontophoresis	opiate receptors in LC	Korf et al,1974 Aghajanian,1978

Table 3.2 (see over) summarises evidence from previous studies suggesting that some of these drugs - clonidine,chlordiazepoxide, and desipramine - also reduce NA function as measured biochemically. The measures used have been discussed in section 11 of Chapter 3 . They are identified in Table 3.2 as follows:

AMT-NA represents measurement of NA depletion after synthesis inhibition; this has been designated as an index of utilization of NA.

DDCI-DOPA represents measurement of DOPA accumulation after decarboxylase inhibition,and is a measure of the rate-limiting step in synthesis of NA.

3H-TYR-NA involves the measurement of 3H-NA newly synthesised from injected 3H-tyrosine.

FLA63-NA represents measurement of NA depletion after inhibition of dopamine-beta-hydroxylase,and is another index of utilization of NA, but in the presence of dopamine accumulation.

MOPEG measurement; this is one of the major metabolites of NA in brain; its accumulation can be measured , and its egress is blocked by probenecid in some studies.

TH represents enzyme activity in vitro measured by adding 3H-tyrosine as substrate to suspensions containing the enzyme and counting the tritiated products of hydroxylation.

3H-DA metabolism refers to studies of 3H-NA and metabolites in brain after a bolus intracerebral injection of 3H-dopamine.

TABLE 3.2

DRUGS WHICH ALTER NORADRENALINE SYNTHESIS AND TURNOVER IN THE RAT BRAIN.

DRUG	BRAIN REGION	ASSAY METHOD AND RESULT	REFERENCE
CLONIDINE (0.1 mg/kg)	whole brain	DDCI-DOPA decreased	Anden et al 1976 Strombom et al 1976
		AMT-NA less depletion	Anden et al 1976
		MOPEG decreased	Tang et al 1979b Braestrup et al 1976
		FLA-63-NA less depletion	Pugsley et al 1979
CHLORDIAZEPOXIDE (10 mg/kg)	midbrain cortex, cerebellum	3H-DA metabolism decreased	Taylor & Laverty 1969
	cortex & cerebellum (not hypothal)	FLA-63-NA less depletion	Corrodi,Fuxe et al 1971
DESIPRAMINE (10 mg/kg)	cerebral hemispheres	DDCI-DOPA decreased	Carlsson & Lindquist 1978
	whole brain	AMT-NA less depletion	Sugrue,1980
	whole brain	MOPEG decreased	Bareggi 1978,Sugrue 1980 Nielsen, 1975
MORPHINE (10-20 mg/kg)	hypothalamus	3H-TYR-NA increased	Johnson et al 1974
	hypothalamus cerebellum brainstem	MOPEG increased	Roffman et al 1977
	hypothalamus midbrain	TH unchanged	Cicero et al 1973

It will be noted that the inhibitory effect of morphine on the spontaneous firing rate of single units in the LC is not paralleled by evidence of a reduction in NA synthesis. On the contrary, there is some evidence of increased synthesis of catecholamines; although NA synthesis and DA synthesis were not distinguished in the study by Cicero et al (1973), Johnson et al (1974) found a small increase in NA synthesis in the hypothalamus . Roffman 's (1977) finding of increased MOPEG in hypothalamus, cerebellum and brainstem is the only definite evidence I have found for increased NA synthesis outside the hypothalamus in rats treated with a single dose of morphine.

Dopamine synthesis and utilization were also increased in several studies; indeed some authors consider that the main effect of morphine on catecholamine neurons is an activation of DA neurons, and an increase in intraneuronal monoamine catabolism. Cicero (1973) measured TH activity in different brain regions as well as looking at total catecholamine synthesis; he found no changes after injections of 10 - 20 mg/kg morphine, nor were there any changes in kinetic properties of enzyme incubated in vitro with morphine at concentrations up to 10^{-3}M. It therefore seemed unlikely at the time that morphine was acting directly on TH activity (but see Chapter 3, section 9).

The following experiments were performed to test the relationship between changes in firing rate (see Table 3.1), changes in synthesis rate(Table 3.2), and synaptosomal hydroxylation rate of exogenous labelled tyrosine . Some of the results were reported in a preliminary communication (Fillenz and Graham-Jones, 1980). Clonidine- , desipramine- and chlordiazepoxide-treated rats were expected to have reduced TH activity in synaptosomes from noradrenergic areas of the brain. From the evidence of LC firing rates, but without backing from previous biochemical studies, morphine was also expected to reduce synaptosomal TH activity.

Method:

This series of experiments was performed on naive male Sprague-Dawley rats weighing approximately 150-250g. Drug solutions at the concentrations specified below were freshly prepared for intraperitoneal injection using normal saline as solvent, and the rats were always injected between 8 and 10 am , to minimise any instability in control levels due to diurnal variation. The injected rats were left in their home cages in the laboratory for 1-3 hours before killing. Each experiment included a treated group of 4-10 animals and a control group. Controls were either injected with saline or not injected at all . Drugged and control rats were killed alternately, and dissection, sample preparation and assay were performed as described in Chapter Four . Some of the experiments were performed twice. Two way analysis of variance was used to analyse the data from these experiments, with 'REPLICATION' as a factor. Appropriate t tests, unpaired or paired, were used to test the significance of difference between group means.

Synaptosomal TH activity at a tyrosine concentration of 20 μM was the main variate in all the experiments in this section.

In some experiments, solubilized TH activity under subsaturating conditions was also assayed, to assess whether changes in synaptosomal hydroxylation rates were likely to be accompanied by changes in kinetic properties of the solubilised enzyme. The subsaturating assay was performed at a tyrosine concentration of 40 μM as substrate and DMPH4 at 0.5 mM. Results from the subsaturating assays are given in APPENDIX I.

SECTION 3 a : EFFECT OF A SINGLE INJECTION OF NORMAL SALINE.

Synaptosomal tyrosine hydroxylation rates in rats injected intraperitoneally with saline, 1 ml/kg, one hour before killing, were compared with those in animals left in home cages until the killing session. There was no difference between the rates in hippocampus or hypothalamus, as shown below. Group mean results are shown with standard error of the mean ; numbers in brackets are the numbers of rats per group.

	CONTROLS	SALINE-INJECTED
HIPPOCAMPUS	161 ± 24	159 ± 11
	(n=10)	(n=12)
HYPOTHALAMUS	374 ± 50	417 ± 38
	(n=6)	(n=7)

fmol/min/mg P2 protein

SECTION 3 b : EFFECT OF A SINGLE INJECTION OF CLONIDINE.

This experiment was performed in two replications. Clonidine hydrochloride (Boehringer-Ingelheim) was dissolved in saline and injected at 0.025 or 0.1 mg/kg , 1-2½ hours before killing. In the first experiment,half the experimental group were injected with 0.025 mg/kg and half with 0.1 mg/kg of clonidine. In the second experiment, all the experimental group had 0.025 mg/kg. The rats tolerated the injection well and no obvious behavioural effects were noted afterward. Uninjected control rats were left in the home cages until the killing session, and were then killed alternately with the injected rats . Synaptosomal tyrosine hydroxylation rates are expressed in fmol/min/mg P2 protein.

RESULTS:

See Table 3.3 . Analysis of variance (two way,including REPLICATIONS as a factor) showed that the main effect of DRUG was significant ($F=9.53$, df 1/21, $p< 0.005$). Overall there is an increase in hydroxylation rate in hippocampus of clonidine-injected animals with respect to controls; the increase amounts to 28% of control rates. REPLICATIONS was also significant as a main effect ($F=51.59$, df 1/21, $p< 0.001$) but there was no significant DRUG X REPLICATIONS effect.

There was no significant difference between the groups treated with different doses of clonidine in the first experiment (0.025 mg/kg, 259 $\pm$ 7 fmol/min/mg P_2 protein; 0.1 mg/kg, 273 $\pm$ 15 fmol/min/mg P_2 protein); in the second experiment, all the drugged rats received the same dose, 0.025 mg/kg. Table 3.3 shows the mean values, taken over both experiments.

The hypothalamus (assayed in one experiment only; see Table 3.3) showed no difference in hydroxylation rate between control rats and those

injected with clonidine at 0.025 mg/kg. The difference between the group means only amounted to 2% of the control mean.

SECTION 3 c : EFFECT OF A SINGLE DOSE OF CHLORDIAZEPOXIDE

Chlordiazepoxide (Roche) was dissolved in normal saline at 5 mg/ml and injected at 5 mg/kg intraperitoneally 1-3 hours before killing. Hippocampal synaptosomal TH activity was measured; no other regions were assayed.

RESULTS:

Hippocampal tyrosine hydroxylation rates were no different in the treated group from those in controls.(see Table 3.3). The mean hydroxylation rate in the chlordiazepoxide-injected group was 98% of the control mean.

TABLE 3.3

TYROSINE HYDROXYLATION IN SYNAPTOSOMES AFTER DRUGS WHICH REDUCE FIRING RATE IN NA NEURONES.

	CONTROL	DRUG	
HIPPOCAMPUS			
CLONIDINE (0.025-0.1 mg/kg)	167 ±23 (10)	214 ±16 (15)	**
CHLORDIAZEPOXIDE (5mg/kg)	202 ±20 (9)	198 ±23 (5)	ns
DESIPRAMINE (10mg/kg)	208 ±13 (10)	214 ±9 (10)	ns
MORPHINE (15mg/kg)	148 ±8 (17)	128 ±9 (18)	*
HYPOTHALAMUS			
CLONIDINE (0.025 mg/kg)	374 ±50 (6)	383 ±18 (7)	ns
DESIPRAMINE (10 mg/kg)	451 ±41 (11)	540 ±36 (11)	ns
MORPHINE (15 mg/kg)	332 ±15 (10)	328 ±22 (10)	ns

fmol/min/mg P2 protein.

** p< 0.005

* p< 0.05(paired)

(numbers of subjects in each group are shown in brackets)

SECTION 3 d : EFFECT OF A SINGLE DOSE OF DESIPRAMINE

In two experiments, rats were injected with saline or desipramine, (Geigy Phamaceuticals) 10mg/kg, 1-2 hours before killing. Hippocampus and hypothalamus samples were assayed for synaptosomal tyrosine hydroxylation rate (see Table 3.3) .

Analysis of variance of the synaptosomal results (after $\log_{10}$ transform) was performed with DRUG, BRAIN REGION and REPLICATION as factors. The main effect of DRUG did not reach significance ($F= 3.69$, df 1/34, $p< 0.1$) although the main effects of BRAIN REGION ($F=160.8$, df 1/34, $p< 0.001$) and REPLICATION ($F=9.19$, df 1/34, $p < 0.005$) were significant. None of the interaction terms were significant. Table 3.3 shows that, although there was no difference in mean hydroxylation rates in hippocampal synaptosomes from the desipramine-treated and control groups, there appears to be an increase in the desipramine group in hypothalamic synaptosomes. A paired t test (paired comparisons between control and drugged rats killed at the same time) was therefore performed on the hypothalamic results ; but this failed to reach significance ($t= 1.98$, df 10, $p< 0.1$).

SECTION 3 e : EFFECT OF A SINGLE DOSE OF MORPHINE

Morphine sulphate was dissolved in normal saline and injected at 15 mg/kg, 1-2 hours before killing. This experiment was repeated on three occasions. The synaptosomal TH results are shown in Table 3.3 . Two-way analysis of variance does not reveal a significant main effect of DRUG ($F=3.95$, $df=1/29$, $p< 0.1$) although hippocampal synaptosomes show a mean reduction in tyrosine hydroxylation rate to 88% of the control value . However, paired comparisons of morphine treated and saline-injected controls gives a significant fall in hydroxylation rates after morphine (paired $t= 2.14$, df 16, $p< 0.05$). In this case the paired-comparison technique is more sensitive than the ANOVA although generally the two methods give fairly similar results (see above, section 3 d). The REPLICATION factor in the ANOVA for hippocampal synaptosomes was highly significant ($F=10.33$, $df=2/29$, $p < 0.001$) but the interaction term did not reach significance ($F=0.48$).

Morphine does not affect TH rate in hypothalamic synaptosomes (Table 3.3). The values for morphine-treated and saline-treated rats were almost identical (morphine group mean was 99% of control mean) in each of two replications.

SUMMARY: EFFECTS OF SINGLE DOSES OF DRUGS WHICH REDUCE FIRING RATE IN NA NEURONES.

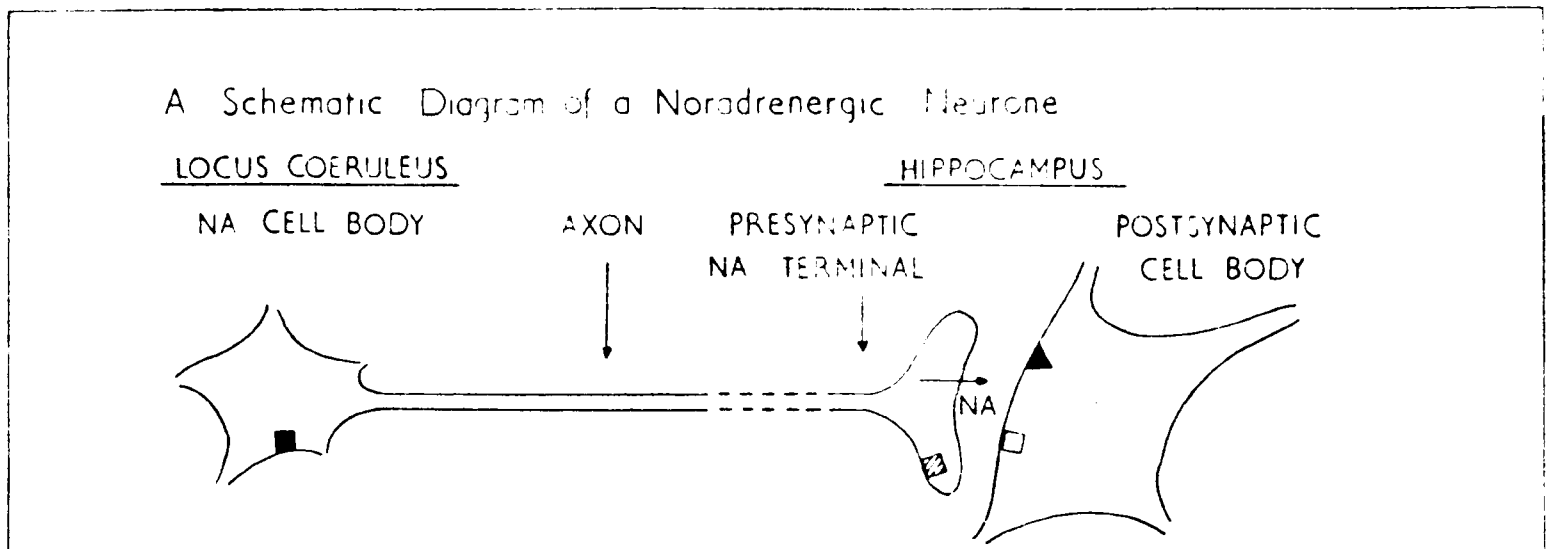
It was established that saline injections do not alter synaptosomal tyrosine hydroxylation rates in hippocampus or hypothalamus samples. Doses of different drugs which have been reported to reduce firing rates of cells in the locus coeruleus of anaesthetized rats , and to reduce the rate of synthesis of NA in the brain, were given to rats 1-3 hours before death.

In the hippocampus, clonidine caused a significant increase in synaptosomal TH rate; chlordiazepoxide and desipramine had no effect; and morphine gave a small reduction in synaptosomal TH rate.

In the hypothalamus, clonidine and morphine had no effect on synaptosomal TH rates. Desipramine, however, caused an increase which nearly reached statistical significance.

These results will be discussed at the end of section 4 of this Chapter.

FIG. 3a Noradrenergic receptor agonists and antagonists.



	■	▨	□	▲
RECEPTOR:	"autoreceptor"	"presynaptic" α_2-receptor"	"postsynaptic" α_1-receptor"	"postsynaptic" β_1-receptor"
AGONISTS:	NA CLONIDINE	NA CLONIDINE	NA CLONIDINE	NA ISOPRENALINE
ANTAGONISTS:	YOHIMBINE PIPEROXANE	YOHIMBINE PIPEROXANE TOLAZOLINE PHENTOLAMINE	PHENOXYBENZAMINE PHENTOLAMINE PRAZOSIN (HALOPERIDOL ?)	PROPRANOLOL

EXPERIMENTS : SECTION 4

TYROSINE HYDROXYLATION AFTER SINGLE DOSES OF DRUGS WHICH INCREASE THE FIRING RATE OF NA NEURONS.

Rationale:

This series of experiments is complementary to the previous section and follows further the hypothesis that synaptosomal tyrosine hydroxylation rate will tend to reflect changes in the firing rate of locus coeruleus neurons.

The drugs used here - yohimbine and 2-deoxyglucose - are both thought to cause activation of noradrenergic neurones. In the case of yohimbine, which is a specific alpha$_2$ receptor antagonist, the mechanism of action is probably a selective action at a particular receptor . Yohimbine blocks the actions of clonidine on presynaptic or autoreceptors of the alpha$_2$ type on central NA neurons; in addition, when given alone it accelerates locus coeruleus cells firing rates (Svensson and Usdin, 1978; Grant, Redmond and Huang, pers. comm.) and increases the utilization of NA as measured by NA depletion after synthesis inhibition (see table 4.1).

2-deoxyglucose serves as a substrate for the enzymes which promote glucose utilization in brain . When administered in sufficiently large doses to rats or other experimental subjects, the result is a failure to metabolize glucose; there is intracellular glucose deficiency, accompanied by hyperglycaemia and behaviour known as 'glucoprivic feeding' (Hokfelt and Bydgeman, 1961). The hyperglycaemic response (which can also be elicited by electrical stimulation of the hypothalamus) is produced by infusion of 2-deoxyglucose (2-DG) into the hypothalamus or by administration of large doses parenterally; and it is prevented by adrenal medullary denervation (Himsworth,

1968; Balagura and Kanner,1971). The mechanism is believed to be activation of the adrenal gland via a pathway from the ventromedial nucleus of the hypothalamus. The stimulus to the adrenal results in increased output of both noradrenaline and corticosteroids; and in this respect the animal responds to an injection of 2-DG as it would react to a stressful stimulus. The mode of action of 2-DG in the hypothalamus is not known.

Ritter and Neville (1976) have shown that the short term response to 2-DG involves a rise in hypothalamic, but not telencephalic NA utilization (i.e. rate of NA depletion after synthesis). The glucoprivic feeding response and the increased adrenal activity are dependent on intact innervation of the adrenal (Muller,1972; Nijima, 1975).

Acute dosage of 2-deoxy-D-glucose has been shown to increase discharge rate in splanchnic nerve filaments (preganglionic ; see Nijima,1975), but there is as yet no corresponding study in CNS tissue. It was however shown by Ritter and Neville (1976) that 2-DG increased NA utilization in the hypothalamus,though not in the telencephalon (see Table 4.2) ; intraperitoneal administration of 2-deoxyglucose produced increased DOPAC levels (Kennedy et al, 1979) in frontal cortex, and this may also be due to noradrenergic mediation via the projection from the locus coeruleus to the dopaminergic nucleus, A 10 (Simon et al, 1979) . It was therefore of interest to see whether regional differences would be apparent in tyrosine hydroxylase activity under similar conditions.

TABLE 4.1

DRUGS WHICH INCREASE SYNTHESIS AND UTILIZATION OF NORADRENALINE IN THE BRAIN

DRUG	BRAIN REGION	ASSAY METHOD	REFERENCE
YOHIMBINE (10 mg/kg)	whole brain	NA levels decreased AMT-NA; depletion increased	Anden et al,1976
2-DEOXYGLUCOSE (500mg/kg)	hypothalamus (not telen-cephalon)	AMT-NA; depletion increased	Ritter & Neville 1976

Method:

As in section 3, all drugs were dissolved in normal saline unless otherwise specified. Intraperitoneal injections took place between 8 and 11 am, and rats were killed approximately 2 hours later. Synaptosomal assays of TH activity were performed as set out in Chapter 4. Results from subsaturating assays of soluble tyrosine hydroxylase are given in APPENDIX I.

SECTION 4 a : EFFECT OF A SINGLE DOSE OF YOHIMBINE

Yohimbine HCl (Sigma) was dissolved in warm distilled water (expt I) or saline (expt II) at 10 mg/2 ml, and injected at 10 mg/kg intraperitoneally to male Sprague-Dawley rats.There were no obvious gross behavioural effects of the drug. Non-injected control rats were left in the home cages until the killing session. The experiment was performed twice; the hippocampus was assayed in both experiments, but the other regions were assayed on one occasion only.

The results for synaptosomal TH in hippocampus and hypothalamus are given in table 4.2. The hippocampus shows a fall in TH in the yohimbine group to 80% of the control mean rate; and DRUG condition was significant as a main effect in a two-way analysis of variance ($F=11.3$, df 1/16, $p< 0.005$) . REPLICATIONS was also significant as a main effect ($F=22.52$, df 1/16, $p< 0.001$), but the DRUG x REPLICATIONS interaction was not significant. In the hypothalamus and cerebral cortex there was no significant change from control level in the yohimbine group (mean hydroxylation rates in the treated groups were 101 % and 103 % of control means in hypothalamus and cortex respectively).

SECTION 4 b: EFFECT OF A SINGLE DOSE OF 2-DEOXYGLUCOSE

2-deoxy-D-glucose (2-DG, BDH Chemicals) was dissolved in normal saline and injected to rats at 500 mg/kg intraperitoneally. Controls were injected with saline at the same time. Two hours later all rats were killed. The deoxyglucose group tolerated the injection well and no gross behavioural differences between the groups were noted.

The results from the synaptosomal assays are shown in table 4.3 . Analysis of variance of the results with BRAIN REGION and DRUG as factors was performed after $\log_{10}$ transformation of the data. The main effect of DRUG did not reach significance (F=0.87, df 1/16), although the main effect of BRAIN REGION was significant (F=29.6, df 1/16, $p < 0.001$). The interaction term was not significant (F=0.38) .

The deoxyglucose group showed a drop to 81 % of mean control hippocampal synaptosomal TH rate (paired t= 0.79,df 4), which , although not a statistically significant effect in this experiment, is a similar result to that obtained in the yohimbine experiment (see above, section 4 a). This 2-DG experiment was performed once only, so the group sizes are small (n=5). As in the yohimbine experiment, there was no difference between groups in the hypothalamus (deoxyglucose group mean hydroxylation rats was 95 % of control mean).

TABLE 4.2

TYROSINE HYDROXYLATION IN SYNAPTOSOMES AFTER DRUGS WHICH INCREASE FIRING RATE IN NA NEURONES.

	CONTROL	DRUG	
HIPPOCAMPUS			
YOHIMBINE (10 mg/kg)	153 ±10 (10)	122 ±9 (10)	**
2-DEOXYGLUCOSE (500 mg/kg)	234 ±41 (5)	189 ±38 (5)	ns
HYPOTHALAMUS			
YOHIMBINE	278 ±17 (5)	281 ±34 (5)	ns
2-DEOXYGLUCOSE	453 ±27 (5)	431 ±24 (5)	ns
CORTEX			
YOHIMBINE	151 ±18 (5)	156 ±25 (5)	ns

fmols/min/mg P2 protein. ** $p < 0.005$
(numbers of rats in each group are shown in brackets)

SUMMARY: EFFECTS OF SINGLE DOSES OF DRUGS WHICH INCREASE FIRING RATE OF NA NEURONS.

Administration of yohimbine and 2-deoxyglucose , in doses which increase firing rate in NA neurons, did not increase TH activity in hippocampal or hypothalamic synaptosomes. On the contrary, there was a significant reduction in synaptosomal TH in the hippocampus of yohimbine-treated rats; this was not found in the hypothalamic samples of the same animals. Administration of 2-deoxyglucose was associated with a non-significant reduction of hippocampal synaptosomal TH; there was no sign of the predicted activation in the hypothalamus.

DISCUSSION:

ACUTE EFFECTS OF DRUGS ON TYROSINE HYDROXYLATION RATE.

The experiments in sections 3 and 4 were designed to test several predictions :

a) that drugs which had been shown to increase the firing rate of cells in the locus coeruleus would also increase the postmortem synaptosomal tyrosine hydroxylation rate in terminals of LC neurons :

b) that drugs which reduced the spontaneous firing rate in the LC would show an inhibition of postmortem synaptosomal tyrosine hydroxylase activity in projection areas of the LC.

c) that drugs which had been shown to increase NA synthesis in vivo would be associated with increases in synaptosomal tyrosine hydroxylation; and

d) that administration of drugs which reduce synthesis rate of NA in vivo would cause a fall in synaptosomal tyrosine hydroxylase activity.

e) that , if there were drug-induced changes in enzyme properties sufficient to change in vivo synthesis rate significantly, these would also be manifested as changes in hydroxylation rate achieved in an assay of solubilised enzyme incubated with a fixed subsaturating concentration of cofactor and of tyrosine.

For all the drugs used, except morphine, it was possible to assemble evidence from biochemical studies in other laboratories which demonstrated evidence of altered NA synthesis or metabolism within a few hours of drug injections, congruent with the changes in firing rates already noted (see Tables 3.1, 3.2, 4.1,). The methods used in those studies generally involved measuring accumulation of newly synthesised NA or metabolites over a short time interval , in vivo. (In selecting these studies, little reliance was placed on reports of changes in levels of NA as these are insensitive as measures of NA function under normal circumstances.)

The methods in common use for measuring the in vivo synthesis rate of NA are often cumbersome and may involve intracerebral injections and other invasive procedures , including anaesthesia, which may in turn cause interference with the variables or responses being measured (e.g. drug effects, or even the effects of immobilization stress). The present study was carried out to see whether the measurement of TH in synaptosomes prepared from fresh brain tissue of treated and untreated rats would provide an index of NA function which would reflect the rate of NA synthesis and rate of firing of NA neurons in vivo immediately before death.

Salzman and Roth (1980) have measured the activity of TH enzyme isolated and solubilized from hippocampus and cerebral cortex of rats killed up to 20 minutes after a period of electrical stimulation of the LC (see Chapter 3,section 10). There are kinetic changes in the properties of the enzyme under these circumstances which could underlie the increased 3H-NA synthesis measured in vivo in these animals. For the purposes of the present experiments, it was proposed that synaptosomal TH rate might additionally reflect changes in synthesis rate due to regulatory factors other than kinetic changes in the enzyme, since synaptosomes , although cut off from the axon and cell body, still retain enough integrity as 'pseudo-cells' to maintain and control synthesis , storage, release and uptake of NA in a normal manner.

Synaptosomal preparations from different brain regions did show alterations in hydroxylation rate of 3H-tyrosine after in vivo administration of drugs, but not after saline injections. Results were fairly reliable, in terms of experimental replications with the same drug. But the alterations of synaptosomal TH rate did not, in general, follow the direction of the reported change in firing rate or in vivo synthesis rate. Thus the results obtained in these experiments do not support the 5 predictions outlined above.

Synaptosomal TH was significantly increased in the hippocampus after clonidine injections . Reduced synaptosomal activity was found in the hippocampus after yohimbine and after morphine, and there was a suggestion of a similar effect with 2-deoxyglucose . No significant changes in either direction were found with chlordiazepoxide or desipramine in the hippocampus. There were no significant changes in hypothalamic tyrosine hydroxylation rates with any of the drugs used. The only drug which produced a change in the predicted direction for firing rate was morphine, for which there had been no agreement between the direction of changes in firing rate and the changes in synthesis rate reported in previous biochemical studies.

Subsaturating TH assay results , which are reported in APPENDIX I, p. 344 did not concord with the findings in the synaptosomal assays; the only significant finding with this method was an increase of TH activity in hippocampus after treatment with desipramine (i.e. a response in the opposite direction to that predicted) . This discrepancy remains unexplained.

Clearly, the LC firing rate and in vivo NA synthesis rate (as measured by product and metabolite accumulation) are not the only or even the principal determinants of postmortem NA synthesis rate in brain tissue from drug-treated rats. If we relinquish the hypothesis that synaptosomal tyrosine hydroxylation rate will reflect the firing rate of NA neurons, can we explain the results of sections 3 and 4 above by reference to other effects of the drugs on NA neurones?

In the last few years, new techniques have been developed which have radically changed our thinking about the modes of action of drugs in relation to neurochemical processes. Can recent work in these newer areas throw any

light on the discrepancy between firing rate, in vivo product and metabolite accumulation, and activity of TH in synaptosomes? We need to consider the in vivo effects of the various drugs and also any mechanisms which may affect the function of isolated synaptosomes.

MORPHINE:

Morphine was the only drug which fulfilled the prediction that synaptosomal TH rate would drop , in line with reports of a reduction in firing rate in the LC . Morphine-induced inhibition of firing rate in the LC has been demonstrated in several studies. The inhibition is reversed by the opiate antagonist naloxone. The LC has a high concentration of opiate receptors (Fields and Basbaum review, 1979). The limbic areas are well supplied with opiate receptors; the cortex has relatively few. The opiate receptors in all parts of the brain are inhibitory to firing rate of the post synaptic neuron (Iwamoto 1978).

The effects of a single dose of morphine on catecholamine synthesis have been outlined above; although there is a lack of evidence for noradrenergic areas, there is evidence for increased synthesis in dopaminergic neurones in areas including cerebral cortex and hypothalamus, and Roffman (1977) showed increased MOPEG accumulation in cerebellum and hypothalamus which suggested that these changes might also occur in noradrenergic neurones. This evidence contrasts with the present finding of reduced TH activity in hippocampal, but not hypothalamic , synaptosomes.

Other effects of morphine include a non-specific inhibition of NA uptake into slices and synaptosomes of mouse brain at high concentrations (10^{-3}M) of morphine (Carmichael and Israel, 1973); and Taube et al (1977) showed that morphine and met-enkephalin inhibited stimulus-evoked release of labelled NA from slices of rat cortex, cerebellum and hypothalamus after pre-

loading with the labelled transmitter. This is evidence in favour of an effect at terminals in addition to the in vivo effects on the cells of the locus coeruleus (Langer et al, 1980). Because of the discrepancy between the reports of electrophysiological effects and catecholamine synthesis in vivo, such a local effect might have to be invoked to explain the increased CA synthesis reported in other laboratories; whereas the synaptosomal TH results, which do not depend upon release of NA from the terminals, are congruent with the electrophysiological results. It is not clear, however, how inhibition of release as a local effect of the drug could be related to increased NA synthesis or metabolism . Eidelberg (1976) refers to the possibility that calcium binding may be affected by morphine, in such a way as to interfere with release; may this perhaps alter the intraneuronal metabolism of NA in favour of MOPEG production? Clearly further work on in vitro and in vivo effects of morphine is needed in order to understand this discrepancy.

CLONIDINE AND YOHIMBINE: MODES OF ACTION

Clonidine was synthesised in 1962 by Stahle as a likely vasoconstrictor agent. Early clinical trials demonstrated its sedative and hypotensive effects (see Schmitt,1977 for review) . In clinical practice it has been used as a second line drug in hypertension, but it has side effects such as bradycardia, impotence, nausea and dizziness. A more dangerous complication is the development of rebound hypertension when the drug is abruptly withdrawn after continued use.

At a lower dose than that used in hypertension, clonidine is used for prophylaxis of migraine attacks. Recently another use has been found for clonidine in clinical practice; Gold et al (1978) found that small doses of clonidine alleviated withdrawal symptoms in opiate addicts, and further evaluation in this field is under way. Redmond (1979), who has worked with Gold in this field, suggests that yet another use of clonidine will be as an anxiolytic drug. There is insufficient evidence as yet to evaluate this suggestion (but see Bullock et al, 1978).

The pharmacological bases for these different clinical uses of clonidine are not fully understood. However, there is substantial evidence that clonidine acts as a noradrenergic agonist at alpha-adrenergic receptors in the CNS as well as in the periphery. In higher doses (0.5 mg/kg or more), clonidine seems to act as a post synaptic agonist , possibly acting on $alpha_1$ receptors. It has synergistic actions with dopamine receptor stimulants such as apomorphine, in animals treated with reserpine and noradrenaline-depleting drugs. It is not clear, however, which clinical effects are due to this post synaptic action.

At lower doses (0.01-0.1 mg/kg), clonidine is a potent agonist at other alpha-noradrenergic receptors, possibly of two or more types. In the CNS, there are two types of alpha-adrenergic receptors on noradrenergic neurons; 'autoreceptors' on the soma and dendrites of cells in the locus coeruleus, and 'presynaptic alpha$_2$ receptors ' on noradrenergic terminals in hippocampus, cortex and possibly other areas of the brain (see FIG.3a) p 175. The exact distribution of these two classes of receptors has yet to be described, but clonidine is established as a potent and selective agonist, probably at both types; indeed tritiated clonidine is used as a marker for presynaptic alpha$_2$ receptors (Langer, 1980).

The principal function of these receptors seems to be the regulation of noradrenaline release; this is also found in the periphery . Clonidine in very low concentrations mimics the action of NA in lowering release rate, or 'overflow' of NA from noradrenergic tissue, during stimulation or depolarization which would normally produce an increase in the rate of release. The greatest effects of clonidine are obtained at low stimulation frequencies and lower concentrations of depolarizing agents such as potassium; there is thus a U-shaped dose-effect curve. It is not clear whether this is due to the postsynaptic effects of higher doses of clonidine itself, or a 'partial agonist' effect due to competition between clonidine and NA for receptor sites (Lowenstein, 1980).

Yohimbine appears to be an equally specific antagonist at these alpha$_2$ receptors, and promotes release of NA, besides blocking the presynaptic effects of clonidine or NA. Several studies have attempted to rank a whole series of agonists and antagonists in order of potency at noradrenergic receptor sites in various tissues, and some differentiation is evident between drugs active at post synaptic sites and those active at presynaptic sites. Mulder et al (1979) suggest that the mechanism of action of

the presynaptic receptor in the regulation of NA release may be via a reduction in calcium availability for the excitation-secretion coupling process. It is not known whether this is a similar effect to the interaction of morphine and calcium (see above).

Less is known about the autoreceptors found in the LC; but from the studies in Table 3.1, indicating work carried out using iontophoresis of drugs onto cells of the LC, it seems that clonidine inhibits the rate of firing of these cells, as does application of NA. Yohimbine, on the other hand, increases the rate of firing. In the intact animal, it may be that these autoreceptors are activated by recurrent collaterals from the axons of LC cells (Aghajanian, 1978; Cedarbaum and Aghajanian, 1977); or, as has been suggested by other authors (Bolme, 1974), these may be activated by adrenaline-containing neurons. It is interesting to speculate that these receptors may contribute to the characteristic firing pattern of LC cells after an intense or noxious stimulus.

Inhibition of release at noradrenergic terminals in other areas might have a role in limiting the amount of NA available at the postsynaptic NA receptor after impulse-evoked release, although reuptake of NA is thought to be a very efficient mechanism, and it is difficult to envisage the nature of the interaction between uptake inhibition and inhibition of release without further information.

The mechanism of action of clonidine at the autoreceptors is not clear; presumably the agonist action of clonidine is similar to the effect of increased release of NA during activation of a collateral axon , and acts in some way to reduce the firing rate of the cell body. Table 3.2 showed that clonidine given in vivo tends to reduce the accumulation of NA and its metabolites; this could be due either to inhibition of release at the terminals

or to inhibition of firing rate. The converse is true for yohimbine and other alpha$_2$ antagonists, which increase 'turnover ' or 'synthesis ' measures.

The results reported in section 3 b are a direct test of the action of these receptor-active drugs on the rate-limiting hydroxylation step in the synthesis of NA , in isolated noradrenergic terminals of cells projecting from the LC. The prediction made at the outset was that the apparent inhibition of NA function by clonidine (manifested by reduced firing and reduced accumulation of metabolites) would be mirrored in a reduced enzyme activity in the terminal; and the converse prediction was made for the effects of yohimbine. These predictions were flatly contradicted by the results obtained. The opposing effects of clonidine and yohimbine on the synaptosomal preparation suggest that the drugs are indeed acting on alpha$_2$ receptors ; but the effect of clonidine (administered in vivo) was to <u>increase</u> hydroxylation rate significantly, and that of yohimbine was to <u>decrease</u> it. It is not clear from this experimental design whether the drug effects were the consequences of recent firing patterns, or effects of clonidine at the nerve terminals in suspension after separation from axons. But the implication is that the presence of a drug which inhibits release of NA is not necessarily associated with reduced synthetic potential. Braestrup and Nielsen (1976) showed that clonidine affects NA release and metabolism even when synthesis is inhibited with AMT or when infusions of labelled DOPA are used , thus bypassing the tyrosine hydroxylation step. The effects of clonidine on synthesis and on release may be independent.

Recent evidence from in vitro experiments in this laboratory (Nicholass, unpublished) have failed to show any significant changes in synaptosomal TH rate when clonidine is added at 10^{6} - 10^{-5}M to the incubation medium. Braestrup and Nielsen (1976) did find inhibition of synaptosomal tyrosine hydroxylation , but only with higher concentrations of

clonidine (10^{-3}M). However, clonidine in vitro does not impair the acceleration of hydroxylation obtained during depolarization with high potassium solutions. This effect of depolarization has been shown (see Chapter 3, section 8) to be independent of the presence of calcium in the medium, ie independent of release. So the membrane events associated with depolarization do increase synthesis rate, despite the presence of an alpha$_2$ agonist which tends to reduce release; whereas when the CNS has been exposed to the agonist drug in low dosage for 1-2 hours in vivo, the activity of the synaptosomal enzyme is increased.

It is noteworthy that hypothalamic synaptosomes were unaffected by morphine, clonidine or yohimbine. This might be due to the mixture of dopaminergic and noradrenergic TH, or to differences in noradrenergic terminals in the two regions (hippocampus contains more specialised noradrenergic synapses than other noradrenergic areas ; Koda & Bloom (1977) ; but this remains speculation at present.

CHLORDIAZEPOXIDE

Chlordiazepoxide, at a dose of 5 mg/kg, had no effect on hippocampal synaptosomal TH activity. Although Grant et al (pers.comm.) have shown that this drug does affect LC firing rate, the widely-quoted experiments showing reduction of NA turnover after CDP (Table 3.2) used large doses; although the benzodiazepines were effective at preventing stress-induced increases in catecholamine turnover , they were not potent inhibitors of turnover in unstressed animals. Rastogi et al (1978) found no changes in whole-brain synaptosomal synthesis, uptake or catabolism of NA after treatment with two benzodiazepines, but suggested that the benzodiazepines reduced the rate of transmitter release and decreased MOPEG production.

Recent work on benzodiazepines and their interaction with specific receptors in the brain has suggested that their effects upon the monoamines may be mediated by GABA-ergic mechanisms (Costa and Guidotti, 1979). GABA is an inhibitory transmitter in brain ; its effect is facilitated by benzodiazepines. The nature of the interaction is not yet clear but it is probably localized to the GABA neuron. Effects on noradrenergic mechanisms may therefore be secondary to the effects of GABA in the LC, such as inhibition of firing rate. As we have seen, synaptosomal TH activity does not faithfully reflect changes in LC firing rate; so , although further work is needed to confirm the single negative result reported here, it seems unlikely that synaptosomal TH would be a sensitive index of the effects of benzodiazepines on noradrenergic mechanisms if these effects are in fact secondary to changes in GABAergic neurons.

DESIPRAMINE AND TRICYCLIC ANTIDEPRESSANTS.

Single doses of desipramine (DMI) have been reliably shown to decrease firing rate of LC neurons and to reduce synthesis and metabolism of NA in vivo. One mechanism of action of DMI in acute dosage and in vitro on noradrenergic terminals is an inhibition of the specific, high-affinity uptake of NA . In certain circumstances this can appear to be associated with increased release (Gilbert and Wyllie, 1980). Another effect which might affect NA metabolism in terminals is monoamine oxidase inhibition; although this has only been demonstrated as an in vitro effect by Roth and Gillis in rabbit brain (1974).

As for the reduction in firing rate of the LC cells by DMI , this is probably mediated by the autoreceptors discussed above. The effects of systemic DMI administration on LC firing rate are similar to the effect of iontophoresis of NA onto LC cells. The effects of DMI on LC firing rate are antagonised by alpha$_2$ antagonists such as dibozane (German et al,1979), yohimbine (Svensson et al, 1978; Dresse et al, 1980), and piperoxane (Cedarbaum and Aghajanian, 1977), and by LC electrical stimulation.

There was no significant effect of DMI on synaptosomal TH in hippocampus in the present study, and again the conclusion can be drawn that TH activity in the synaptosomal preparation does not reflect LC firing rate. German et al (1979) showed evidence of a similar dissociation of effects in that low doses of DMI reduced firing rate of LC cells but did not reduce MOPEG levels.

It is still not clear whether synthesis is linked to the reuptake mechanism.

From the negative results of the present investigation, it seems unlikely that presynaptic alpha$_2$ receptor stimulation at

terminals is a complete explanation of the activation of synaptosomal tyrosine hydroxylation by clonidine. The inhibitory effect of DMI on NA reuptake would also tend to increase presynaptic alpha receptor stimulation; but in this case there was no enzyme activation.

2-DEOXYGLUCOSE

In section 4 b , evidence was cited for increased utilization or turnover of NA after administration of 2-DG in the hypothalamus, and increased firing rate of NA neurons in the periphery and possibly in the CNS. The results described in section 4 b indicate a non-significant fall in synaptosomal TH activity in the hippocampus but no change from control activity in the hypothalamus; there was no significant change in TH activity in the solubilised enzyme under subsaturating conditions in either region.

The mechanism of action of 2-DG on NA neurons is not understood. The results in synaptosomes after in vivo administration of the drug are similar (clonidine) to those obtained with yohimbine, which gave an apparently paradoxical fall in hippocampal synaptosomal TH activity in contrast with the acceleration of LC firing rate and in vivo synthesis rate or turnover (Table 4.1). With both these drugs, and with clonidine, the effects were confined to the hippocampus . Hypothalamic synaptosomes were unaffected . This raises the possibility that the mechanism of action of 2-DG is related to that of yohimbine, but it is not clear whether this effect on synaptosomal TH activity is mediated by NA receptors or whether some other unknown mechanism is involved.

The general conclusion from the experiments in sections 3 and 4 is that the changes found in synaptosomal tyrosine hydroxylase activity after acute administration of drugs are not yet explicable in terms of the known effects of the drugs which have been briefly reviewed here.

EXPERIMENTS : SECTION 5

THE EFFECT OF REPEATED ADMINISTRATION OF DRUGS ON TYROSINE HYDROXYLASE ACTIVITY.

Rationale:

Long term changes in TH activity in noradrenergic neurones in the CNS were discussed in section 12 of Chapter 3. Reserpine has been shown to cause induction of TH with increased enzyme protein in the cells of the LC, and 'delayed augmentation' of TH activity in noradrenergic projection areas (Reis et al, 1977). Oxotremorine causes 'delayed activation' of TH without a change in amount of enzyme protein; and neurotoxic lesions of NA neurons resulted in long lasting reduction of TH activity. Synaptosomal TH activity did not follow the changes in solubilised activity after reserpine.

Other stimuli, such as cold, immobilisation, and electroconvulsive shocks, were found to produce increased brain TH activity in a range of assays, with varying concentrations of tyrosine and cofactors (see Table H). Mandell (1978) discussed the difficulties in interpreting results obtained under very different conditions. Stimuli associated with activation of NA synthesis have also given increases in NA turnover, firing rate in NA neurons, and in plasma levels of NA (see Stone, 1975; and Benedict et al, 1978).

The experiments to be described in this section were designed as an investigation of TH activity during long term administration of drugs which alter the firing rate of NA neurons . As will be shown later,repeated administration of drugs can produce changes that may be different from acute effects in ways that merit further study , especially in NA neurones. For

example, if acute and chronic administration of a drug produce different effects on firing rate, the question arises whether changes in NA synthesis rate or synthesising capacity accompany the adaptation of the organism to, say, opiate or tricyclic administration.

REPEATED ADMINISTRATION OF DRUGS.

The effects of repeated daily administration of morphine and desipramine on synaptosomal and soluble tyrosine hydroxylase activity were investigated in the experiments reported here, looking for evidence of changes in synthesis rate in synaptosomes and/or changes in maximal synthesis potential as measured in solubilized enzyme preparations with saturating conditions. As Mandell et al (1975) pointed out, if firing rate were maintained over long periods at a lower than normal rate, there might be negative induction of the rate-limiting enzyme .

The effects of clonidine administration have also been studied over a similar period because this is another drug which reduces firing rate in locus coeruleus cells and may also cause adaptive changes in NA neurons, as shown in a study by Kane and Johnson, (1979). These authors administered clonidine in drinking water. Since it has been reported (Reis, Hess and Azmitia,1970) that repeated saline injections led to a significant reduction of TH activity in the caudate nucleus, compared with activity in non-injected controls, both saline-injected and non-injected control groups were incorporated in the design. TH activity in both noradrenergic and dopaminergic brain regions was measured after repeated clonidine administration either by intraperitoneal injection or in drinking water.

An investigation of the effects of chronic 2-deoxyglucose administration was included here. Ritter et al (1977) have shown that repeated

administration of this drug prevented the acute depletion of NA in the hypothalamus after a single session of footshock. This suggested an increased potential for synthesis and/or release of NA in these animals, localized to the hypothalamus rather than the telencephalon. We therefore used Ritter's schedule of 2-deoxyglucose injections and studied synaptosomal TH and solubilized TH activity to test the hypothesis that either synthesis rate or synthesising potential would be increased in the hypothalamus rather than in the hippocampus.

WITHDRAWAL AFTER REPEATED EXPOSURE TO DRUGS.

The 'withdrawal syndrome' is the term used to describe the constellation of effects following abstinence from drugs such as the opiates, barbiturates, and alcohol. Unpleasant withdrawal effects (nightmares, insomnia, and restlessness amongst others) are also found in clinical practice following abstinence from many different hypnotic and sedative drugs including the benzodiazepines and tricyclic antidepressants. Recently a syndrome of 'rebound hypertension' has been identified following cessation of clonidine administration as a hypotensive agent (see Lowenstein, 1980), and this too has been termed a withdrawal effect.

One component of the withdrawal syndrome , certainly in morphine-abstinent rats (Aghajanian, 1978) and possibly also with other drugs and species (see Redmond,1979), is an increased rate of firing in the locus coeruleus. This may be due to diminished inhibitory receptor effectiveness (reduced numbers of receptors?) at the locus coeruleus after long term reduction in firing rate. Clonidine,an $alpha_2$ agonist, can reduce firing rates in the locus in opiate-abstinent rats (Aghajanian, 1978) despite the presence of naloxone, the opiate antagonist; and clonidine has been shown to reduce some of the symptoms of withdrawal in heroin and methadone addicts (Gold et al, 1978).

The synaptosomal and soluble TH rate in brain regions were studied in rats undergoing withdrawal from chronic clonidine or morphine treatment, to investigate the relationship between synaptosomal enzyme activity and synthesis potential (saturating TH assay) at a time of increased firing rate in the locus coeruleus . Little is known about the relationship of firing rate to changes in synthesis rate and synthesising potential in the CNS during chronic drug administration . We do not know, for example, whether a reduction in amount of TH ('negative induction') will lead to a limitation of transmitter synthesis rate in the terminals of NA neurones, and what relationship this bears to firing rate. (See page 90)

A definitive study in this field would require simultaneous monitoring of electrophysiological phenomena and biochemical measures of in vivo synthesis rate, such as, for example, the conversion rate of infused 3H-tyrosine to 3H-NA or its metabolites. Such comparisons as we can make with the data accumulated in the present study and previous investigations using electrophysiological and biochemical methods (see Tables 5A and 5B) are complicated by differences in methodology (see Korf,1980, and Chapters 2 and 3). For example, electrophysiological studies have until recently used only acute preparations with anaesthetised rats.

Also, most studies on synthesis and turnover of NA report on the accumulation of NA or precursors or metabolites over periods of 15 - 240 minutes in vivo. The studies presented here, on the other hand, report enzyme activity measured post-mortem in synaptosomes freshly prepared, or in solubilized enzyme activity measured after freezing and thawing. Given the recent evidence (Salzman and Roth, 1980a; summarized in section 10 of Chapter 3) of changes in forebrain TH which were perceptible post-mortem and paralleled in vivo measurements of synthesis and turnover after electrical stimulation of the locus coeruleus, we have grounds for pursuing the hypothesis

that TH activity will serve as an indicator of important aspects of functional activity in NA neurones.

SECTION 5 a: EFFECTS OF REPEATED DESIPRAMINE ADMINISTRATION.

INTRODUCTION

Electrophysiology:

McMillen's recent study (1980) showed a significantly reduced rate of firing of LC cells (mean 1.09 $\pm$0.24 Hz) in female rats anaesthetized after 7-9 days treatment with desipramine (DMI) at 5 mg/kg twice daily. The firing rates were intermediate between those of untreated controls (1.73 $\pm$ 0.14 Hz) and those after a single dose in a naive rat (0.5 $\pm$ 0.11 Hz) . There was tolerance to a small acute dose of DMI in the chronically treated rats,in that the firing rate dropped only slightly in response to the injection; but the alpha$_2$ antagonist dibozane (1 mg/kg) increased the firing rate to some extent in all groups of rats, showing that autoreceptor inhibitory control of firing rate was still operating during treatment with DMI.

Biochemical measures:

The acute effects of a single dose of DMI on TH activity and NA metabolism have been discussed in Section 3 of this Chapter. These effects seem to last less than 24 hours, since studies measuring TH activity and NA metabolism after this time show no change with respect to controls (Nielsen and Braestrup,1977; and Mandell et al, 1975).

Biochemical measures of NA levels,synthesis and turnover after chronic treatment with DMI yield a very confusing picture. The earlier versions of the 'catecholamine hypothesis of depression' held that antidepressants acted by increasing 'available' NA and alleviating the lack of NA thought to be at the root of depressive symptomatology. Some results have upheld this hypothesis, and have shown increased NA synthesis or 'utilization '

after repeated DMI treatment (Rosloff and Davies, 1974; Sugrue, 1980). Other studies, however, using similar methods in some cases, have shown the opposite; in fact a large number of studies, using various measures of NA synthesis and metabolism, have shown either no change or a reduction in NA functional activity after 4 - 21 days of repeated daily or twice-daily injections of DMI (see Table 5.1).

The timing between injection of the last dose and recording of experimental measures may be an important factor in interpreting these studies; studies which measure long term effects by allowing an interval of more than 12 hours to elapse after the last dose of DMI avoid this pitfall. Changes seen at this time can presumably be taken , if they remain stable, as manifestations of a new resting state ; one that is a result of adaptation to DMI, but maintained by repeated dosage of the drug.

Another possible explanation for the variation in these results is that NA synthesis rate changes gradually in the course of chronic DMI treatment, ie that there are adaptive changes which result eventually in tolerance to the acute effects of DMI . Nielsen and Braestrup (1977) propose 3 stages in adaptation to DMI treatment. The results of injecting rats for 4,10 or 20 days in their study show a significant reduction in 3H-MOPEG accumulation after intraventricular injection of 3H-tyrosine after 4 and 10 days treatment, but no reduction after 20 days.

Changes in the sensitivity of alpha-2-receptors may also occur during chronic administration of DMI, and must be taken into account to interpret the biochemical and electrophysiological studies discussed above. As we have seen, these receptors may be involved in the control of synthesis and release of NA .

TABLE 5.1

CHRONIC DESIPRAMINE TREATMENT AND NORADRENERGIC FUNCTION IN RAT BRAIN

No. days	Dosage (mg/kg)	Hrs	Region	Assay used	Result	Reference
3	10,bd	2	whole brain	MOPEG	no change	Bareggi et al 1978
4	20		whole brain	MOPEG	reduced	Tang et al 1979
4	10,bd	24	whole brain	MOPEG	reduced	Nielsen et al 1977
	"			3H-MOPEG	reduced	"
4	10,bd	2	whole brain	3H-TYR-NA	3H-NA reduced	Rosloff et al 1978
7	5,bd	16	whole brain	MOPEG	no change	McMillen et al 1980
				AMT-NA	no change	"
8	10,bd	?	hipp	SUBSAT TH	reduced	Segal et al 1974
10	10,bd	24	whole brain	MOPEG	no change	Nielsen et al 1977
				3H-MOPEG	reduced	"
14	10,bd	2&7	whole brain	3H-TYR-NA	3H-NA reduced	Rosloff et al 1978
14	10	24	whole brain	MOPEG	increase	Sugrue et al 1980
14	10	27		AMT-NA	depletion increased	"
14	?	6		MOPEG	increased	Schildkraut 1976
20	20	?	whole brain	MOPEG	reduced	Tang et al 1979
21	10,bd	24	whole brain	MOPEG	no change	Nielsen et al 1977
21	10,bd	24	"	3H-MOPEG	no change	"
21	10,bd	24	"	DOPEG	reduced	"

Hrs = time after last dose.
Once daily dosage except where bd indicates twice daily dosage.

The tools for measuring changes in number and binding affinity of alpha-2-receptors are the agonist drug clonidine and the antagonist yohimbine.

Recently it has been proposed that chronic DMI treatment may result in reduced alpha-2-receptor sensitivity. Crews and Smith (1978) used a peripheral preparation - atrial strips contracting in response to electrical stimuli of different frequency - to show that whereas NA uptake was consistently inhibited during chronic DMI treatment, the frequency-response curve shifted to the left and the efflux of 3H-NA (both impulse-evoked and impulse-independent) progressively increased over 3 weeks of treatment.Alpha-blockade by phenoxybenzamine produced the increased responsiveness early in the treatment period. The authors propose a gradual desensitization of the alpha-2-receptor as the explanation for the chronic effect of DMI. This desensitization , whe ther it takes the form of reduced numbers of receptors or reduced affinity for ligands, could allow high concentrations of NA to be maintained despite lower than normal rates of firing in the LC, by dissociating release from mechanisms sensitive to extracellular NA concentration.

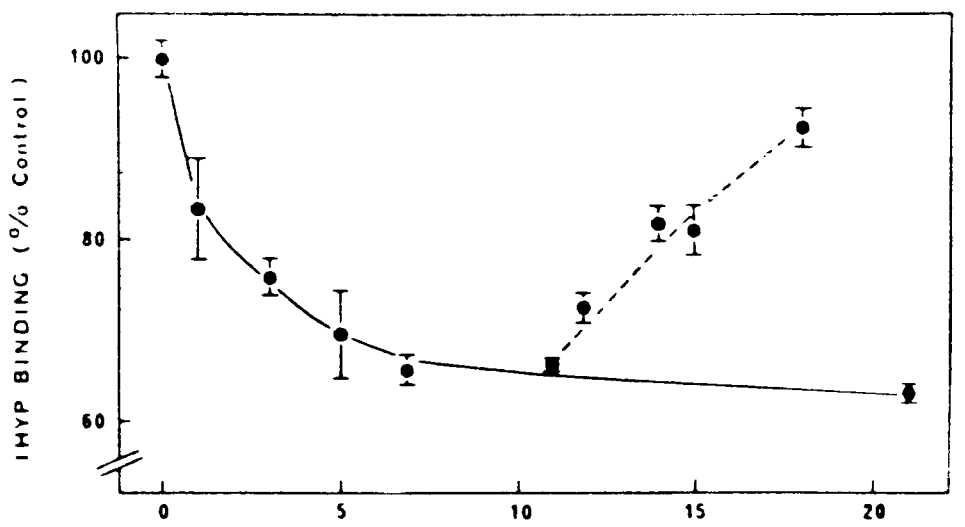
Several studies in the CNS yield further evidence for this proposition. Tang et al (1979a) showed that the reduction in MOPEG after a single dose of clonidine was attenuated after 4 or 21 days of DMI treatment,although DMI alone also reduced MOPEG. McMillen et al (1980) also found reduced sensitivity to clonidine after 12 days of DMI, in terms of the dose required to produce a reduction in MOPEG levels. Ursillo et al (1980) have shown an increase in alpha-2-binding sites after DMI treatment, which is difficult to reconcile with decreased sensitivity to clonidine. However, they have also reported preliminary results which suggest a synergistic effect of DMI on the duration of the rise in MOPEG after yohimbine administration,which agree with other reports of synergistic effects of DMI and yohimbine (Quinton,1963; Sanghvi and Gershon,1977).

Although some of these results are difficult to interpret unambiguously, the notion of alpha-2-desensitization during chronic treatment with antidepressants like DMI may be useful in the development of a satisfactory model for the clinically relevant modes of action of these drugs, which take several weeks to produce a therapeutic effect. Uptake blockade of monoamines is an immediate and maintained effect of some, but by no means all, of the antidepressants. It is therefore encouraging to find long-latency changes which correspond more closely with the clinical time-course of improvement in depressive symptomatology.

Such changes are also found in postsynaptic beta-adrenergic receptors in the CNS. It now appears that reductions in numbers of both $alpha_2$ and beta receptors in various brain regions are likely to be important (see FIG.5a) in mediating the common antidepressant effects of such varied therapies as medication with tricyclic drugs, monoamine oxidase inhibitors, iprindole, mianserin, and electroconvulsive shock (Bergstrom and Kellar, 1979; Vetulani et al,1976; Ursillo et al, 1980; Huang, 1979; Schweitzer, 1979; and see review by Sulser, 1978). A recent development is the discovery that the antidepressant-induced decrease in postsynaptic numbers of beta-receptors in the brain can be accelerated by blocking the alpha-2 receptors, ie by allowing 'flooding' of the postsynaptic membrane by a maximum amount of NA in the extracellular space (Paul and Crews, 1980; Ursillo et al,1980).

As Mandell et al (1975) speculated , the effects of the tricyclic drugs on the functioning of the noradrenergic neuron itself may merely exemplify one component of a complex adaptation process. The common element in antidepressant therapies may be the change in receptor activity at the postsynaptic neuron, for example. The overall result, whatever the mechanism of action of the different treatments, is presumably to counteract whatever is

FIG. 5a Effect of DMI administration on beta-receptor density in rat cerebral cortex.
(from Wolfe et al, 1978)



Effect of DMI administration on the density of *beta* adrenergic receptors in rat cerebral cortex. The amount of IHYP specifically bound (in femtomoles per milligram of protein) was determined in homogenates of rat cerebral cortex at different times after initiation of treatment with DMI. Each point is the mean ± S.E.M. of values from 12 to 33 animals combined from eight experiments. IHYP binding was measured in assays that contained one concentration of IHYP varying from 0.04 to 0.13 nM. All values on the solid line are significantly different from control ($P < .01$). Results are expressed as percentage of control binding (●——●). After 11 days of DMI treatment the recovery of binding sites was determined at 1, 3, 4 and 7 days (●- - -●). The ordinate is broken at 55% of control values.

maintaining the 'depressive' state in the brain, and to produce a new set of relationships between firing rate, release rate, transmitter synthesis rate,and receptor function in noradrenergic neurons and also in interacting parts of the brain. The interactions between these various parameters of noradrenergic function are as yet poorly understood. The present study is an attempt to assess synthesis rate in synaptosomes and solubilised TH activity in various brain regions after 3 weeks treatment with DMI, using a long injection - assay interval. These results will then be compared with those of other drug treatments which are thought to affect alpha-receptors and/or firing rate of NA neurons.

METHOD:

Desipramine (Geigy Pharmaceuticals) was dissolved in warm saline at 10 mg/2 ml (pH 6.0) . A group of 5 male Sprague Dawley rats were injected daily with 10 mg/kg of this solution intraperitoneally. Injections were given between 10 am and midday for 20 days. A group of control rats was injected daily with saline. During the injection period , food availability was gradually reduced to a 23-hour deprivation schedule for both groups, to cut down weight gain. Water was available ad lib.

About 20 hours after the last injection, all rats were killed; hippocampus, hypothalamus and locus coeruleus (LC) were dissected on ice. Synaptosomal and solubilized TH assays were performed as described in Chapter 4, and the results are described below. Subsaturating TH assays were also performed, and these results are to be found in APPENDIX I, Table C).

RESULTS:

The injections of DMI were well tolerated for the first three days. On the 4th and subsequent days, the DMI group appeared more irritable on handling and injection than the controls; they also dropped a larger number of faecal pellets during the injection session. From about the 7th day onwards, the DMI group were noted to have some slight loss of tone on handling, and a tendency to sit immobile and to explore much less than the saline group when placed in the weighing scales. Also, a characteristic behaviour pattern was seen in the DMI animals ,starting immediately after the daily injection and lasting for a few minutes. The abdominal musculature appeared to contract and the rats crawled slowly around the cage as if squeezing themselves through a narrow opening, sometimes pressing up against the side of the cage. After a few minutes this stopped and all the rats looked normal, if a little quieter than the controls.

WEIGHTS:

There was no significant difference in weight between the two groups at the beginning of the experiment (Saline group, mean 253 $\pm$ 15 g; DMI, mean 264 $\pm$ 5 g). DMI animals lost more weight during food deprivation than controls, possibly owing to the sedative effects of the drug. At the end of the experiment, the DMI rats' mean weight was 88% of their original weight, whereas the saline group had a mean weight representing a net gain of 8% with respect to their original mean weight.

However,there was no significant correlation between weight and TH activity in hippocampus or hypothalamus.

TH ACTIVITY :

The results for hippocampus and hypothalamus in 2 different assays (synaptosomal and soluble saturating TH) are shown in Table 5.2. TH rates are recorded as fmol/min/mg P2 protein, and the results are also expressed as a percentage of the appropriate control value ± S.E.M.

TABLE 5.2

EFFECT OF 20 DAYS DESIPRAMINE TREATMENT

ON TH ACTIVITY IN RAT BRAIN.

	SYNAPTO. TH	SATURATING TH
HIPPOCAMPUS		
SALINE (n=5)	211 ± 14	2354 ± 218
DMI (n=5)	196 ± 12	1775 ± 206
HYPOTHALAMUS		
SALINE (n=5)	644 ± 12	16,350 ± 920
DMI (n=5)	658 ± 45	13,260 ± 1150
LOCUS COERULEUS		
SALINE (n=5)	-	5381 ± 410
DMI (n=5)	-	4826 ± 595

fmol/min/mg P_2 protein

Synaptosomal TH activity was unchanged by DMI treatment for 20 days; neither hippocampus nor hypothalamus showed any difference from controls injected with saline. The mean TH activity in the DMI group was 93% of control in the hippocampus, and 102% of control in the hypothalamus. But there was a significant reduction in maximum synthesis potential, as measured by TH activity with saturating cofactor and substrate concentrations. A two way ANOVA with TREATMENT and BRAIN REGION as factors showed significant main effects of TREATMENT ($\log_{10}$ transforms of data: $F = 7.06$, df 1/24, $p < 0.025$) and of BRAIN REGION ($F=205.9$, df 2/24, $p < 0.001$). The interaction term was not significant. The mean saturating TH activity was reduced by 25% in the hippocampus in the DMI group, and by 19% in the hypothalamus; in the LC, the DMI group mean was reduced by 10 % of the control activity.

DISCUSSION:

Desipramine is a drug which lowers the firing rate in the locus coeruleus, both acutely and after chronic administration (McMillen, 1980). If synaptosomal TH activity is an indicator of noradrenergic activity in vivo, then we would expect to see a reduction in this indicator as a result of chronic DMI treatment. (It was reported in Section 3 that a single dose of DMI produced no change in hippocampal synaptosomal TH, and that there was some indication, albeit not statistically significant, of a rise in the hypothalamic synaptosomal TH rate.)

In this experiment, measuring TH activity in synaptosomes from animals killed about 20 hours after the last of 20 daily injections of DMI, no evidence was found for any difference between DMI and saline groups. If there is indeed a lowered firing rate in the LC at this time , then we must

postulate a dissociation between firing rate in vivo and TH activity in post mortem brain issue.

The considerable reduction in TH activity in the saturated enzyme preparation of both hippocampus and hypothalamus bespeaks a reduction in total NA-synthesising capacity, such as would accompany a reduction in the total amount of enzyme in the neuron. The question of a reduction in the quantity of protein as such must await confirmation with immunoprecipitation techniques; if this were successful, we could reasonably term this phenomenon 'negative induction'. If it arises by a process analogous to the enzyme induction described by Thoenen(1970) in the adrenal medulla , and by others in the brain (eg Crespi et al, 1980; and see Chapter 3, section 12), then the sequence of events is probably as follows. A sustained change in firing rate at the locus coeruleus, probably caused by some trans-synaptic input, is followed after 24-48 hours by a measurable change in TH enzyme synthesised in the LC. This is followed in turn by a delayed change in enzyme activity under saturating conditions in terminal regions, in parallel with a change in enzyme protein in these terminal regions, delay time being related to distance from the LC because the change is dependent on transport of newly synthesised TH from cell body to terminals (Black, 1975). There is some evidence that induction can take place within 12-48 hours in the LC, after reserpine, with a gradual return to normal levels over two or three weeks.

In the experiment reported here, the LC shows a slightly reduced saturated TH level after 20 days of DMI treatment, and the activity in terminal regions was markedly lowered at this time. This could mean that after a period of reduced synthesis in the cell bodies, the neuron has undergone further adaptation and enzyme synthesis may have returned toward normal, although the delay in axonal transport time to terminals means that the enzyme quantity in terminals is still low.

The finding of a fall in saturated enzyme activity in the absence of a change in synaptosomal activity is compatible with the hypothesis of negative induction of TH; previous work in this laboratory has demonstrated that changes in synaptosomal activity do not necessarily accompany changes in saturated TH activity (Boarder and Fillenz, 1979). Another finding which supports negative induction as a mechanism in this experiment is that both terminal regions assayed showed a fall in saturated TH activity. We have already seen (sections 3 and 4) that the various terminal regions innervated by the LC do not necessarily respond alike to stimuli which change LC firing rate and other indices of NA function.

The studies outlined in Table 5.1 are fairly evenly divided between those showing no change in NA function during repeated administration of DMI and those showing a reduction, with two studies suggesting an increased turnover of NA. Schildkraut's experiments monitored the disappearance of injected 3H-NA from the brain after intracisternal injection. This technique is clearly not a direct measure of NA synthesis, and involves assumptions which are tenuous at best, as discussed above in Chapter 3 . The work of Sugrue et al (1980) stands against a number of other studies in showing an increase, rather than a decrease , in the rate of accumulation of MOPEG in the brain. The chief problem with this technique, which is becoming increasingly popular, is that MOPEG only accounts for the metabolism of less than half the total NA in the brain (Korf,1980; Nielsen and Braestrup, 1977). A further disadvantage is that DMI has some activity as an inhibitor of monoamine oxidase (Roth and Gillis,1974) , and may therefore exert some influence on the metabolic fate of NA , in addition to effects on synthesis rate and neuronal firing rate. This will complicate the comparison with control animals.

The experiments by Tang et al (1979a) and by Nielsen and Braestrup (1977) in Table 5.1 both include groups of animals with 4 days and 20 days of

DMI administration. Both studies used 20 mg/kg/day which is twice the dose used in the experiment under discussion here. At 4 days of treatment, there is agreement on a reduction of MOPEG accumulation and Nielsen and Braestrup also report a reduction in 3H-MOPEG synthesis after injection of 3H-tyrosine intraventricularly. After 20 days of DMI treatment, Tang et al still report a reduced MOPEG accumulation; Nielsen and Braestrup do not find a reduction in MOPEG or 3H-MOPEG at this time, though they find a small decrease in DOPEG accumulation and support the possibility that DMI affects both synthesis rate and metabolic routing of NA .

The present results are compatible with Nielsen and Braestrup's finding of a normal synthesis rate for NA , 24 hours after the last of 20 days of DMI treatment. The present findings do not contribute constructively to the confusion about alteration of metabolic pathways for NA. But they do constitute an additional finding, namely reduced synthesis potential for NA in hippocampus and hypothalamus at this time.

We do not know what mechanism is responsible for initiating the reduction in TH enzyme synthesis, if such is the case. One possibility is that it is associated with a prolonged decrease in firing rate in the LC , which is indicated as a possibility by McMillen et al (1980); by analogy with the experiments discussed in Chapter 3 (section 12), a decreased Vmax would be expected with no change in Km for tyrosine or cofactor . Thus synthesis of TH itself might be under the control of factors which also influence firing rate, although synaptosomal TH activity does not reveal any change after DMI treatment. Segal et al (1974) found reduced TH activity in the hippocampus and the locus coeruleus after 8 days of DMI 10 mg/kg, injected twice daily. Dopaminergic TH in the caudate showed a slightly reduced activity. The assay system in these experiments , however, contained labelled tyrosine at 3 μM, and DMPH4 at 1 mM; therefore it is not yet clear from this reduction in TH

activity under subsaturating conditions whether negative induction has occurred with a single week of DMI treatment.

Another possible initiating stimulus for a reduction of TH synthesis in the cell body is provided by the receptor changes discussed above . Early in treatment, the effect of reduced NA uptake on autoreceptors may contribute to lowering the rate of firing in the LC. Later the autoreceptors seem to become 'desensitized' as a population, and receptor numbers drop, as evidenced by a fall in Bmax for alpha$_2$ receptors.

A third possible mechanism for reducing TH levels in the noradrenergic neurone is the operation of a trans-synaptic feedback loop from the postsynaptic neurons in LC projection areas. Ongoing changes which could theoretically contribute from this site are exemplified by changes in cyclic AMP responses (eg Stone, 1979) and in populations of beta-receptors, which could provide output for a polysynaptic pathway impinging on the LC. In terms of available mechanisms, then , there is no shortage of possibilities. At present we cannot distinguish between these mechanisms.

SECTION 5 b : REPEATED ADMINISTRATION OF MORPHINE AND SUBSEQUENT WITHDRAWAL,PRECIPITATED BY NALOXONE.

INTRODUCTION:

Electrophysiology. Aghajanian (1978) has demonstrated that in anaesthetized rats, implantation of morphine pellets in the subcutaneous tissues results in a longlasting inhibition of firing rate in the locus coeruleus. However, after regular daily pellet implantation for 4 or 5 days, the firing rate in the LC returns to normal. This is an example of tolerance to the inhibitory effect of morphine. On injection of naloxone, either iontophoretically onto cells in the LC or intravenously into the tail vein, there is a rapid increase of firing rate up to 200% of the previous resting rate. These results were taken to indicate the likely presence of inhibitory opiate receptors on the cells of the LC. Bradley and Dray (1974) showed no change in single unit responses to NA during development of tolerance to and withdrawal from morphine .

Biochemical studies of the effects of chronic morphine administration on noradrenaline metabolism in the brain have yielded conflicting results, somewhat at variance with the concept of turnover and synthesis being related to firing rate. Maynert and Klingman (1962) found brain NA levels to be normal or slightly raised in morphine-treated rats. But Johnson et al (1974) report increased conversion of 14-C-tyrosine into labelled NA and DA into brains of rats on chronic morphine treatment. Reis,Hess and Azmitia (1970) found increases in TH activity (assayed according to Nagatsu, 1964b) in adrenal gland and in caudate nucleus after 15 days of morphine injections with respect to saline-injected controls. Clouet et al (1973) also found evidence of increased TH activity in hypothalamus .

However, in contrast to these studies, Branchey & Friedhoff (1976) found a delayed reduction in soluble TH in striatum several days after implantation of morphine pellets subcutaneously, and Branchey et al (1974) found a significant reduction in caudate TH activity at a tyrosine concentration of 100 µM after 3 weeks of morphine treatment as well as after chronic ethanol. Most of these and similar studies found that the most marked changes occurred in dopaminergic regions, such as the striatum, and mixed dopaminergic and noradrenergic areas such as the hypothalamus, although Cicero et al (1973) found no change in caudate or midbrain-pons TH, several days after implantation of a morphine pellet .

Roffman et al (1977) examined MOPEG levels after chronic morphine administration ; 16 hours after the last injection, MOPEG was reduced in hypothalamus and cerebellum. A further dose of morphine produced an increase in MOPEG in the hypothalamus, though not in other regions. They suggested that tolerance to the acute effects of morphine is not manifested in the hypothalamus. The mechanism of action of chronic morphine on catecholaminergic neurons is not known. The result of Reis et al (1970) has been quoted as an example of enzyme induction , but this is unlikely, at any rate as regards the caudate nucleus in which induction has never been demonstrated by immunoprecipitation (Reis and Joh, 1978). It seems possible that the changes in dopaminergic neurons may represent changes in turnover and metabolism of DA independently of changes in the activity or amount of TH (Marshall and Smith, 1974; Iwamoto and Way, 1979).

Although Clouet and Iwatsubo, in a review in 1975, felt that both NA and DA mechanisms were important in mediating the effects of the narcotics such as morphine, a more recent review by Way and Glasgow (1978) suggests that NA and DA are probably not specifically involved in the development of tolerance to and dependence upon the opiates. They point out recent studies in

which increases in synaptosomal or vesicular calcium accumulation increases have been shown after chronic morphine treatment ; naloxone is a specific antagonist of this, as of many other effects of morphine. But such changes as these could affect a variety of neuronal systems in the brain.

However, there is little doubt that the effects of withdrawal from opiates include a definite impact on NA neurons (Iwamoto and Way, 1979). Several studies have used naloxone, a relatively pure opiate antagonist ,to precipitate withdrawal, and have found a marked and specific activation of noradrenergic neurons, not only in terms of firing rate (Aghajanian , 1978) but also including an increased accumulation of metabolites such as MOPEG (Crawley et al,1979)and increased utilization of NA as measured by the rate of depletion of NA in hippocampus and cerebral cortex after synthesis inhibition with alpha-methyl tyrosine (Laverty and Roth, 1980). A different line of evidence (Schwartz, *et al* 1978) suggests that postsynaptic adrenergic receptors may play a part in opiate withdrawal. Postsynaptic beta receptors may, it is hypothesised, be subject to disuse hypersensitivity during chronic morphine treatment; upon withdrawal, when the neuron firing rate increases, the exaggerated postsynaptic responses from these receptors may contribute to the withdrawal syndrome.

The experiments in this section were performed in order to clarify the effect of morphine administration on TH activity in noradrenergic brain regions (including the hypothalamus with its mixed NA and DA innervation), both during the development of tolerance to morphine administration when LC firing rate is normal or lower than normal, and during withdrawal precipitated by naloxone when firing rate is markedly increased.

METHOD:

Experiment I : In the first experiment, two groups only were used. Five male Sprague Dawley rats were injected with morphine sulphate (obtained from commercial sources) three times a day with doses of morphine gradually increasing from 10 mg/kg to 120 mg/kg, giving large doses at night to ensure maintenance of high drug levels throughout. The injection schedule was as follows (adapted from a previous study, Graham-Jones 1974, unpublished). Doses are in mg/kg; morphine sulphate powder was dissolved in normal saline daily to give a volume for each rat of between 0.4 and 1.0 ml,injected intraperitoneally.

Day	9am	1pm	7pm
1	-	10	15 mg/kg
2	20	35	50
3	30	50	70
4	40	70	100
5	60	90	120
6	100	-	-

On the sixth day, approximately 1 hour after the last injection of morphine, these rats were injected with 4 mg/kg of naloxone (Winthrop Laboratories) intraperitoneally. 15 minutes later, each rat was stunned and decapitated and brain dissection into regions was performed on ice. The control group consisted of 5 rats left undisturbed in home cages intil the killing session, when they were paired with injected rats and killed along with them.

Experiment II : In this experiment, 20 male Sprague Dawley rats , housed in cages of 4 or 5 rats, were randomly allocated to one of 4 groups; untreated CONTROLS, SALINE injected group, MORPHINE injection group, and MORPHINE-WITHDRAWAL (MWD) group. The first and last groups were

treated exactly like the two groups in the first experiment. The saline group was given injections of saline matched to the injection volumes of the morphine group, and half this group were given naloxone as the last injection, whilst half had another saline injection. The third group was treated with morphine on the same schedule as before but was given saline instead of naloxone as the last injection.

The morphine sulphate used in this experiment was obtained from the Radcliffe Infirmary pharmacy and was made up as a solution of 10 mg/ml or 50 mg/ml for vetinerary use as intramuscular injection.

Thus the comparison of rats undergoing naloxone-precipitated withdrawal with untreated controls could be made in both experiments, whereas the effects of chronic morphine administration per se in comparison with saline injected controls could only be tested in the second experiment. This schedule of injections induces tolerance to morphine, as shown by the large doses tolerated after a few days. These doses would be lethal in naive rats. A one-way analysis of variance was performed on the four treatment groups of the second experiment, and two way analysis of variance with replications as a factor on the CONTROL and WITHDRAWAL groups taken from both experiments.

RESULTS :

Morphine injections were well tolerated on the whole, although one rat died on the 3rd day of injections in Experiment II ,and several animals seemed sedated at times. The naloxone injection had a marked and rapid effect in Experiment I; the rats became irritable to handle, with diarrhoea and red-rimmed eyes.In the second experiment, the saline-naloxone rats seemed unaffected by naloxone, whereas the morphine-naloxone group showed some, though not all, of the signs noticed in the first experiment.

SYNAPTOSOMAL TH: The results of synaptosomal assays of TH activity in hippocampus and hypothalamus are shown for the two experiments in Table 5.3 . There was no difference in Experiment II between saline and control groups, nor between these groups and the morphine-treated groups, nor between saline-saline and saline-naloxone groups. This applies to both brain regions studied. In the first experiment, however, both hippocampus and hypothalamus showed increased TH activity in synaptosomes from the morphine-withdrawal group. Two-way analysis of variance (with REPLICATIONS as a factor) of the data from the control and withdrawal groups from both experiments gave a variance ratio of 2.031 (df 1/19) for the main effect of TREATMENT, which did not reach significance ($p< 0.2$). A similar analysis of hypothalamic data gave a significant effect of TREATMENT ($F=4.40$,df 1,20,$p< 0.05$) ; REPLICATIONS was also significant as a main effect ($F=56.23$,df 1,20, $p< 0.001$) But there was also a significant interaction term ($F=8.18$, df 1,20,$p< 0.01$), since the difference was not found in both experiments. Because of this discrepancy between the two experiments, paired t tests confined to the question of a difference between control and morphine-withdrawal animals in the first experiment were performed, ignoring the data from the second experiment. These showed a 21% increase in synaptosomal TH in the hippocampus which did not reach significance ($t=1.64$, df 4, $p< 0.2$) and a 58% increase in the hypothalamus which was significant ($t=2.99$. df 4, $p< 0.05$).

TABLE 5.3

EFFECT OF REPEATED MORPHINE ADMINISTRATION AND WITHDRAWAL ON SYNAPTOSOMAL TH ACTIVITY IN RAT BRAIN REGIONS.

	CONTROL	SALINE	MORPHINE	MWD	
Hippocampus					
Expt I	217 ± 28 (5)	-	-	262 ± 25 (5)	
Expt II	122 ± 16 (6)	126 ± 7 (6)	121 ± 14 (7)	142 ± 20 (7)	
Combined	162 ± 25 (11)	-	-	192 ± 23 (12)	
Hypothalamus					
Expt I	626 ± 61 (5)	-	-	992 ± 88 (5)	*
Expt II	313 ± 50 (7)	291 ± 55 (8)	271 ± 56 (8)	292 ± 68 (7)	
Combined	444 ± 59	-	-	583 ± 116	

fmol/min/mg P_2 protein
MWD - Morphine Withdrawal.

* $p < 0.05$ (paired)

SATURATED, SOLUBILISED TH ACTIVITY: The discrepancy between the results of the two replications of this experiment was also seen in the soluble TH results,which are shown in Table 5.4. In the first experiment, a two way analysis of variance of the data after $\log_{10}$ transform to achieve normality, with BRAIN REGIONS as a factor , showed a significant main effect of TREATMENT ($F=7.43$, df 1,24, $p< 0.025$). The main effect of REGIONS was highly significant ($F=296.7$, df 2,24, $p< 0.001$). The interaction term ($F=0.05$) was not significant. A reduction in solubilized TH activity was found in all regions studied; the activity in morphine-withdrawn groups was 75 % of control in hippocampus, 82% of control in hypothalamus, and 80 % of control in the locus coeruleus.

In the second experiment,however, the saturated soluble TH in the locus coeruleus showed no significant variation across groups (one way ANOVA showed no significant effect of TREATMENT, $F=0.34$, df 3/26), and there was no significant difference between naloxone-treated rats and controls.

Other regions were not assayed for soluble TH activity in Experiment II.

TABLE 5.4

SOLUBLE TH ACTIVITY UNDER SATURATING CONDITIONS

AFTER REPEATED MORPHINE ADMINISTRATION AND WITHDRAWAL.

	CONTROL	SALINE	MORPHINE	MWD	MWD/CONTROL %
Hippocampus (fmol/min/mg P_2 protein)					
Expt I	1097 ± 12 (5)	-	-	821 ± 73 (5)	75 ± 7 %
Hypothalamus (pmol/min/mg P_2 protein)					
Expt I	14.14 ± 1.3 (5)	-	-	11.64 ± 1.8 (5)	82 ± 13 %
Locus Coeruleus (pmol/min/mg P_2 protein)					
Expt I	7.37 ± 0.6 (5)	-	-	5.87 ± 0.6 (5)	80 ± 8 %
Expt II	14.0 ± 1 (7)	14.8 ± 1 (8)	15.5 ± 1 (8)	15.6 ± 0.8 (7)	111 ± 6 %

numbers in brackets refer to number of rats in each group.

MWD refers to the morphine-withdrawal group.

DISCUSSION :

As indicated in the Introduction to this section,it was difficult to make a clear prediction as to the likely effect of chronic morphine administration on synaptosomal TH . As far as firing rate of LC neurones is concerned, there was evidence for a return to normal rates after a few days of morphine administration (Aghajanian, 1978; compare McMillen, 1980, on LC firing rates after chronic DMI treatment); but the biochemical results outlined gave conflicting results as to whether chronic morphine treatment results in an increase or decrease of catecholamine turnover .

However, there is a firm prediction of increased firing rate , turnover and release, and decreased NA levels shortly after morphine withdrawal precipitated by naloxone (Starke et al, 1977). We were able, in Experiment I, to demonstrate the predicted rise in synaptosomal TH activity in the hypothalamus and to a lesser extent in the hippocampus. But the hypothalamic rise was not replicated in the second experiment .

Unfortunately, the results from Experiment II,which was designed to investigate the effects of chronic morphine administration, show no differences between controls and morphine-tolerant groups in synaptosomal TH in hippocampus or hypothalamus , and throw no light on this question. The only known difference between the two experiments which was likely to contribute to this failure to replicate a significant finding lay in the drug used. Morphine sulphate was obtained from different sources for the two experiments . The reaction to naloxone as seen in the behaviour noted in the second experiment was less extreme than in the first experiment. If the morphine solution used in the second experiment was relatively less potent than the solutions prepared daily during the first experiment, this could also explain the failure to distinguish between morphine-treated and control groups in the second experiment.

Saturated solubilised TH activity was reduced in morphine withdrawn animals to 80% of control activity in all regions studied , in the first experiment. (No differences were found in the LC in the second experiment.) This reduction denoted a possible change in the quantity of TH enzyme in the cell body and terminals at this time. If this is so, then it is unlikely to represent a reaction to withdrawal since this has occurred so recently; it may be a result of chronic morphine administration. Now, a change in enzyme protein (negative induction) would be found first in the cell body and only several days later in terminal regions . Thus the change , if due to changes in enzyme production, would be expected to have developed in the LC at this time but not in the hippocampus or hypothalamus. This suggests that the mechanism involved may be of the long-lasting type without enzyme induction as such in the cell body.

The results of Experiment I suggest that there is a rise in synaptosomal TH activity during morphine withdrawal which may be greater in the hypothalamus than in the hippocampus; and that this is superimposed on a reduced total soluble enzyme activity in cell bodies and terminals. Unfortunately the question of whether the reduction in maximal soluble activity is a consequence of chronic administration, or of the morphine-abstinence syndrome, remains unresolved due to the failure to replicate these results in the second experiment using a different morphine preparation.

SECTION 5 c : REPEATED ADMINISTRATION OF CLONIDINE: AND SUBSEQUENT ABSTINENCE

INTRODUCTION

There have been no studies , to date, of the effect of prolonged administration of clonidine on the firing rate of the neurons of the locus coeruleus. But several reports of the behavioural and biochemical effects of clonidine administered daily over a period of a week or more are available.

Laverty and Taylor noted in 1969 that the hypotensive and sedative effects of clonidine were less marked after 8 days of clonidine administration (in drinking water at 3-6 ug/ml, and by injection of 0.2 mg/kg on days 1 and 8) than after a single injection. The chronically treated rats were more aggressive than controls; but behavioural testing for specific effects such as the reduction of conditioned avoidance responses was subject to great variation and no conclusion was reached as to the effect of chronic clonidine administration. There were no differences in NA or DA levels, nor in normetanephrine levels, between untreated controls and rats given 8 days of clonidine treatment.

Draper et al (1977) gave twice-daily clonidine injections at 0.05-0.1 mg/kg for 7 days to normotensive and hypertensive rats. Whole brain NA and DA utilization was measured as the rate of depletion of catecholamine after AMT; the effect of chronic clonidine was a reduction in this measure, as with a single clonidine injection (see Table 3.2). Svensson and Strombom (1977) showed that a dose of 0.1 mg/kg of clonidine reduced DOPA accumulation after DOPA-decarboxylase inhibition after 12 consecutive days of clonidine administration, and Tang et al (1979b) found that the ability of clonidine to reduce MOPEG levels in brain was not altered after 3 weeks treatment with saline or clonidine injections. These studies do not give any evidence for

tolerance to the effects of clonidine on NA turnover or synthesis, although tolerance to the behavioural effects has been found in other studies (Paalzow et al, 1978; Meyer et al, 1977).

Tyrosine hydroxylase activity was measured after 3 weeks of clonidine administration in drinking water (5 ug/ml) by Kane and Johnson (1978). Solubilised, triton-treated enzyme was assayed at 40 µM tyrosine concentration and 825 µM DMPH4, at pH 7.5; there was no change in TH activity in the superior cervical ganglion or in the LC, but there was a reduction of activity in the coeliac ganglion, suggesting a peripheral action possibly related to vasomotor control rather than to central noradrenergic activity.

However, the effect of abstinence from clonidine for 3-5 days after 3 weeks of clonidine treatment was an increase in TH activity ; the subsaturating assay described above showed an increase of 74% in the LC, 40% in the superior cervical ganglion, and 140% in the coeliac ganglion. Dix and Johnson (1977) showed a similar increase in adrenal TH. Since this was dependent on intact splanchnic nerves, it may be an example of trans-synaptic induction of TH in noradrenergic cell bodies. However, this remains to be established using saturating conditions for the assay and, of course, by immunoprecipitation studies.

Several other studies support the notion that withdrawal from chronic clonidine treatment results in activation of noradrenergic neurons. Increased DOPA-accumulation after DOPA-decarboxylase inhibition was shown in the brainstem, limbic forebrain and cerebral hemispheres, though not in the striatum, at 14-18 hours after a last dose of clonidine in the Svensson and Strombom (1977) study mentioned above ; MOPEG accumulation was increased at 28-48 hours after a last dose in rat brain in Tang et al (1979b); and

Lowenstein (1980), reviewing the clinical use of clonidine, refers to increases in plasma and urinary catecholamine levels after cesssation of clonidine treatment, as well as rebound hypertension. Svensson and Strombom, however, did not find an increase in utilization of NA after withdrawal, at the time of increased NA synthesis ; this may be evidence that changes in NA synthesis are not faithfully reflected in other measures of NA function, or a reflection of the possible distortions introduced by the use of enzyme inhibitors to measure NA synthesis rate (see Chapter 3, section 11).

In the experiment described in this section, clonidine was administered by intraperitoneal injection or in drinking water for two weeks, and the effects of this regime on synaptosomal and saturating soluble TH were measured. The two methods of administration were included in order to control for possible effects of repeated saline injections . In addition, some rats were kept abstinent from clonidine for a further 3 days and similar assays were carried out on these and simultaneous untreated controls to assess the effects of clonidine withdrawal.

METHOD:

30 male Sprague-Dawley rats were caged individually from the beginning of the experiment and fed ad lib. Drinking water was available ad lib but water bottles were weighed daily. The mean daily volume drunk in the preliminary week of the study was 29.4 ml/day, and the mean weight of the rats at this time was 230 g.

In the second week, the rats were divided into 6 groups of 5 rats.

C-DRINK rats were given a solution of clonidine (pure substance, Boehringer-Ingelheim) in distilled water (5ug/ml) in place of tap water to drink for a period of 15 days. Drinking bottles were weighed daily, but the rats were handled only as necessary for cleaning cages and for weighing on day 14.

C-INJECT rats were individually matched by weight to C-DRINK rats and served as a yoked group. The clonidine intake of each C-DRINK rat was measured daily, and this amount of clonidine was administered by subcutaneous injection to the appropriate C-INJECT rat (twice daily in divided doses at 10 am and 8 pm; clonidine ampoules,0.15mg/ml, Boehringer Ingelheim) on days 2-16.

SALINE rats were matched by weight to C-INJECT rats and were injected subcutaneously with similar volumes of normal saline twice daily on days 2-16.

CONTROL I rats were untreated, though their water consumption was recorded daily, and handling was kept to a minimum.

CLON-WD rats were treated exactly like the C-DRINK rats until day 16,when the clonidine solution was replaced by tap water. The rats were kept for a further 3 days and were weighed on day 18.

CONTROL II rats served as untreated controls for the CLON-WD group. They were weighed on days 14 and 18.

All rats were weighed on day 14. The first 4 groups of rats were killed on day 16, 2-4 hours after the last injection of clonidine or saline. Hippocampus, hypothalamus, corpus striatum and locus coeruleus were dissected and synaptosomal preparations were undertaken immediately. The locus and the P2 suspensions remaining after the synaptosomal assay were stored at -17°C for later assay of solubilized TH.

CONTROL II and CLON-WD rats, however, were kept in their cages for a further 3 days of abstinence from clonidine after the substitution of tap water for clonidine solution. These rats were all weighed on day 18 and killed on day 19.

RESULTS: CLONIDINE ADMINISTRATION FOR 15 DAYS.

Table 5.5 shows the group mean weights, mean intake volume and total clonidine dose over the 16-day period. The clonidine groups gained less weight than controls. One way analysis of variance showed a significant between-group variation in weight at day 14 ($F=4.13$, df 5/24, $p< 0.01$). Appropriate t tests showed that all clonidine groups weighed less than their respective controls on day 14 ($p< 0.005$).

However, there were significant positive correlations , in the clonidine-treated groups, between weight at day 14 and total clonidine dose ($r=0.64$, df=13 , $p< 0.02$), and between weight at day 14 and the mean volume of clonidine solution drunk daily ($r=0.79$, df=8, $p< 0.01$).

Overall, weight was also positively correlated with saturated TH activity (per mg protein) in the LC ($r=0.49$, df=17, $p< 0.05$). The separate correlation coefficients for clonidine-treated and control groups were 0.26 and 0.51 respectively; neither of these was significant at the 5% level.

TABLE 5.5

CLONIDINE ADMINISTRATION AND WITHDRAWAL EXPERIMENT

GROUP :	CLON INJECT	CLON DRINK	SALINE	CONTROL I	CONTROL II	CLON WD
GROUP MEAN WEIGHT,DAY 1: (g)	232 ±4	230 ±8	234 ±5	226 ±10	232 ±6	234 ±8
DAY 14:	283 ±7	283 ±9	335 ±15	321 ±20	336 ±13	282 ±13
DAY 18:	-	-	-	-	356 ±13	295 ±14
GROUP MEAN VOLUME DRUNK/DAY: (ml/day)	31.7 ±1.0	42.3 ±0.3	30.2 ±0.5	33.8 ±1.0	33.0 ±0.7	31.4 ±0.9
GROUP MEAN TOTAL DOSE CLONIDINE:	2.1	2.2	-	-	-	2.0 (mg)

The mean daily dose of clonidine was 0.52 mg/kg/day, which was similar to that in the Kane and Johnson (1978) study. The mean volume drunk per day was similar in all groups except the C-INJECT group, which drank more than the SALINE group throughout.

The results of this period of clonidine administration are shown in Tables 5.6 and 5.7 for synaptosomal TH and saturated solubilized TH in hippocampus, hypothalamus, corpus striatum and locus coeruleus. A summary of the results from different regions in terms of percentage difference from control values is shown in Table 5.9. As is clear from the SUMMARY table, no marked differences were found between controls and clonidine-treated animals. Nor was there any evidence of decreased TH activity in striatum (cf Reis et al, 1970) or any other region after 2 weeks of daily SALINE injections. The rats in Reis et al's study were immobilized in Plexiglass tubes for the daily injections, and possibly this immobilization , rather than the saline injection, was the cause of the change in TH activity found by these authors. In the present study , injections were carried out without restraining apparatus.

One way analysis of variance by treatment groups was carried out on each region for each assay; none of the variance ratios were significant at the 5% level. Also, a two way ANOVA with brain regions and treatment as factors gave no significant effect of TREATMENT ($F=1.07$, df 3/45).

Paired t tests for matched pairs were also performed on the synaptosomal and solubilized TH data, and this method revealed a significant decrease in the saturated TH activity in the striatum of clonidine treated animals ($t=3.346$, $df=9$, $p< 0.02$), but not in other regions. This is of interest since many studies have found that enzyme induction occurs in noradrenergic neurones rather than dopaminergic ones; less is known about negative induction (see section 5 a). The subsaturating enzyme activity in the striatum (at 40 μM tyrosine) was not significantly reduced in the clonidine treated animals ; the mean for the C-DRINK group was identical to the control mean, and the mean for the C-INJECT group was 97% of control (subsaturating assay only done on striatum,and not shown in the table).

TABLE 5.6

SYNAPTOSOMAL AND SATURATING SOLUBILIZED TH ACTIVITY IN HIPPOCAMPUS AND HYPOTHALAMUS AFTER REPEATED CLONIDINE ADMINISTRATION.

	SYNAPTOSOMAL TH		SATURATING TH
HIPPOCAMPUS	fmol/min/mg P_2 protein	n	fmol/min/mg P_2 protein
CONTROL I	157 ± 7	(5)	857 ± 163
SALINE	168 ± 11	(5)	833 ± 171
Combined	162 ± 6	(10)	844 ± 112
C-DRINK	157 ± 10	(5)	928 ± 59
C-INJECT	164 ± 23	(4)	695 ± 107
All clonidine	160 ± 11	(9)	824 ± 67
HYPOTHALAMUS	fmol/min/mg P_2 protein		pmol/min/mg P_2 protein
CONTROL I	217 ± 12	(5)	12.9 ± 0.6
SALINE	256 ± 20	(5)	11.9 ± 0.8
Combined	236 ± 13	(10)	12.4 ± 0.5
C-DRINK	244 ± 18	(5)	11.9 ± 1.4
C-INJECT	271 ± 33	(4)	11.6 ± 0.7
All clonidine	256 ± 17	(9)	11.7 ± 0.8

TABLE 5.7

SYNAPTOSOMAL AND SATURATING SOLUBILIZED TH ACTIVITY IN STRIATUM AND LOCUS COERULEUS AFTER REPEATED CLONIDINE ADMINISTRATION.

	SYNAPTOSOMAL TH		SATURATING TH
STRIATUM	fmol/min/mg P_2 protein	n	pmol/min/mg P_2 protein
CONTROL I	4648 ± 417	(5)	143 ± 28
SALINE	5213 ± 330	(5)	153 ± 17
Combined	4934 ± 268	(10)	148 ± 15
C-DRINK	5241 ± 251	(5)	127 ± 4
C-INJECT	5272 ± 75	(4)	134 ± 10
All clonidine	5255 ± 136	(9)	130 ± 5

LOCUS COERULEUS		n	pmol/min/mg P_2 protein
CONTROL I	-	(5)	14.6 ± 0.84
SALINE	-	(5)	14.5 ± 0.89
Combined	-	(10)	14.5 ± 0.98
C-DRINK	-	(5)	12.5 ± 0.65
C-INJECT	-	(4)	14.9 ± 1.29
All clonidine	-	(9)	13.6 ± 0.76

No statistically significant clonidine-induced changes were found in synaptosomal TH assays in any of the regions ,though there is a non-significant rise in the hypothalamus.

If we compare these results with the effects of a single injection of clonidine, (section 3 b) , it seems that tolerance is manifested to the acute effects of clonidine, at least in the hippocampus, since the C-INJECT group show no sign of a rise in synaptosomal TH after the last injection, given 2-4 hours before death.

RESULTS: EFFECT OF CLONIDINE WITHDRAWAL.

Three days after clonidine treatment was stopped,a further group of rats (CLON-WD) was killed along with another group of untreated controls (CONTROL II). The results of synaptosomal and soluble (saturating) assays of TH in various regions are shown in Table 5.8 (see also summary in Table 5.9).

Synaptosomal TH: The data were subjected to $\log_{10}$ transformation . ANOVA by REGION AND TREATMENT showed no significant main effect of TREATMENT ($\log_{10}$ transformed data, F= 0.03, df 1/32), although the main effect of REGION (4 regions: hippocampus, hypothalamus, striatum, cerebellum) was significant (F= 755.8, df 3/32, $p< 0.001$). The interaction term did not reach significance (F= 2.38, df 3/32, $p< 0.1$).

However, a paired t test showed a significant increase in TH activity (to 126% of control level) in the hypothalamus of the withdrawal group. Other regions did not reveal significant changes with this method.

Saturating solubilised TH : the results for hippocampus, hypothalamus, striatum and locus coeruleus are shown in Table 5.8. After $\log_{10}$ transform , two way ANOVA of the results from hypothalamus and hippocampus (the noradrenergic terminal areas) by TREATMENT and REGION showed no significant main effect of TREATMENT (F=0.002, df 1/16), although again the main effect of REGION was significant (F= 249.0, df 1/16, $p< 0.001$). The interaction term did not reach significance.

As for the other regions, which are not terminal areas for noradrenergic fibres; there is a reduction in striatal saturated TH activity, although this is not significant. In the locus coeruleus there is evidence of a change-over from slightly decreased activity compared to controls during clonidine treatment to a small increase in the withdrawal phase. But this increase also fails to reach significance (t=1.38, df 9).

TABLE 5.8

EFFECT OF CLONIDINE WITHDRAWAL

ON SYNAPTOSOMAL AND SATURATING SOLUBLE TH ACTIVITY.

	SYNAPTOSOMAL TH	SATURATING TH
	fmol/min/mg P_2 protein	fmol/min/mg P_2 protein
HIPPOCAMPUS		
CONTROL II	238 ± 33	272 ± 85
CLON-WD	190 ± 9	283 ± 119
HYPOTHALAMUS		pmol/min/mg P_2 protein
CONTROL II	311 ± 28	8.5 ± 0.4
CLON-WD	393 ± 29	9.4 ± 0.3
CEREBELLUM		
CONTROL II	238 ± 18	-
CLON-WD	236 ± 16	-
STRIATUM		pmol/min/mg P_2 protein
CONTROL II	7571 ± 313	102 ± 11
CLON-WD	7153 ± 558	75 ± 12
LOCUS COERULEUS		pmol/min/mg P_2 protein
CONTROL II	-	16.9 ± 1.0
CLON-WD	-	19.5 ± 1.3

(n=5 in all groups)

TABLE 5.9

CHRONIC CLONIDINE ADMINISTRATION AND WITHDRAWAL:
SUMMARY OF RESULTS IN DIFFERENT BRAIN REGIONS.

	Hipp	Hypothal	Striatum	Cerebell	LC
SYNAPTOSOMAL					
CONTROL	100 ± 4	100 ± 6	100 ± 9	100 ± 8	-
SALINE	107 ± 7	118 ± 9	112 ± 7	-	-
CLONINJ	104 ± 14	125 ± 15	113 ± 2	-	-
C-DRINK	100 ± 7	112 ± 8	113 ± 5	-	-
All CLON	99 ± 7	108 ± 7	107 ± 3	-	-
CLON-WD	80 ± 4	126 ± 9	94 ± 7	99 ± 7	-
SATURATING					
CONTROL	100 ± 19	100 ± 5	100 ± 20	-	100 ± 6
SALINE	97 ± 20	92 ± 6	107 ± 12	-	99 ± 13
CLONINJ	81 ± 12	90 ± 5	93 ± 7	-	100 ± 9
C-DRINK	108 ± 7	92 ± 11	89 ± 3	-	86 ± 4
All CLON	83 ± 7	94 ± 6	88 ± 3	-	93 ± 5
CLON-WD	104 ± 43	111 ± 4	73 ± 12	-	115 ± 8

Results are shown as % control ± S.E.M.
None of the changes shown were significant at the 5% level.

DISCUSSION:

In Section 3 it was shown that a single injection of clonidine at 0.025-0.1 mg/kg was associated with increased synaptosomal tyrosine hydroxylation rate in hippocampus, though not in hypothalamus. The present results show no difference in synaptosomal TH in hippocampus between untreated controls, saline-injected controls, and rats injected with or drinking clonidine over a period of 2 weeks. Specifically, there was no difference between the synaptosomal TH of injected rats 2-3 hours after a last dose of clonidine, and that of controls. This suggests that these rats no longer respond to clonidine as naive rats do; that is, they are tolerant to clonidine. This result is unexpected, in the light of the earlier studies discussed in the Introduction to this experiment; but it is possible that the method used in the present study reveals a different facet of the adaptation to clonidine treatment, since tolerance to behavioural effects of the drug has been reported (Paalzow , 1978; Meyer et al, 1977). On the other hand, the dose used in the chronic clonidine experiment (approximately 0.5 mg/kg/day for both C-INJECT and C-DRINK groups, yoked to the volume drunk daily by the C-DRINK group) exceeded the small dose (0.025-0.1 mg/kg) used in the acute clonidine experiment in Section 3 b. The dose used in the long-term experiment was that reported in Kane and Johnson's (1978) study of the effects of clonidine withdrawal on TH activity.

If induction of tyrosine hydroxylase is taking place during withdrawal, as discussed in the Introduction to the experiment, it is likely that such changes would appear in the locus coeruleus rather than in the terminal regions at this time, since induction is thought to occur in the cell body in response to trans-synaptic influences. The rise in maximal activity in terminal regions after enzyme induction is seen after a delay of several days, often long after the precipitating stimulus has ceased to activate the cell body (see Chapter 3, section 12).

A small increase in locus coeruleus TH at saturating concentrations of tyrosine and cofactor was found after clonidine withdrawal. This increase , although it did not reach significance at the 5% level (possibly because of the small numbers of rats involved) represents an extension of Kane and Johnson's (1978) finding of increased TH activity in the locus coeruleus at 40 μM tyrosine, between 3 and 5 days after clonidine withdrawal . This concentration of tyrosine is probably not saturating in the solubilised enzyme preparation, and the present study used a higher concentration (150 μM tyrosine). The present result thus tenuously supports the notion of induction of TH in noradrenergic cell bodies after withdrawal from clonidine treatment. Saturated TH activity in noradrenergic terminal regions was unaffected by withdrawal from clonidine. This is consistent with induction, which is manifested initially by an increase in maximal enzyme activity in the cell bodies.

If there is an accompanying activation of noradrenergic function in intact nerve terminals, it seems to be localised to the hypothalamus . The projections from LC to the cerebellum and hippocampus are not affected , as far as the synaptosomal TH results show. The meaning of the reduced saturating activity in the dopaminergic striatum is obscure, given the lack of evidence for withdrawal-related changes in dopaminergic neurons (Svensson and Strombom, 1977).

SECTION 5 d : EFFECT OF REPEATED ADMINISTRATION OF 2-DEOXYGLUCOSE .

INTRODUCTION :

Long term dosage with 2-deoxyglucose (2-DG) has not been thoroughly investigated; but Ritter and Ritter (1977) made the interesting finding that injections of 2-DG on alternate days for a fortnight attenuated the depletion of NA in hypothalamus and telencephalon after a single session of moderately intense footshock on the day after the last injection (270 footshocks , intensity 3mA, spread over 45 minutes). Thus rats which had undergone the continued metabolic 'stress' caused by 2-DG administration over a period of 2 weeks responded differently from controls to a new acute form of stress, footshock. Since this different response reduces the reaction to the new stress, an analogy can be drawn with the development of tolerance to a repeated stimulus. In fact, 'cross-tolerance ' has been demonstrated between 2-DG administration and footshock, in that repeated daily footshock sessions also reduce the depletion of NA found after a further session of footshock (Ritter et al,1978).

In the experiments of Weiss et al (1975), the protective effect of repeated stress (footshock or cold-swim) has been termed a process of 'toughening up'. In these experiments too, one way of measuring the outcome was the capacity for maintaining near-normal NA levels in the brain in the face of stress which would otherwise cause severe depletion of NA. However, the mechanism by which the protective effect prevents NA depletion is not clear. Weiss et al (1975) found increased TH activity after repeated inescapable shock, using high concentrations of tyrosine and cofactor according to the method of Musacchio et al (1969) . Although Weiss et al suggested this was due to enzyme induction, no immunoprecipitation studies were done. Repeated exposure to cold did not have the same effect on TH

activity as repeated shock in Weiss' study, although cold stress can result in in TH induction in the brain (Zigmond et al, 1974; Zigmond, 1979); so the mechanism of these changes therefore remains unclear (see Chapter 3, section 12).

The present experiments, initiated in collaboration with Dr Juana Gomez,were an investigation of the effects of a course of 2-deoxyglucose injections in rats on a schedule similar to that used by Ritter et al (1977). Various aspects of noradrenergic function were measured, including TH, synaptic vesicular content of NA, and uptake of labelled NA in synaptosomes. Only the TH results will be reported here.

METHOD:

The experiment was performed twice. In each replication, a group of 5 male Sprague Dawley rats were injected on alternate days with 2-deoxy-D-glucose (BDH chemicals),dissolved in normal saline at 350 mg/3 ml and injected at 350 mg/3 ml/kg, subcutaneously. A control group of animals, caged separately, were injected on a similar schedule with normal saline only. Twenty four hours after the last of the seven injections, all rats were stunned and decapitated. Crude synaptosomal pellets were prepared from hippocampus and hypothalamus . The locus coeruleus was dissected , homogenized in buffer containing Triton,and stored in the freezer.

Assays of tyrosine hydroxylase were performed on hippocampus and LC in the first experiment, and in hippocampus, hypothalamus and LC in the second experiment.

RESULTS:

Weight gain . The injections were well tolerated by all animals. There was less overall weight gain in the 2-DG-treated animals than in controls in the first experiment, but this difference was not found in the second experiment, when saline-injected animals put on 14% of their starting bodyweight and 2-DG-treated animals 17% . Ritter et al (1977) refer to a slower weight gain in their 2-DG-treated animals.

Synaptosomal TH results are shown in Table 5.10 ; hippocampal measures were obtained in both experiments and the results were subjected to two way analysis of variance with REPLICATIONS as a factor. The main effect of TREATMENT failed to reach significance at the 5% level ($F=3.44$, df 1,16, $p< 0.1$) and the same was true of the main effect of REPLICATIONS ($F=3.2$, df 1,16, $p< 0.1$). A paired t test was also performed on the difference between the results of the 2-DG group and their matched saline controls killed at the same time. This comparison gave a difference which was just significant ($p< 0.05$,one tailed). Thus the effect of repeated 2-DG injections is to produce a barely significant rise in synaptosomal TH in the hippocampus; the mean increase is 13% with respect to the mean of the saline controls (110% of control in the first experiment; 117% in the second experiment).

Hypothalamic synaptosomal TH, assayed in the second experiment only, showed no difference between 2-DG and saline groups .

TABLE 5.10

EFFECT OF REPEATED INJECTIONS OF 2-DEOXYGLUCOSE ON SYNAPTOSOMAL TYROSINE HYDROXYLASE.

	CONTROL	2-DG	
HIPPOCAMPUS			
Expt I	189 ± 12	208 ± 14	
Expt II	162 ± 13	189 ± 11	*
Combined	176 ± 10	199 ± 9	*
HYPOTHALAMUS			
Expt II	569 ± 32	569 ± 33	

fmol/min/mg P_2 protein

* $p < 0.05$

(all groups: n=5)

TH activity in solubilized preparations from hippocampus after lysis and freezing of synaptosomes was measured in the usual manner at saturating tyrosine and cofactor concentrations. A homogenate of locus coeruleus tissue was also assayed, in both experiments. (Solubilized assays were not done on the hypothalamus.) The results are shown in Table 5.11. (Subsaturating assay results on the same tissue are in APPENDIX I, Table D.)

No difference between groups was found in hippocampal TH activity under saturating conditions in the first experiment (2-DG group mean was 88% of control mean; paired t= 1.11, df 4). The locus coeruleus, however, showed a significant increase of 45 % above control levels of saturated TH activity in the 2-DG-treated animals in the first experiment (t= 2.41, $p < 0.05$); but this increase was not found in the second experiment.

TABLE 5.11

SOLUBILIZED TH ACTIVITY AFTER REPEATED 2-DG ADMINISTRATION:

SATURATING TH ACTIVITY

	CONTROL	2-DG
HIPPOCAMPUS		
Expt I	2059 ± 228	1819 ± 170
LOCUS COERULEUS		
Expt I	1472 ± 150	2148 ± 237
Expt II	2849 ± 442	2780 ± 140

fmol/min/mg P_2 protein

(all groups: n=5)

DISCUSSION:

In these experiments, rats were repeatedly injected with 2-deoxyglucose over a period of two weeks. This was intended as a mild continuing metabolic 'stress' identical to the schedule used in the experiments of Ritter et al (1977) to produce 'resistance to stress', i.e.resistance to depletion of brain NA after footshock. Following Weiss et al (1975) it was hypothesised that a change in the saturating enzyme preparation might indicate induction of TH in relation to prolonged stress.

Although the locus coeruleus showed an increase in saturating TH , compatible with the possibility of enzyme induction, in Experiment I, this was not replicated in Experiment II. The results do not therefore provide much support for the hypothesis of enzyme induction after chronic 2-DG injection. Indeed, no significant effects of treatment with 2-DG were found on two way analyses of variance of hippocampal or hypothalamic data. By using a t test for matched pairs, a small significant activation of synaptosomal TH in the hippocampus was found . There are no a priori reasons for the differences in the two replications of the experiment. Nevertheless, these results stand against the hypothesis that the main effect of 2-deoxyglucose on noradrenergic mechanisms is to be found in the hypothalamus.

The resistance to stress found by Ritter et al (1977) may therefore not be due to induction of TH in these brain regions; we have not uncovered any facilitation of NA synthetic capacity in the hypothalamus, although the activation in the hippocampus may represent an increased capacity to maintain NA levels during further stress.

There may , of course , be other changes after 2-DG treatment which remain latent until the animal is under further stress which activates NA

neurons acutely (cf Anisman and Sklar, 1979); changes of this nature could take the form of alterations in , say, adrenergic receptor populations, and would not necessarily be manifested as a change in TH activity at the end of the treatment period.

EFFECTS OF LONG TERM DRUG ADMINISTRATION : GENERAL DISCUSSION.

The experiments in this section were intended to test the proposition that long term administration of drugs which affect noradrenergic neurones might result in changes in the synthesis rate of noradrenaline which are reflected in post mortem TH activity, since this enzyme catalyses the rate-limiting step in synthesis. It was also suggested at the beginning of this section that changes after long term drug administration might differ from the short term effects of the drugs, because of adaptive mechanisms in the brain which could include induction or delayed activation of TH (or processes with the opposite effects). Withdrawal of certain drugs after chronic administration seemed likely to stimulate further changes in noradrenergic neurones which might throw light on the mechanisms by which long term adaptation takes place.

The phenomenon of <u>tolerance</u> represents the adaptation of an organism to a repeated stimulus (such as injection of a particular drug),in that repeated stimulation produces effects that become progressively less marked in comparison with the original effect. Both physiological and pharmacological tolerance are well documented in respect of psychotropic drugs. Tolerance to opiates and barbiturates is a clinical problem associated with drug dependence, abuse of hypnotics, and pain relief. Increasing amounts of these drugs are taken in order to achieve the desired subjective effects.

Tolerance to antidepressant medications, on the other hand, may be important in their clinical actions. For example, tolerance develops within 1-3 weeks to the undesirable anticholinergic side-effects of some tricyclics (eg constipation, blurring of vision). This particular effect may be independent of the antidepressant properties of these drugs. But another form of adaptation, or tolerance, is probably crucial to the central effect of antidepressants on noradrenergic neurons. This mechanism involves a reduction in the number of beta receptors on postsynaptic neurons in hippocampus, cortex and other forebrain areas (see Schwartz et al, 1978; and Ursillo et al,1979), and a reduction in the number of presynaptic alpha-2-receptors on noradrenergic nerve terminals (and probably also a reduction in number of autoreceptors on locus coeruleus cell bodies). The receptor-mediated effects of a further dose of tricyclic on the firing rates of both these types of cell are reduced. Other effects of the drug may not be subject to tolerance; for example the NA uptake inhibition caused by desipramine is unchanged by repeated administration (Crews and Smith, 1978).

Three of the drugs used in the chronic studies have the effect of slowing LC firing rates when administered in a single dose. Desipramine and clonidine may act via the same receptor (autoreceptors in the LC) to bring about this effect (see section 3) ; morphine probably acts on opiate receptors in the LC.

Acute-on-chronic drug administration : synaptosomal TH assays performed shortly after the last of a series of injections of DMI, clonidine and morphine showed no difference from drug-free controls in synaptosomal tyrosine hydroxylation rate in the hippocampus and hypothalamus. Since hippocampal synaptosomal TH activity was increased by a single dose of clonidine , and reduced by a single dose of morphine , given at a similar

interval before death, this implies the development of tolerance to the effects of these drugs on synaptosomal NA synthesis.

The mechanism for the development of tolerance is not clear; changes in the autoreceptor, alpha$_2$ receptor and opiate receptor populations are possibilities. However, clonidine has not previously been shown to cause biochemical tolerance (see introduction to section 5 c); in vivo synthesis measures have shown a reduction in synthesis and turnover even after continued daily administration.

In the hypothalamic assays, no changes in synaptosomal TH activity after chronic morphine or clonidine administration were found.

Enduring effects of drugs on synaptosomal TH activity : after prolonged treatment with desipramine and 2-deoxyglucose, rats were killed 24 hours after the last injection, in order to detect enduring effects of the drug on the synaptosomal NA synthesis rate. Neither of these drugs had caused significant changes in synaptosomal TH after a single injection (see sections 3 and 4 of this Chapter). In the case of DMI, there was no change from control synaptosomal enzyme activity at 24 hours after the last injection; after 2-DG, there was no change in the hypothalamus, which had been proposed as the principal site of action of ~~DOG~~ 2 DG in the brain; but in the hippocampus there was a significant increase in synaptosomal TH activity. Some treatments, then, such as 2-DG and handling (see section 2) , can bring about a long-lasting elevation in synaptosomal TH in hippocampus; this has not been detected in other brain regions.

Withdrawal from chronic drug administration : there is substantial evidence (see sections 5 b and 5 c) for withdrawal-induced activation of noradrenergic neurones after abrupt cessation of treatment with clonidine , or,

in the case of morphine, of injection of a morphine antagonist. The mechanism of the withdrawal reaction is not established, but changes in autoreceptor , opiate receptor, or postsynaptic receptor binding are probably involved (i.e.changes either in affinity or numbers of effective receptor sites, as in Collier's (1969) hypothesis of 'disuse hypersensitivity' of post synaptic receptors) . Firing rate of the LC has previously been shown to increase in naloxone-precipitated morphine withdrawal, and TH activity has been shown to increase after clonidine withdrawal, along with other signs such as rebound hypertension and irritability (see sections 5 b and 5 c).

Synaptosomal measures of TH activity were increased in hypothalamic samples after withdrawal from both morphine and clonidine, and in the hippocampus after withdrawal from morphine but not clonidine. The rise in the hypothalamus after clonidine withdrawal is not accompanied by any significant change in striatal synaptosomes, so it is probably not due to changes in dopaminergic neurons. Thus the increased rate of firing of noradrenergic neurons during precipitated withdrawal from morphine is accompanied by an increase in synaptosomal TH activity which is not found after withdrawal from clonidine. This provides further evidence that the relationship between firing rate and TH activity is a complex one.

Effects of long term drug administration on saturated TH activity :

assays of saturated TH activity were performed with the aim of detecting long lasting activation, inactivation, induction or negative induction after chronic drug administration. Changes due to induction were expected to be most obvious in the locus coeruleus where TH is itself synthesised. Chronic 2-DG treatment resulted in a significant increase of LC saturated TH activity in one experiment but this was not replicated; and there was also evidence of a small, non-significant increase in LC saturated TH activity 3 days after cessation of

clonidine administration. Therefore , although the studies reported here included small numbers of animals in each group, and were therefore often beset by considerable within-group error, there was evidence for increased total TH activity, consistent with induction of the enzyme in the locus coeruleus, after the 'stress' of chronic 2-DG administration , and also during 'rebound' activity following withdrawal of the sedative and hypotensive drug, clonidine.

These changes in the LC were not accompanied by significant changes in other brain regions. But after chronic administration of all three drugs which lower LC firing rate, saturated TH activity was reduced in hippocampus and hypothalamus. The effect was clearest with DMI, which caused a significant reduction in all regions. The effect with morphine was a reduction of TH activity in all regions in animals which had had an injection of naloxone 15 minutes before death; it was suggested in section 5 b that this was more likely to be an effect of repeated morphine administration over 5 days than a rapid effect of naloxone. This , however, contrasts with the increased TH activity found (with 100 μM tyrosine concentration) in hypothalamus by Clouet et al (1973). The effect of clonidine on saturated TH in the present study did not reach significance, although a small reduction was found in all regions.

It is now clear that changes in solubilized TH assayed under saturating conditions are to a considerable extent independent of changes in synaptosomal TH. The results of the saturating assay bear a closer relation to the postulated long term effects of these drugs on firing rate of LC cells than do those from the synaptosomal assay. This may denote a relationship between synthesis capacity and other aspects of neuronal function, for example the state of post synaptic receptors on neurones in regions containing noradrenergic terminal regions (see FIG 3.14 in Chapter 3, from Mandell et

al,1975, and section 5 a). The relationship between changes in enzyme activity in the LC and changes in terminal regions needs further investigation , since it appears that some of the changes found after chronic treatment cannot be accounted for in terms of induction. The nature of other mechanisms of long-lasting activation may require an exploration of receptor changes, both pre- and post-synaptic .

It seems probable that the synaptosomal assay reflects poorly understood mechanisms acting on the enzyme in nerve terminals, possibly via receptors other than the $alpha_2$ adrenergic receptors which regulate release of NA. Clearly synaptosomal TH activity does not bear a simple relationship to drug effects on LC firing rate in vivo, in the short or long term. Noradrenergic neuronal adaptation to the effects of drug administration probably involves changes in tyrosine hydroxylase activity in cell body and terminals, changes in the synthesis rate of the enzyme itself, and changes in the number (and possibly the affinity for ligands) of pre- and post-synaptic receptors. The time courses of these changes are not uniform. Other changes will probably be revealed by further work; this may enable us gradually to build up a more coherent and comprehensive picture of noradrenergic neuronal function.

SECTION 6: EFFECTS OF PARACHLOROAMPHETAMINE ON SYNAPTOSOMAL TYROSINE HYDROXYLATION.

Rationale:

In this section I shall describe a preliminary attempt to investigate one aspect of the interaction of the noradrenergic and serotoninergic systems in the brain.

Anatomical relationship between the locus coeruleus and the raphe nuclei.

Brodie and Shore (1957) first proposed antagonistic functions for NA and 5HT in the brain, before the anatomy of the two systems was established. They thought that NA might provide the central nervous system basis for the peripheral sympathetic nervous sytem, and that 5HT neurons in the brain might correspond to the parasympathetic division of the autonomic nervous system. Although this picture has not been confirmed there is now plentiful evidence that the NA and 5HT fibre systems are interlinked. Anatomical evidence from lesion studies, electron microscopy, retrograde horseradish peroxidase transport and staining, immunocytochemistry, and autoradiography in cats and rats (Samanin and Garattini,1975; Conrad et al,1974; Sakai et al,1978; Bobillier et al,1978; Descarries and Leger,1978;) have shown that both the locus coeruleus cells in the pons and the raphe nuclei of the midbrain have widely diverging projections to the forebrain.

They also show reciprocal innervation between the two groups of cell bodies mentioned. Noradrenergic varicosities and terminals ,and the noradrenaline-synthesising enzymes TH and dopamine beta hydroxylase, have been located in close proximity to 5HT cell bodies in the raphe nuclei, and conversely 5HT terminals and synthesising enzymes have been found in the LC (Descarries and Leger, 1978). The raphe nuclei are interconnected groups of

5HT-containing cell bodies (areas B1-B9 in the rat, according to Dahlstrom and Fuxe, 1964; corresponding areas have been mapped in the cat brain by Sakai et al, 1978) which form a diffuse network compared to the compact LC (see FIG.6a). The raphe projections have been mapped ; work with small discrete lesions (Sakai et al, 1978; McRae-Degueurce, 1980) has suggested that the serotoninergic innervation of the LC and adjacent areas such as the locus sub-coeruleus comes mainly from the nucleus raphe dorsalis and nucleus raphe centralis superior (B7; Lewis et al, 1976) whereas the innervation of the hippocampus is from B8, the nucleus raphe medianis. The hypothalamus also has a projection from B8.

Specialised synaptic junctions are sparse in both raphe and locus coeruleus; these types of close synaptic junction are no longer thought to be characteristic of these two monoamine systems (Descarries et al, 1977) : it is impossible to say from the anatomical evidence whether the function of the reciprocal innervation of LC and raphe nuclei is based on direct monoamine-receptor interactions on the cell bodies, or whether interneurones are interposed. There may also be important species differences in the organization of these connections. Morgane and Stern (1974, see FIG 6b) suggest that there is a cholinergic interneurone interposed between serotonergic fibres and the cells of the LC.

Functional relationship between LC and raphe nuclei.

The work of Jouvet and Pujol (1974) and Pujol et al (1973) on the effects of lesions of these two systems on patterns of arousal in cats gave support to the idea of reciprocal inhibition of firing rate as the role of the connections between LC and raphe nuclei. More recently, electrophysiological studies in rats (Segal,1979) have demonstrated this. Stimulation in the dorsal raphe of anaesthetized rats interrupts the spontaneous firing of the locus coeruleus and inhibits the characteristic LC response to a noxious stimulus.

FIG. 6a Serotonergic pathways in the rat brain
(from McGeer et al, 1978)

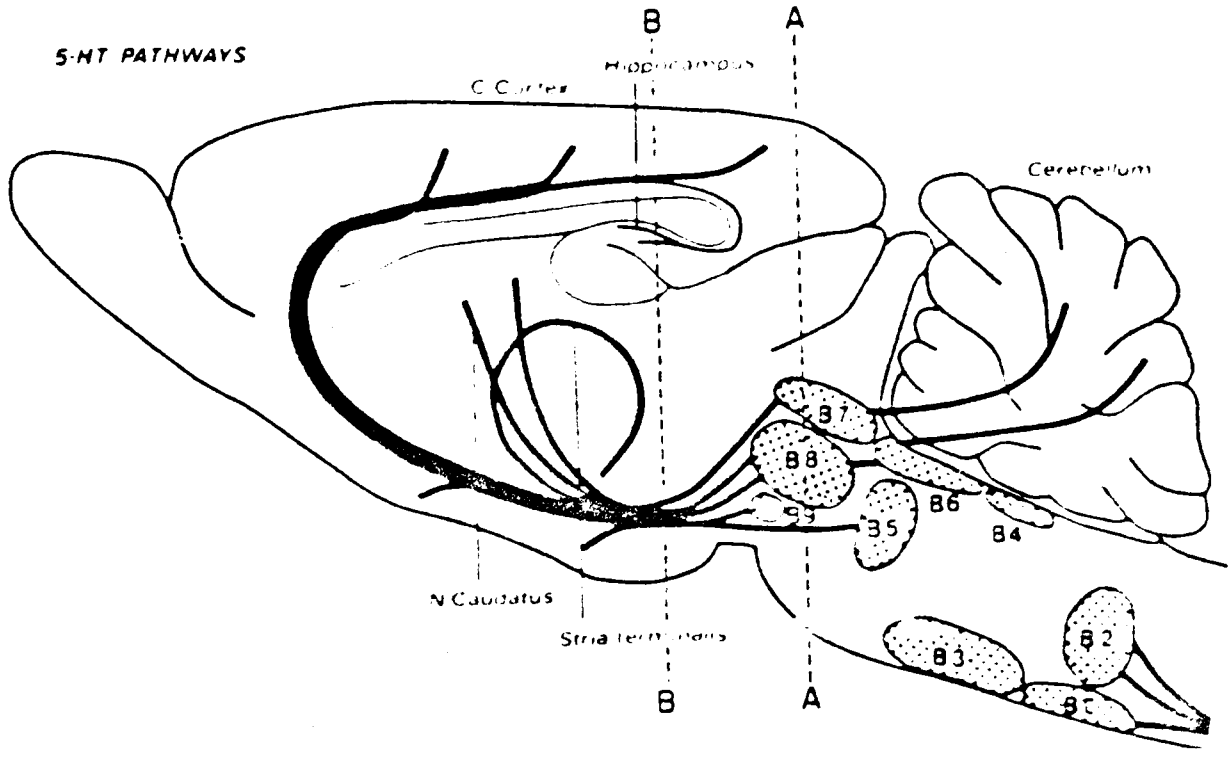
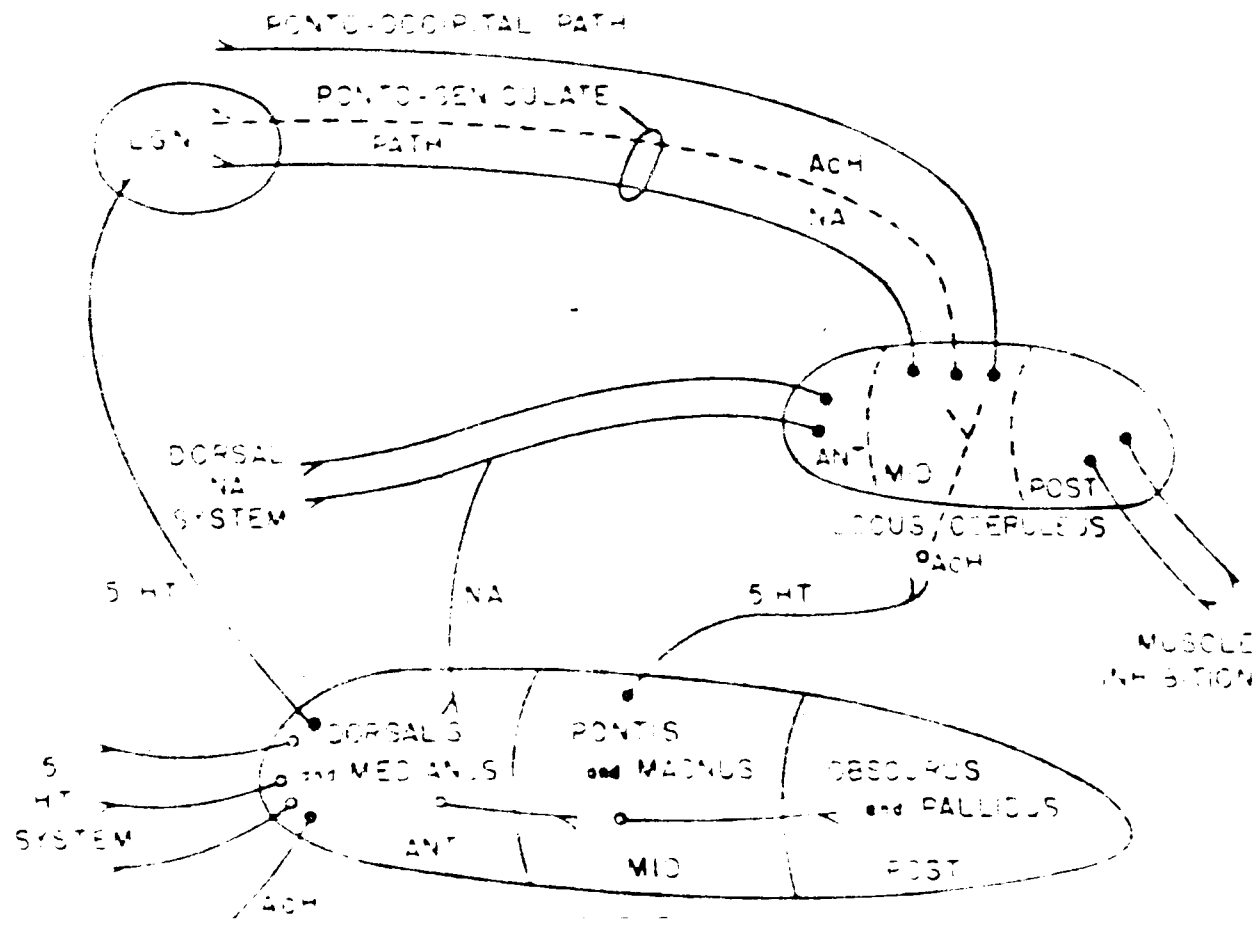


FIG. 6b Possible interconnections between the locus coeruleus and the raphe nuclei . Note the short serotonergic neurons linking the anterior, middle and posterior raphe nuclei. 5HT neurones from the middle raphe are shown synapsing on possible cholinergic neurones which in turn synapse on noradrenergic neurons in the locus coeruleus.
(from Morgane & Stern, 1974)



The effect of raphe stimulation is blocked by pretreatment with PCPA, an inhibitor of 5HT synthesis. Conversely, Morgane and Stern (1974) showed that LC stimulation blocked firing of units recorded from the anterior raphe. More recently, Baraban et al (1978a,1978b) have investigated the nature of the LC - raphe interactions using single unit recording.

Further evidence of a reciprocal inhibitory connection comes from biochemical studies. Not only are the synthesising enzymes and the transmitters themselves present , but the levels and enzyme activity can be manipulated by damage or stimulation in the appropriate cell body region. Lesions of noradrenergic neurons with 6OHDA produce large increases in 5HT metabolites in the forebrain (Blondaux et al, 1974; Kostowski et al,1974 ; see review by Pradhan and Bose, 1978). Raphe lesions, on the other hand, are followed by an increase in NA metabolite accumulation (MOPEG - see Kostowski et al,1974), and NA utilization (Pujol et al,1973; Jouvet and Pujol,1974); and several studies have demonstrated an increase in TH activity in the LC and other regions. Lewis et al (1976) found an 82% increase in TH activity 4 days after a lesion of the raphe centralis, and a 30% increase after lesioning the nucleus raphe dorsalis. Renaud et al (1975) likewise found a rise in TH activity (at 20 μM tyrosine concentration,with 'saturating cofactor concentration', i.e. DMPH4 at 1.14mM) in the LC , 4 days after an intraventricular injection of the serotonergic neurotoxin 5,6,dihydroxytryptamine (5,6,DHT) . TH in frontal cortex was also affected, with a bimodal timecourse. Both these experiments used saturating concentrations of cofactor in the TH assay, although tyrosine concentration in Renaud's study was probably subsaturating.

Therefore it is not clear whether the changes were due to activation or increase in amount of enzyme, although Renaud emphasises the possibility of enzyme induction in the LC, with transport of newly synthesised enzyme to

terminals to explain the second peak of activity in the frontal cortex . Keane et al (1978) used 50 μM tyrosine and showed an increased TH activity at 24 hours after intracisternal 5,6,DHT injection, rising to a maximum at 4 days. This study included kinetic analysis and supported the possibility of enzyme induction, in that the velocity of the reaction, rather than the affinity for substrate or cofactor,was increased.

Transmitter synthesis inhibition.

PCPA, the inhibitor of 5HT synthesis, gives some interesting and somewhat conflicting results. With respect to the reciprocal inhibitory relationship between NA and 5HT neurones, Peters et al (1972) showed that PCPA administration led (after 2 hours and 24 hours) to a reduction in total catecholamine synthesis from labelled tyrosine injected intravenously, and McGeer et al (1968) found that PCPA could inhibit TH in vitro, at high concentrations (10^{-4}M).

However, Crespi et al (1980) obtained substantial increases in TH activity (using an assay with tyrosine at 20 μM) after a series of 4 daily injections of PCPA; though a single injection of PCPA decreased TH activity as well as depleting 5HT. This latter effect may be related to the in vitro finding of McGeer, though such high levels as were necessary to achieve inhibition of TH in vitro are unlikely to be reached in vivo. It seems that in order to demonstrate the activation of NA neurons after inhibition of 5HT synthesis, PCPA must be administered over several days.

McRae-Deguerce (1980) compared the effects of selective lesions of raphe nuclei, destruction of 5HT terminals with 5,6,DHT and a 4-day course of PCPA. The effects at 4 days after these treatments were very similar, each treatment leading to a parallel reduction in 5HT terminals,depletion of 5HT, and an increase in TH activity in the LC . With lesions of nucleus raphe

dorsalis (B7), all the increase in TH could be accounted for by enzyme induction; whereas with lesions of nucleus raphe pontis (NRP) and nucleus raphe centralis superior (NRCS) there was an additional activation of enzyme as shown on the immunotitration plot. The mechanism of the additional activation is not clear. Possibly it may be linked with Sakai's finding (1978) that NRCS and NRP project to the LC-alpha and locus subcoeruleus regions (A5, A7) rather than to the LC itself (A6); it is possible that the effects of changes in the innervation of areas around the locus coeruleus activate neurons in the locus in different , perhaps additive ways.

Since PCPA seems to have a direct effect on NA synthesis, it is not an ideal drug for investigating the effects of serotonergic blockade on noradrenergic function. An alternative tool for this purpose was found in the form of parachloroamphetamine (PCA), a chlorinated analogue of amphetamine which has marked and relatively specific effects on 5HT neurons. An account of these effects and of associated changes in NA neurons follows.

In vitro effects of PCA on 5HT and NA neurons.

In vitro, PCA has been shown to be a potent inhibitor of the high-affinity uptake process for 5HT (Wong et al,1973) and it promotes carrier-mediated efflux of 5HT from the cytoplasm of synaptosomes of reserpinised rats, presumably without actually entering synaptosomes (see Ross and Kelder,1977). PCA (10^{-4}M) is also an effective inhibitor of monoamine oxidase with 5HT as substrate (Morgan et al,1972) . Sulser et al (1973) found no in vitro inhibition of 5HT synthesis by PCA at concentrations up to 10^{-5}M; but this has more recently been demonstrated with PCA at higher concentrations (10^{-3} -2 x 10^{-2}M) by Koe and Corkey (1976). A 75% inhibition of the enzyme tryptophan hydroxylase from a medulla-pons preparation , assayed at

saturating substrate and cofactor levels , was achieved. A smaller effect (25% inhibition) was found for TH from the striatum in this study. However, it seems unlikely that concentrations as high as this are found in vivo at the doses (5 mg/kg) adequate to produce profound changes in 5HT neurons; so the conclusion may be drawn that PCA probably does not act as a direct inhibitor of the monoamine-synthesising enzymes.

As for the investigation of in vitro effects of PCA on noradrenergic neurons, Morgan et al (1972) and Wong et al (1973) have shown inhibition of uptake of labelled NA into slices and synaptosomes . Wong showed that the high-affinity uptake process has a lower affinity (higher Km) for NA than for 5HT. Amphetamine and PCA are equipotent as blockers of NA uptake, whereas PCA is more potent than amphetamine as a blocker of 5HT uptake. It seems that there is some selective effect of PCA on 5HT uptake, though NA uptake may also be inhibited to some extent; this must be born in mind in interpreting results of in vivo studies.

In vivo administration of PCA.

When PCA is administered in vivo to rats in doses of 5-10 mg/kg, its effects on 5HT neurones are rapid, long-lasting and profound. Intravenous injection at 0.25-2.5 mg/kg slows the firing rate of neurons in the midbrain raphe area with a latency of 30 seconds (Sheard,1974). There is no analogous study on NA neurons ; other drugs which inhibit uptake of NA have been shown to inhibit firing rates in the LC (e.g. Nyback et al,1975). Another rapid effect of PCA takes the form of a behavioural syndrome which in several experiments has been likened to the 5HT-receptor stimulation syndrome (Trulson and Jacobs,1976), and is accompanied by an increase in the '5HT signal' obtained by voltammetric recording from striatum and hippocampus in awake rats (Marsden, 1979). Sheard (1976) and Steranka et al (1977) have

noted biphasic effects on behaviour after a first dose of PCA. They propose that there is an initial phase in which 5HT is released at terminals and affects post-synaptic 5HT receptors; the behavioural accompaniments of this are variously manifested as characteristic head-weaving and forepaw treading, decreased reactions to aversive stimuli, and disruption of learned behaviour in certain reinforcement schedules. This phase lasts only for about an hour; after which the second phase is manifested by a reduction in gross motor activity, decreased thresholds for aversive stimuli, and facilitation of performance on the same behavioural paradigm. This phase , according to Steranka (1977), is due to activation of catecholaminergic neurons.

There has been considerable controversy as to whether these effects are related primarily to changes in NA , DA , or 5HT neurons; an attractive though simplistic hypothesis compatible with most of the experimental evidence is that the earliest effects are due to release of 5HT, followed after about 2-3 hours by an activation of NA neurons (see below).

Effect of PCA on levels and synthesis of 5HT.

There has been a great deal of work on the biochemical effects of PCA on 5HT in the brain (see Sanders-Bush and Steranka, 1978 for review). 5HT levels in the brain are lowered as early as 2 hours after a large dose (25 mg/kg; Sanders-Bush et al,1975); many other studies have shown depletion at 4 hours with doses of 5 mg/kg (Morgan et al ,1972; Strada et al,1970). Levels stay low for a few days and show some recovery by 4 days (Gal et al,1975; Sanders-Bushand Steranka,1977). Morgan et al (1972) found that the hypothalamus was less sensitive than the telencephalon to 5HT depletion. As shown in the electrochemical study cited (Marsden, 1979), 5HT release appears to increase after PCA administration. If intraneuronal synthesis can accelerate to keep up with release, then transmitter levels can remain steady; this may be the situation for the first few hours after PCA injection. However,

there is some evidence that in vivo synthesis inhibition may be occurring at this time ,which would account for the depletion observed.

Despite earlier failures to demonstrate enzyme inhibition (Plescher et al, 1964), Sanders Bush et al (1972) showed a biphasic inhibition of tryptophan hydroxylase activity in a soluble enzyme preparation from brain tissue ; there was a rapid effect at less than 2 hours after first administration, followed by a return to near-normal activity; then there was a gradual decline with maximum depletion of 5HT levels between 1 and 6 days, starting 16 hours after injection of 10 mg/kg PCA. This effect is not reversed by dialysis , which suggests a non-competitive mechanism. No changes in affinity of the enzyme for cofactor or tryptophan substrate were found. The in vitro findings cited earlier (Koe et al,1976; Sulser and Sanders-Bush, 1973) also stand against an explanation in terms of a direct effect on the enzyme; and furthermore, Gal et al (1975) found no change in Km or Vmax of the enzyme in brainstem during 3 days following PCA administration intraventricularly.

Mechanisms other than inhibition of tryptophan hydroxylase have therefore been sought to explain the prolonged reduction in synthesis and fall in 5HT and 5HIAA levels in brain. Extensive studies have failed to reveal a PCA metabolite which inhibits synthesis of 5HT. Uptake of tryptophan was mentioned by Sanders-Bush et al (1972) as a possible regulatory factor in 5HT synthesis, but this was abandoned after the discovery that cerebral tryptophan levels were unchanged after PCA at doses which did produce synthesis inhibition. Knapp et al (1974) studied tryptophan uptake in synaptosomes from the striatum and found no change after PCA. But they did show a rapid and enduring fall in synaptosomal tryptophan hydroxylation starting at 15 minutes after injection and dropping to 79% of control values at 2 hrs after a dose of 10 mg/kg PCA Soluble tryptophan hydroxylase activity was also reduced in lateral midbrain samples,and striate soluble activity also fell, lagging behind

the drop in synaptosomal activity. In confirmation of results cited above, these authors found no change in tryptophan hydroxylase activity with in vitro addition of PCA.

The mechanism by which PCA brings about inhibition of 5HT synthesis, then, is still a mystery. Further work has been done to elucidate the long term effects of this drug, including the development of neutotoxic effects at about 6 days after a single injection of the drug (Gal,1975; Sanders-Bush,1977) but these studies are not directly relevant here.

Effects of PCA on noradrenergic neurons.

In 1976 a Workshop on PCA was held under the auspices of the National Institutes of Mental Health; there seemed to be a consensus that since NA levels were not significantly affected after PCA treatment (Pletscher et al, 1964; Costa et al, 1971) noradrenergic effects of PCA were unimportant . However, levels of neurotransmitters are far from adequate as indicators of function , as has been emphasised in the introductory chapters of this thesis ; and two years after the Workshop, Sanders Bush and Steranka (1978) pointed out that aside from levels, 'every other biochemical parameter of catecholamine function is substantially modified' after PCA administration.

Noradrenaline uptake is inhibited in synaptosomal preparations after in vivo injection of PCA (El-Yousef et al ,1977) as well as with in vitro addition of the drug as mentioned above. Strada et al (1970;1971) have shown increased release of labelled NA in a hypothalamic perfusate after intraventricular injection, with increased production of normetanephrine and decreased de-aminated metabolites. The effects have a time course of 2-6 hours after a first injection of PCA. They are compatible with mechanisms of action on NA neurons parallel to those in 5HT neurons - uptake inhibition, leading to increased apparent release; and inhibition of monoamine oxidase. As

for evidence for increased synthesis of NA, Sulser and Sanders-Bush (1973) found no increase in tyrosine hydroxylase activity according to the method of Waymire et al (1971) using saturating (100 μM) levels of tyrosine after in vitro addition or in vivo injection of PCA with enzyme prepared from striatum or diencephalon. This suggests that there is no change in Vmax after PCA. The small inhibition of striatal TH found in the striatum by Koe and Corkey (1976) with very high concentratons of PCA in vitro has already been mentioned. Direct inhibition of the enzyme seems unlikely after in vivo treatment,though further kinetic studies with subsaturating conditions are needed to confirm the absence of in vitro inhibition.

However, Steranka and Sanders-Bush (1977) have recently shown that the conversion of 3H-tyrosine to labelled catecholamines, and the accumulation of DOPA after decarboxylase inhibition, are both increased after PCA treatment with a maximum effect at 2-3 hours after a 5 mg/kg injection. (This is associated with the facilitation of behavioural performance mentioned above.) So it seems likely that synthesis rate is changed ; but there is no evidence that this occurs by a direct effect on the kinetic properties of TH.

The effects of PCA on NA parameters are accompanied in many cases by changes in dopaminergic measures; in some cases the dopaminergic effects of PCA are more striking than effects on NA (Hattori et al,1976; Massari et al, 1978). In some experiments the catecholamines have not been separated from each other. More work is still needed to separate direct PCA effects on catecholamine neurons from those effects which are secondary to changes in 5HT neurons; this is a difficult task. Some attempts have been made using stereoisomers of PCA . Steranka and Sanders-Bush (1977) found that (s)(+)-PCA improved behavioural performance and catecholamine turnover, as well as depleting 5HT; whereas the (R)(-)-isomer is equally effective in depleting 5HT but less effective in these other experiments.

Pretreatment with 5HT or NA-depleting drugs is another method of attempting to separate drug effects on different transmitter systems. Regional studies may also help to elucidate effects of PCA specific to particular patterns of innervation,such as the neurotoxic effects found solely in B9 and not in other raphe nuclei (Harvey et al, 1975).

To summarise : PCA is a potent inhibitor of 5HT uptake and synthesis and causes a rapid and long-lasting diminution of 5HT function in the brain. This differs from the effects of PCPA in that the rate-limiting enzyme tryptophan hydroxylase is probably not directly inhibited by PCA . The effects of PCA on 5HT neurons last longer than those of PCPA; a neurotoxic mechanism is suspected for PCA but is as yet unproven. Noradrenergic neurones also show inhibition of transmitter uptake and monoamine oxidase after PCA; but the principal effect of PCA on this system takes the form of a short-lasting activation with little residual effect after 4-6 hours. It is not clear whether these short lasting effects on NA neuronal function are due to a direct action of PCA or whether they are secondary to effects on 5HT neurons. If they are secondary effects, they may be due to dis-inhibition of the locus coeruleus from the inhibitory control of raphe neurons, or to effects of 5HT release at terminals near noradrenergic terminals.

The experiments to be presented in this section were performed in order to establish whether synaptosomal NA synthesis is altered in animals killed at times of a) increased 5HT release and behavioural activation (early effects of PCA) and b) decreased 5HT levels and synthesis in the brain (second phase of PCA effects). Knapp et al (1974) showed that synaptosomal tryptophan-5HT conversion rate in the striatum was a sensitive indicator of the progressive fall in 5HT synthesis rate after PCA injection. These present experiments extend this technique to the noradrenergic system, using two different terminal regions to allow for region-specific effects.

METHOD:

Male Sprague Dawley rats were injected with normal saline (controls) or with parachloroamphetamine (PCA, Sigma) at 5 mg/kg. Parachloroamphetamine was dissolved in saline at 5 mg/ml and injected intraperitoneally. After an interval of 45 minutes (one experiment) or 3 hours (two experiments) the rats were killed in pairs , alternating saline-injected controls with PCA-injected animals. Synaptosomal pellets were prepared as usual (see Chapter 4) and synaptosomal TH activity was measured in hippocampus in all 3 experiments, and in hypothalamus in two experiments.

RESULTS:

The results are shown in Table 6.1 . Two way analysis of variance for the hippocampus, including REPLICATIONS as a factor, showed a significant main effect of PCA TREATMENT ($F=5.27$, df 1/30, $p < 0.05$). PCA treatment was associated with an increase of 16 % in hippocampal synaptosomal TH activity with respect to controls. The main effect of REPLICATIONS was not significant, and neither was the TREATMENT X REPLICATIONS interaction. Paired t tests were performed using the PCA rats paired with saline controls killed at the same time; these showed a significant rise after PCA, with a 15%

rise in the 45-minute experiment ($p < 0.05$) and an 18% rise in the 3-hour experiments combined ($p < 0.05$) . The increase in hippocampal synaptosomal TH therefore appeared to be both rapid and sustained.

TABLE 6.1

EFFECT OF PCA ON SYNAPTOSOMAL TYROSINE HYDROXYLATION.

	CONTROL	PCA	PCA/CONTROL
HIPPOCAMPUS			
PCA + 45 mins	146 ± 11 (8)	168 ± 16 (8)	115 % *
+ 3 hours (I)	144 ± 10 (5)	171 ± 9 (5)	119 %
+ 3 hours (II)	128 ± 5 (5)	140 ± 8 (5)	116 %
+ 3 hours (I + II)	136 ± 6 (10)	160 ± 7 (10)	118 % *
HYPOTHALAMUS			
PCA + 45 mins	298 ± 26 (8)	363 ± 26 (8)	122 %
+ 3 hours	384 ± 36 (5)	455 ± 28 (5)	118 % **

fmol/min/mg P_2 protein (numbers in brackets indicate group size)

* paired $p < 0.05$

** paired $p < 0.02$

Hypothalamic synaptosomal results were also subjected to two-way analysis of variance. The main effect of TREATMENT was significant ($F=5.45$, df 1/22, $p < 0.05$) and so was the main effect of REPLICATIONS ($F=9.06$, df 1/22, $p < 0.01$) There was a significant overall increase in TH after PCA (20% higher in PCA rats than controls, $p < 0.05$, t test). This increase did not reach significance in either of the two experiments individually. But paired t tests using the matched pairs showed a near-significant rise in the 45-minute experiment ($p < 0.1$) and a significant rise in the 3-hour experiment ($p < 0.02$).

DISCUSSION:

A very similar activation was found in synaptosomal TH activity in hippocampus and hypothalamus in all the experiments with PCA. The activation was small but consistent, amounting to a rise of 15-22 % above control TH activity . The results were very similar in both regions at both 45 minutes and 3 hours after PCA injection. There is therefore no suggestion of a biphasic effect on NA synthesis in synaptosomes to correspond with the in vivo biphasic early effects of PCA described by Sheard (1976) and Steranka et al (1977). Even if there is increased release of 5HT at 45 minutes, as suggested from the electrochemical experiments of Marsden (1979), there is no associated inhibition of TH in noradrenergic synaptosomes measured post mortem in this experiment.

Many reports have shown a difference in sensitivity of different brain regions to effects of PCA, with the hypothalamus often portrayed as less sensitive than other areas (Morgan et al,1972 ; Steranka and Sanders-Bush,1977) ; the hypothalamus also has a heterogeneous NA input and has been shown to differ from hippocampus in some of the other experiments reported in previous sections of this thesis. But in these experiments the

hippocampus and hypothalamus were equally affected . Both these regions have a direct serotonergic input from the nucleus raphe centralis superior, B8 , in the rat (Bobillier et al ,1978).

Synaptosomal TH, in the present experiments, responds to a single injection of PCA in a manner consistent with other , in vivo measures of catecholamine synthesis. The period of increased noradrenergic activity as measured in this way overlaps with the period of increased 5HT release reported by Marsden(1979), among others , and with the onset of depletion of 5HT (Sanders-Bush et al , 1975). The mode of action of PCA in activating NA synthesis is not clear. Several alternatives are discussed below.

1. Direct activation of tyrosine hydroxylase by PCA : this is an unlikely mechanism, given the negative results of in vitro assays (see above) and the lack of any evidence that PCA is taken up into neurons or terminals (Steranka and Sanders-Bush,1977) .

2. Direct effects of PCA on noradrenergic terminals. The effects of PCA on NA uptake and turnover have similar time courses, with a maximum effect 2-4 hours after in vivo administration, and a return to normal within 24 hours (Sanders-Bush, 1977; Steranka,1977). Although neither uptake inhibition nor monoamine oxidase inhibition are known to trigger changes in synaptosomal NA synthesis, a direct effect of this nature cannot be ruled out. Receptors such as $alpha_2$ receptors or other presynaptic receptors could theoretically mediate such effects, though little evidence has been found for a consistent increase in synaptosomal TH activity after uptake-inhibiting drugs in previous experiments (section 3).

3. The principal effect of PCA on noradrenergic neurons may be indirect. Interruption by PCA of the putative inhibitory control of the LC by

neurons projecting from the raphe may result in activation of cells in the locus. It is not known whether this postulated loss of inhibitory input is accompanied by increased firing in the LC neurons. TH activity in the LC was not measured in these experiments; changes in TH in the LC have not been reported at intervals of less than 24 hours after treatment. Our results showing activation of TH in terminals do not necessarily imply an increase in firing rate in the LC; dissociation of changes in vivo firing rate and postmortem synaptosomal TH activity seems probable after several different drug treatments, as indicated previously. However, dis-inhibition of LC cells may influence TH activity in synaptosomes in some way, whether or not through measurable changes in firing rate . But the evidence that 5HT release is increased shortly after PCA administration suggests that the inhibitory influence of the raphe would be potentiated at least temporarily, so that LC neurons would be more than usually subdued at this time.

4. Effects on NA terminals due to PCA-induced changes at serotonergic terminals in the same projection areas. Very little is known about terminal-terminal interactions and what effect changes in 5HT terminals might have on synthesis . Knapp et al (1974) show that striatal synaptosomal tryptophan hydroxylation is decreased 2 hours after PCA administration, and this may be the case in hippocampus and hypothalamus too. Could the combination of uptake inhibition and decreased 5HT synthesis in serotoninergic terminals affect NA terminals in these regions so as to stimulate TH activity? However, one problem with this possibility, as with (3) above, is the finding of a stable increase in TH activity during a period when 5HT release at terminals is thought to be first increased and later decreased (Sheard,1976; Marsden,1979).

Much further experimental work is needed to clarify the mechanism of action whereby PCA increases TH activity in synaptosomes. Methods which relate release of NA and 5HT to the time course of changes in uptake inhibition and synthesis will be particularly valuable in evaluating changes localised to nerve terminals. Further work is in progress in this laboratory using an in vivo technique for measuring release of neurotransmitters by voltammetry (see W.Beck, unpublished D.Phil thesis, 1981), and some preliminary work with PCA has suggested that parallel studies of synthesis and release would indeed be a fruitful area for further experiments on both long- and short-term interactions of the NA and 5HT systems.

SECTION 7.

PARTIAL REINFORCEMENT ACQUISITION TRAINING.

THE PARTIAL REINFORCEMENT PARADIGM.

Rationale:

The characteristics of the noradrenergic pathways in the brain , and their similarity with the peripheral nervous system, have led many workers to search for a noradrenergic basis to certain aspects of behaviour. This notion was introduced in Chapter 1, as the starting point for the investigations reported in this thesis .

Several authors have speculated as to whether noradrenergic neurons in the ascending dorsal bundle might play a part in the patterning of rewarded behaviour (Crow,1972; Stein,1971; discussed by Gray, 1979) . An experiment by Anlezark et al (1973) suggested that acquisition of a rewarded response might be impaired by lesions of the locus coeruleus. But later experiments failed to show any impairment of acquisition of a rewarded response in straight alley runway experiments after dorsal bundle lesions (Mason and Iversen, 1975; Owen et al, 1977), provided that sufficient handling and pretraining in the runway was given, to dissipate the effects of novelty (Owen, 1979).

It was then suggested that NA might have more of a part to play in extinction processes (Mason and Iversen, 1978) or in responses to the omission of expected rewards (non-reward; Gray,1977). These possibilities have been explored in this laboratory using the partial reinforcement paradigm. Partial reinforcement schedules involve the presentation of a reward to subjects on a proportion (usually 50 %) of acquisition trials; and the effect is to increase the probability of performance of the learned response during extinction (when

reward is never presented) compared to the performance of subjects continuously rewarded during acquisition. As noted in Chapter 1, Gray (1967) advanced the proposition that a common physiological mechanism, involving the dorsal noradrenergic bundle and the septo-hippocampal system, is concerned in the development of tolerance to all forms of stress , including the partial reinforcement extinction effect.

Anti-anxiety drugs such as the benzodiazepines and barbiturates were found to counteract the partial reinforcement extinction effect (PREE) in the runway at long inter-trial intervals (Feldon and Gray, 1981). But dorsal bundle lesions did not abolish the PREE when acquisition training was given at one trial per day (Owen, 1979). However, Owen et al (1977) showed that resistance to exinction in partially rewarded rats was abolished by lesions of the dorsal noradrenergic bundle when the acquisition phase was run with 50 trials (10 trials /day,intertrial interval 4-7 minutes). The effect of the dorsal bundle lesion was to increase resistance to extinction in the continuously rewarded (CR) group, and to decrease resistance to extinction in the partially rewarded (PR) group; this accorded with the predictions of the non-reward hypothesis of NA function.

On the other hand, the PREE was not abolished by the dorsal bundle lesion in an experiment with 100 acquisition trials (Owen, 1979). This result of 'overtraining' suggested that with practice and massed trials, animals deficient in forebrain NA showed the normal differential responses in extinction according to the reinforcement schedule applied during acquisition. The finding of a more robust PREE with increased number of acquisition trials was also reported by Feldon and Gray (1979) and Henke (1974), with septal lesions and (Feldon, personal communication) with the benzodiazepine chlordiazepoxide, both of which impaired the PREE after 48 acquisition trials.

Although there is some evidence suggesting noradrenaline pathways as a possible substrate for the maintenance of responding , in extinction conditions, of animals with some experience of non-reward, the different results obtained as a function of length of training and of inter-trial interval complicate the issue.

A collaborative experiment involving members of the Physiology Laboratory and the Department of Experimental Psychology pioneered the use of TH assays to investigate NA function during the PR paradigm, as an alternative to lesion studies. The non-reward hypothesis implied that NA function might be increased in the brains of rats undergoing PR training. It was decided to investigate this possibility using synaptosomal TH as a measure of NA synthesis rate in the neurons, and saturated TH to determine whether long term activation or induction had taken place during acquisition training. Because of the different results obtained when drugs are injected using different acquisition schedules, two schedules were used in this experiment. Acquisition was either run for 12 days at one trial per day (one trial: OT) or for 8 days at 6 trials per day (multitrial : MT). A group of handled but behaviourally naive animals was run with each experiment.

The results of this first experiment using TH in conjunction with behavioural techniques were unexpected. Synaptosomal TH (measured at pH 6.2 and at a concentration of 10 µM tyrosine) was elevated in hippocampal samples from CR rats in both long and short inter-trial interval groups; handled controls and PR groups had similar synaptosomal TH activities. Saturated TH showed no difference between groups.

Thus the predicted activation of TH in the 'stressed', partially rewarded group did not occur. On the contrary, activation of TH was found in the hippocampal synaptosomes of the CR group, relative to both handled

controls and partially rewarded rats. Unhandled controls were not included in this experiment. This activation was apparent in both long- and short-intertrial intervals. These results suggested that the 'reward' hypothesis of NA function should be reconsidered in relation to runway experiments.

This was the starting point for the experiments described in this section. These experiments were performed with the help and collaboration of Joram Feldon, Michael Boarder, Doreen West, and Cliff Preston. The aim was to replicate and expand our first finding (above; published as a preliminary communication , Boarder et al, 1979) and to sample TH activity in both acquisition and extinction phases of runway experiments using CR and PR schedules in the straight alley.

Control groups of behaviourally naive, handled or unhandled rats were included wherever possible, but the main emphasis was on the detection of differences between CR and PR rats, and accumulation of evidence concerning the role of NA in rewarded and frustrated rats. In addition to synaptosomal and saturated TH activity, noradrenaline levels were assayed in some experiments , using a sensitive radio-enzymic method.

EXPERIMENTAL METHOD AND DESIGN.

The experimental procedure for acquisition training is detailed for Experiment B in APPENDIX II. Other experiments in this series were based on this design with minor modifications and are briefly described in APPENDIX II. All rats were handled for at least 13 days before the start of runway training. Behavioural procedures in Experiments A and B were carried out by J.Feldon, and in Experiment C by C.Preston. Experiments D and E were run by the author. Twenty-four hours after the last trial,all rats were killed, and assays were carried out as shown in Table 7.1.

All experiments involved 3 groups of rats; those continuously reinforced (CR: food reward on every trial in the runway), those subjected to partial reinforcement (PR: food reward on 50 % of trials), and a group of handled controls (HC: no runway experience, but daily handling for the duration of the experiment) for comparison.

The five experiments A-E, as shown in Table 7 1 , all contain groups assayed after acquisition training, either on a multitrial or a one trial a day schedule. Taken together, these experiments comprise a test of the hypothesis that acquisition training which results in resistance to extinction in PR rats might be associated with

1) activation of synaptosomal TH at a time when the rats has grounds for anticipating a further acquisition trial; and

2) a rise in total TH activity in noradrenergic neurones such as would occur after TH induction.

TABLE 7.1

PARTIAL REINFORCEMENT EXPERIMENTS: REGIONAL ASSAYS.

EXPT	Paradigm		synaptosomal TH assay	saturating TH assay	NA assay
A	OT	ACQUISITION	hippocampus	hypothalamus	hypothalamus
A	MT	ACQn	hippocampus	hippocampus hypothalamus cortex	- - -
B	OT	ACQn	hippocampus	hippocampus hypothalamus cortex	hippocampus hypothalamus cortex
B	MT	ACQn	hippocampus	hippocampus hypothalamus cortex	hippocampus hypothalamus cortex
B	OT	ACQn + 7 days	hippocampus	hippocampus hypothalamus cortex	hippocampus hypothalamus cortex
B	MT	ACQn + 7 days	hippocampus	hippocampus hypothalamus cortex	hippocampus hypothalamus cortex
C	OT	ACQn	hippocampus	-	-
C	OT	EXTINCTION	hippocampus	-	-
D	OT	ACQn	hippocampus cortex	-	-
E	OT	ACQn	hippocampus	hippocampus locus coeruleus	- -

Details of assay methods, pH etc. are given in APPENDIX II.

OT 1 trial per day
MT 6 trials per day

RESULTS :

1. Behavioural measures: these , in the form of figures showing runway speeds or times (FIGS.7a - 7e), and analysis of results, are set out in APPENDIX II.

2. The effects of acquisition training on synaptosomal TH activity.

As shown in Tables 7.1 and 7.2 , synaptosomal TH in the hippocampus was measured in all the five experiments run on the one trial per day design, and in the two multitrial experiments. In experiment D, cortical synaptosomes were also assayed. The data from the five OT experiments were subjected to two way ANOVA with REINFORCEMENT condition and REPLICATION as factors. The main effect of REINFORCEMENT did not quite reach significance ($F=2.81$, df 2/87, $p< 0.1$). There was a significant effect of REPLICATION ($F=4.28$, df 4/87, $p< 0.005$) . The interaction term was not significant ($F=1.38$, df 4/87).

To check whether there was significant variation amongst controls from the various experiments, a one way ANOVA was run with all the handled controls from the above-mentioned analysis; there was no significant effect of REPLICATION ($F=1.22$, df 4/27, $p> 0.2$) .

Although there was no significant overall effect of reinforcement condition, each of the individual experiments have been subjected to one way analysis of variance by treatment, to examine the outcome of predictions made from theory and in the light of the first experiment of the series. The F ratios are shown in Table 7.2. In experiment A, as previously reported (Boarder et al, 1979), there was increased synaptosomal activity in hippocampus in CR groups in both OT and MT conditions . Table 7.2 shows that the effect only

reaches significance in the MT experiment, although the same ordering of groups is seen in the OT experiment. Experiment B shows a near-significant treatment effect in the OT paradigm, with a smaller rise in PR than in CR animals. But in the MT paradigm, there was significant lowering of TH activity in PR animals with respect to handled controls (Dunnett's test, $p < 0.05$), but no significant difference between the CR group and controls.

Thus, although the MT paradigm revealed a significant effect of treatment condition in Experiments A and B, this was based on an increase in the CR group with respect to controls, in Experiment A, and a decrease in the PR group with respect to controls, rather than on any significant difference due to reinforcement between the two runway groups.

TABLE 7.2

PARTIAL REINFORCEMENT ACQUISITION:

SYNAPTOSOMAL TYROSINE HYDROXYLATION.

EXPT		HC	CR	PR	CR/HC %	PR/HC %	Statistic F df
Hippocampus							
A	OT	186 (6) ± 22	233 (8) ± 24	162 (6) ± 21	125 %	87 %	2.05, 2/17 (p< 0.2)
B	OT	150 (8) ± 21	241 (6) ± 18	200 (6) ± 34	161 %	133 %	2.96, 2/17 (p< 0.1)
C	OT	190 (4) ± 31	174 (8) ± 17	169 (8) ± 13	92 %	89 %	0.29, 2/17 (ns)
D	OT	223 (7) + 34	243 (7) + 22	241 (7) + 20	109 %	108 %	0.18, 2/18 (ns)
E	OT	186 (7) ± 17	179 (7) ± 20	137 (7) ± 25	96 %	74 %	1.57, 2/18 (ns)
A	MT	156 (6) ± 17	237 (8) ± 17	161 (6) ± 12	152 %	103 %	8.03, 2/17 (p< 0.005)
B	MT	181 (6) ± 14	153 (6) ± 19	120 (6) ± 10	85 %	66 %	3.81, 2/17 (p< 0.05)
Cortex							
D	OT	207 (7) ± 16	236 (7) ± 11	216 (7) ± 23	114 %	104 %	0.74 (ns)

fmol/min/mg P_2 protein
numbers in brackets denote size of group .

results as percentages of handled control means.

OT 1 trial per day
MT 6 trials per day

HC handled control
CR continuous reward
PR partial reward

In the three subsequent experiments, C,D and E, which were performed in order to resolve this question, no significant effects in either direction were obtained; nor was any change in synaptosomal activity noted when cortical synaptosomes were assayed after acquisition training.

The synaptosomal assays in experiments C,D and E were carried out at pH 7.4 using Tris-HCl buffer, at 20 μM tyrosine. This change in assay conditions is unlikely to be the cause of the failure to replicate the earlier finding, since a) the control values with these two assay methods are very similar and did not give rise to a significant replications effect amongst the controls, nor to a significant interaction with reinforcement condition in the two-way ANOVA ; b) the assay at pH 7.4 has been shown in other experiments in this thesis to be capable of manifesting both short term and longer lasting changes in catalytic activity; and c) the results from Experiment G, to be described in section 8, also include a synaptosomal TH assay at pH 6.1, and did not show any difference between CR and HC groups.

Thus the first part of the hypothesis under test, namely the proposition that that a change in synaptosomal TH would accompany the development of anticipation of non-reward (conditioned frustration) is not upheld by the results of these experiments. It seems very unlikely that acquisition training in a runway for up to 20 days reliably alters the activity of TH under conditions found in synaptosomes. This does not , of course, rule out other effects on NA function.

3. Effects of acquisition training on saturated, solubilized TH activity.

Table 7.3 shows an array of experiments using the saturating assay for TH activity, mostly in triton-homogenised brain tissue, but in one experiment (E) this assay was performed on the frozen and triton-treated P_2 suspension. These assays were performed to assess whether long term changes in Vmax would be found after runway training, of the kind found after induction of TH. Table 7.3 shows that no differences of this nature were found in any region studied, in the OT or MT experiments. There are no significant changes in continuously or partially rewarded groups, with respect to controls, and although there is a significant main effect of REPLICATION in an analysis of variance of the data from three hippocampal assays (Experiments B, both paradigms; and Experiment A; $F= 82.6$, df 2/55, $p < 0.001$), the main effect of REINFORCEMENT is not significant ($F=0.48$). The locus coeruleus was assayed in one experiment, since induction occurs in the cell bodies and changes in this region might be expected to precede changes in noradrenergic terminal regions. But no differences in saturated activity were found in this region either.

TABLE 7.3

PARTIAL REINFORCEMENT ACQUISITION:

SATURATED TYROSINE HYDROXYLASE ACTIVITY.

EXPT		HC	CR	PR	CR/HC %	PR/HC %
Hippocampus		(fmol/min/mg wet weight)				
B	OT	69 (8) ± 4	72 (6) ± 8	71 (6) ± 5	104 %	103 %
E	OT	1736 (7)* ± 140	1534 (7) ± 92	1773 (7) ± 181	88 %	102 %
A	MT	83 (6) ± 4	89 (8) ± 3	85 (6) ± 6	107 %	103 %
B	MT	176 (8) ± 15	183 (6) ± 20	160 (6) ± 6	104 %	91 %
Hypothalamus						
A	OT	5043 ± 539	5588 ± 557	5300 ± 299	111 %	105 %
B	OT	6613 ± 306	6845 ± 48	6590 ± 767	104 %	100 %
A	MT	5019 ± 663	4987 ± 480	4400 ± 372	99 %	88 %
B	MT	4507 ± 283	5045 ± 380	5457 ± 606	112 %	121 %
Cortex						
B	OT	299 ± 17	305 ± 42	324 ± 51	102 %	108 %
A	MT	114 ± 7	107 ± 8	88 ± 7	94 %	77 %
B	MT	273 ± 27	276 ± 14	329 ± 40	101 %	121 %
Locus Coeruleus		(pmol/min/mg protein)				
E	OT	12.48 ± 1.4	11.73 ± 1.6	11.42 ± 0.8	94 %	92 %

* fmol/min/mg P_2 protein

no differences are significant.

4. Effect of acquisition training on NA levels in brain regions.

Table 7.4 shows the results of noradrenaline assays performed on perchloric acid supernatants of the triton-solubilised homogenates from various regions of brain. There was no significant overall effect of reinforcement. In experiment B, both the experimental groups in the MT paradigm show a lowering of NA level in the hippocampus (one way ANOVA, $F=4.67, df\ 2/17, p<0.025$). Although a Dunnett test failed to show a significant difference between the controls and either runway group, it is interesting that these rats also showed a lowering of synaptosomal TH in hippocampus at this time (see Table 7.2) which was equally unexpected.

TABLE 7.4

PARTIAL REINFORCEMENT ACQUISITION: NORADRENALINE LEVELS.

EXPT		HC	CR	PR	CR/HC %	PR/HC %
Hippocampus						
B	OT	497 (8) ± 34	512 (6) ± 38	481 (6) ± 30	103 %	97 %
B	MT	226 (8) ± 36	152 (6) ± 14	139 (6) ± 40	67 %	62 % *
Hypothalamus						
A	OT	3749 (6) ± 579	3634 (8) ± 652	3670 (6) ± 313	97 %	98 %
B	OT	2594 ± 268	2085 ± 164	2416 ± 296	80 %	93 %
B	MT	1875 ± 148	2602 ± 437	1698 ± 95	139 %	91 %
Cortex						
A	MT	117 ± 9	128 ± 7	124 ± 7	110 %	106 %
B	MT	210 ± 16	177 ± 16	187 ± 16	88 %	93 %
B	OT	287 ± 14	257 ± 30	258 ± 23	90 %	90 %

ng noradrenaline/g tissue wet weight. * see text

POST-ACQUISITION MEASURES

5. Synaptosomal TH:

Rats which were left in home cages for 7 days after the last aquisition trial, on OT and MT paradigms, showed no between-group differences in hippocampal synaptosomal TH activity ; see Table 7.5. Nor were there any significant changes in animals killed 24 hours after the last of 7 extinction trials in experiment C, although analysis of variance revealed a non-significant drop in TH (F=1.2, df 2/17) as compared to previously handled controls.

TABLE 7.5

PARTIAL REINFORCEMENT:

POST-ACQUISITION SYNAPTOSOMAL TH ACTIVITY.

EXPT		HC	CR	PR	CR/HC %	PR/HC %
Hippocampus						
B	OT					
	ACQn + 7	156 (8) ± 29	146 (6) ± 25	156 (6) ± 25	94 %	100 %
B	MT					
	ACQn + 7	253 (8) ± 17	264 (6) ± 22	293 (6) ± 27	104 %	116 %
C	OT					
	EXTINCTn	151 (4) ± 13	124 (8) ± 15	122 (8) ± 6	82 %	81 %

fmol/min/mg P_2 protein
group size in brackets.

6. Saturated TH:

In experiment B, saturated TH activity was measured in 3 brain regions seven days after the end of acquisition for both MT and OT groups. The results are shown in Table 7.6. A significant effect of treatment was apparent in the hypothalamus of PR rats in the MT condition ($F=4.01, df\ 2/17, p<0.025$) and t test showed a significant reduction in PR rats with respect to handled controls ($p < 0.05$). Apart from this result,no significant between-group differences were found.

TABLE 7.6

PARTIAL REINFORCEMENT:

POST-ACQUISITION SATURATING TH ACTIVITY.

EXPT		HC	CR	PR	CR/HC %	PR/HC %
Hippocampus						
B	OT ACQn + 7	265 (8) ± 9	253 (6) ± 12	233 (6) ± 18	96 %	88 %
B	MT ACQn + 7	157 (8) ± 15	180 (6) ± 16	181 (6) ± 7	115 %	115 %
Hypothalamus						
B	OT ACQn + 7	4624 ± 393	4268 ± 502	5207 ± 187	92 %	113 %
B	OT ACQn + 7	4781 ± 475	3973 ± 545	2934 ± 343	83 %	61 % *
Cortex						
B	OT ACQn + 7	265 ± 17	308 ± 26	268 ± 39	116 %	101 %
B	OT ACQn + 7	136 ± 8	148 ± 40	131 ± 12	109 %	96 %

fmol/min/mg P_2 protein

* see text

7. Noradrenaline levels post-acquisition:

These are shown in Table 7.7. No significant differences are apparent in any of the assays performed in experiment B in hippocampal, hypothalamic and cortical samples.

TABLE 7.7

PARTIAL REINFORCEMENT:

POST-ACQUISITION NORADRENALINE LEVELS

	EXPT	HC	CR	PR	CR/HC %	PR/HC %
Hippocampus						
B	OT ACQn + 7	440 (8) ± 41	371 (6) ± 40	355 (6) ± 20	84 %	81 %
B	MT ACQn + 7	248 (8) ± 16	324 (6) ± 58	257 (6) ± 17	131 %	104 %
Hypothalamus						
B	OT ACQn + 7	1476 ± 74	1394 ± 113	1515 ± 108	94 %	103 %
B	MT ACQn + 7	1329 ± 99	1346 ± 129	1348 ± 68	101 %	101 %
Cortex						
B	OT ACQn + 7	332 ± 21	333 ± 9	339 ± 20	100 %	102 %
B	MT ACQn + 7	193 ± 14	180 ± 13	179 ± 9	93 %	93 %

ng noradrenaline/ g wet weight.
group sizes in brackets.

DISCUSSION

Despite investigations involving 4 separate new behavioural experiments all of which were designed to test the hypothesis that experience of consistent reward and partial non-reward in a runway would be associated with differences in noradrenergic functioning in the brain, it has not been possible to replicate a preliminary finding in this laboratory (raised synaptosomal TH in hippocampus of CR rats, Experiment A), nor to discover any evidence of long term changes suggestive of a change in total activity of TH enzyme in NA neurons during runway training and extinction of a running response. In no experiment have there been consistent and significant differences between the CR and PR groups; although there were minor differences between runway-trained animals and handled controls. This suggests that minor differences in TH activity may come about as a result of runway experience , but there is no firm evidence from these results that reward and partial non-reward have distinct effects. It may be of interest that the extinction data from experiment C show a small reduction of synaptosomal TH activity in the hippocampus of runway-trained rats compared to handled controls, in that this could be analogous to the apparent suppression of activity in the repeatedly-shocked groups in section 2 of this Chapter.

Considering the extremely severe shock regimes employed by several laboratories to achieve NA depletion in the brain (Maynert and Levi,1964;and see Section 1) or the rigorous exercise demanded in the experiment of Gordon et al (1966) it is perhaps not surprising that changes in NA levels have not accompanied the performance of the runway task. Indeed the measurements of NA levels were made more for comparison with the saturated and synaptosomal TH activities , rather than in expectation of significant changes in levels (Bacopoulos and Bhatnagar, 1977). With the data from experiment B,

which included assays of saturated TH and NA levels for three brain regions, it was possible to run correlation coefficients between NA and TH levels for individual animals, and to perform analysis of covariance . However, even after covariance analysis the main effect of REINFORCEMENT remained below the 5% level of significance . This was true of analyses using either NA level or synaptosomal TH in hippocampus as the variate, and covarying total TH.

No significant correlations were found between synaptosomal and total TH activity in the hippocampus during acquisition in experiment B. The calculations were performed on each experimental group of 6-8 animals. There was one significant correlation for NA and total TH activity, in the PR group at one trial per day ($r=0.82$, df 4, $p< 0.05$); but this might have arisen by chance.

Bacopoulos and Bhatnagar (1977) found highly significant linear correlations between NA levels and TH activity (measured at 200 μM tyrosine and 0.9mM DMPH4), and between TH and NA turnover as measured by incorporation of 3H-tyrosine into 3H-NA, in groups of 4 - 6 animals. Possibly these correlations are found in naive rats and are disrupted by handling. (The results of section 2 certainly suggest that handling alone can have marked and long-lasting effects on TH.) Otherwise it is difficult to explain the discrepancy between the present results and those of Bacopoulos and Bhatnagar, since the assay methods for saturating TH seem to be comparable.

SECTION 8.

PARTIAL PUNISHMENT ACQUISITION AND CONFLICT.

Rationale :

Changes in noradrenergic function and TH activity resulting from electrical footshock have been discussed in earlier sections (under activation of TH, in Chapter 3; and in sections 1 and 2 of the present chapter). Besides the short term responses, there appear to be long term changes associated with continued stimulation (beyond some threshold duration?) which results in a delayed increase in synthesis of NA after prolonged stresses. Such changes are manifested as an increased Vmax, or increased total TH activity under saturating conditions, with or without evidence of an increase in immunoprecipitable enzyme protein. NA levels do not reliably increase when TH total activity rises. (Table H, Chapter 3).

Behavioural experiments have shown that although performance on certain tasks is impaired after a session of moderately severe footshock or cold swim ('helplessness',or 'learned helplessness' - see Gray, 1981, for review), the impairment can be reduced or prevented by repeated exposure to stress before the test session. This phenomenon has been termed 'toughening up' by Miller et al (1976), and a connection with noradrenergic mechanisms was made when it was found that NA levels were depleted after the impaired performance and that this acute depletion was prevented by the repeated exposure to stress.

Gray et al (in press) have linked these experiments, which involved escape performance in the rat, with the tolerance to stress which is manifested in the partial reinforcement paradigm (see section 7). Exposure to the 'stress' of non-reward during the acquisition of a response results in resistance to extinction of a running response. Dorsal bundle lesions, which

remove the ascending noradrenergic output from the locus coeruleus, prevent the resistance to extinction in the multitrial paradigm. Section 7 of Chapter 5 reported attempts to show activation of noradrenaline synthesis , as measured by tyrosine hydroxylase activity, after partial reinforcement acquisition. No activation of TH was seen in the PR animals; nor did the 'stress' of frustrative non-reward affect NA levels or total TH activity in various brain regions.

The development of a partial punishment (PP) paradigm in this laboratory by Nicola Davis (1979) provided another opportunity to examine the effects of stress in behavioural experiments on TH activity. As indicated in Chapter 1, the design used by Davis was closely analogous to the partial reinforcement design. Acquisition training in the runway at one trial per day (OT) or at 6 trials per day (MT) is identical for the CR group. The PP group is subjected on half the acquisition trials to a low-intensity footshock delivered to the grid floor of the goal box, where they also obtain a food reward. The details of the procedure are given in APPENDIX 3. In the second phase of the experiment, shock and food are paired on every trial; this is called the 'conflict' phase. The effect of PP training is to maintain the running response at higher speeds than in CR rats , during conflict. Resistance to 'conflict' is analogous to 'resistance to extinction' in the PR design.

Davis (1979) showed that the benzodiazepine , chlordiazepoxide, abolished resistance to conflict in the PP paradigm at one trial per day. Results of dorsal bundle lesions on this effect are not yet clear; as in the PREE, the requirement for intact noradrenergic pathways may vary according to the extent of training and other factors. But since footshock may well be a sufficient stimulus to activate NA neurons, and since evidence of differential responses to acute and repeated footshock had been found , (sections 1 and 2) , the PP paradigm was utilised in the search for noradrenergic changes associated with behavioural training on different reinforcement schedules.

The experiments described in this section are concerned with the measurement of TH activity in synaptosomes, and of total TH activity (saturated) in brain regions containing NA terminals or cell bodies, after acquisition of a running response on a partial punishment schedule at one trial per day (OT) or 6 trials per day (MT); and, in some experiments, during the conflict phase when all rats are shocked. The behavioural experiments were performed in the Department of Experimental Psychology by Nicola Davis (Experiments G,H,I), and Sorrel Brookes (Experiments F,H,J); Experiments D and E were run by the author. Jane Nicholass and Pauline Kelly helped with sample preparation and the TH assays.

EXPERIMENTAL METHOD AND DESIGN

The experimental procedure is described in APPENDIX III for Experiment D; other experiments are briefly described in APPENDIX III, since they are all based on the same design. Table 8.1 provides a summary of the experiments to be described. Experiments D and E involved both partial punishment and partial reward groups, and are included in this section as well as in section 7.

TABLE 8.1

SUMMARY OF PARTIAL PUNISHMENT EXPERIMENTS

	Paradigm	Synaptosomes assayed	Soluble TH	Groups
Expt D	OT ACQn	hippocampus	-	HC/CR/PR/PP
Expt E	OT ACQn	hippocampus	hippocampus	HC/CR/PR/PP
			locus coeruleus	"
Expt F	MT ACQn	hippocampus	hippocampus	CR/PP
			hypothalamus	"
Expt G	OT ACQn	-	hippocampus	HC/CR/PP
			hypothalamus	"
			cortex	"
Expt G	OT CONFLICT	hippocampus	hippocampus	"
			hypothalamus	"
			cortex	"
Expt H	MT CONFLICT	hippocampus	hippocampus	HC/CR/PP
Expt I	OT CONFLICT	-	hippocampus	CR/PP
			hypothalamus	"
			cortex	"
Expt J	OT CONFLICT	-	hippocampus	CR/PP
			hypothalamus	

HC: handled controls
CR: Continuously reinforced
PP: Partial punishment schedule
PR: Partial reward schedule.

RESULTS

1. Behavioural measures: these are described in APPENDIX III (including FIGS. 8c-8f).

2. Synaptosomal tyrosine hydroxylation

Table 8.2 is a summary of the results of synaptosomal TH assays in the partial punishment experiments. The group mean TH activity is given in fmol/min/P_2 protein, and the activity of the PP groups is also expressed as a percentage of the activity in controls and in CR groups. One way analysis of variance was performed separately for each experiment (two way ANOVA, with drug condition as the second factor, in Experiment F). There were no significant main effects of TREATMENT in any of the experiments, as can be seen from the table.

There are no significant differences between CR,PP and handled control groups in hippocampal synaptosomes, in acquisition (Experiments D,E,F); nor are there any significant differences between groups after the conflict phase (Experiments G,H) . Cortical synaptosomes (Experiment D) also reveal no between-group differences .

TABLE 8.2

SYNAPTOSOMAL TH AFTER PARTIAL PUNISHMENT TRAINING

		CONTROL	CR	PP	PP/CR %	PP/CONTROL %
ACQUISITION						
Hippocampus						
Expt D	OT	223 ± 34 (7)	243 ± 22 (7)	255 ± 15 (9)	105 ± 6	114 ± 7
Expt E	OT	186 ± 17 (7)	179 ± 20 (7)	144 ±17 (8)	80 ± 9	77 ± 9
Expt F	MT	-	217 ± 24	262 ± 15	121 ± 7	-
	(saline)	-	(7)	(7)		
	MT	-	240 ± 19	242 ± 12	101 ± 10	-
	(CDP)	-	(7)	(7)		
Cortex						
Expt D	OT	207 ± 16 (7)	236 ± 11 (7)	224 ± 12 (9)	95 ± 5	108 ± 6
CONFLICT						
Hippocampus						
Expt G	OT	205 ± 43 (4)	201 ± 22 (8)	225 ± 31 (8)	112 ± 15	110 ± 15
Expt H	MT	85 ± 19 (5)	93 ± 8 (7)	87 ± 3 (7)	94 ± 3	102 ± 4

results expressed as fmol/min/mg P_2protein

results expressed as percentages

OT : one trial per day
MT : 6 trials per day
PP : partial punishment group
CR : continuous reward group

none of the differences are significant.

3. Saturated TH activity : Acquisition

Table 8.3 summarises the results from enzyme solubilised from triton homogenates of brain regions , or from lysed synaptosomes, after acquisition training. The units used to express the enzyme activity are different for these two assays (fmol/min/mg tissue wet weight for homogenates, and fmol/min/mg P_2protein for lysed synaptosomes), hence there are considerable differences in absolute values; in this table, the mean PP group activity is expressed as a percentage of CR mean activity , and, where appropriate , PP and CR means are expressed as a percentage of the HC mean. There were no significant differences between groups in any region or any individual experiment. One way ANOVA was performed on all experiments with more than 2 groups. Neither one trial per day acquisition , with shock occurring on half of these trials, nor 6 trials per day ,with about 3 shocks each day, led to any increase in total TH activity. Runway experience per se also had no effect, since there were no significant differences with respect to handled controls .

TABLE 8.3

SATURATED TH ACTIVITY
AFTER PARTIAL PUNISHMENT TRAINING.
ACQUISITION PHASE.

		PP/CR	CR/CONTROL	PP/CONTROL
		%	%	%
Hippocampus				
Expt E	OT	112 ± 6	88 ± 5	99 ± 6
Expt G	OT	102 ± 8	113 ± 6	116 ± 9
Expt F	MT	102 ± 8	-	-
Hypothalamus				
Expt G	OT	98 ± 11	92 ± 6	90 ± 10
Expt F	MT	91 ± 8	-	-
Cortex				
Expt G	OT	107 ± 15	109 ± 7	116 ± 16
Locus coeruleus				
Expt E	OT	105 ± 7	94 ± 8	98 ± 6

results expressed as percentages

OT : one trial per day
OT : 6 trials per day
PP : partial punishment group
CR : continuous reward group

4. Saturated TH activity; Conflict phase.

Table 8.4 is a summary of the results of assays of saturated TH in brain regions after the 7-day conflict phase in the runway. During this period the rats are being shocked on every trial and running speeds are dropping. Only one of the individual experiments showed a significant effect of TREATMENT; this amounted to an increased TH activity in CR rats. The statistics are shown in Table 8.4. There was a lower total TH in the hypothalamic homogenate of PP animals than in rats never shocked during acquisition, in Experiment I, significant at the 5% level on a t test. Two way ANOVA of PP and CR data from 3 similar experiments with REPLICATION as a factor showed no significant main effect of TREATMENT either in hippocampus homogenates (Experiments I,J,G : F=0.049,df 2/42) or in hypothalamus homogenates (Experiments I,J,G : F=0.339,df 2/42). One way ANOVA was performed on each experiment which incorporated PP, CR and HC groups, but there were no significant between-group differences.

The only experiment with both acquisition and conflict data for all groups and all brain regions was experiment G, and a Group x Time x Region ANOVA was carried out to incorporate all this data after $\log_{10}$ transformation. There were significant interaction terms for TREATMENT X REGION ($F= 3.02$. df 4/102, $p < 0.025$), TIME X REGION ($F= 6.85$, df 2/102, $p < 0.005$), and GROUP X TIME X REGION ($F= 2.81$,df 4/102, $p < 0.05$) . Subsequent further analysis with t tests showed that in the conflict phase, cortical TH activity was elevated in CR groups above the level of activity in PP rats ($t= 3.14$, $p < 0.01$) and above control level ($t= 4.32$, $p < 0.001$).There were no other significant betweeen-group differences in acquisition or in the conflict phase.

TABLE 8.4.

SATURATED TH ACTIVITY

DURING CONFLICT PHASE.

		PP/CR	PP/CONTROL	CR/CONTROL	Statistic
Hippocampus		%	%	%	
Expt I	OT	113 ± 10	-	-	t=1.129 p < 0.1
Expt J	OT	87 ± 9	-	-	ns
Expt G	OT	118 ± 7	110 ± 7	93 ± 4	F=2.802, p < 0.1
Expt H	MT	93 ± 10	77 ± 8	83 ± 9	F=1.84, p < 0.2
Hypothalamus					
Expt I	OT	61 ± 11	-	-	t=2.425, p < 0.05
Expt J	OT	103 ± 10	-	-	ns
Expt G	OT	118 ± 7	102 ± 6	86 ± 6	F=1.86,p < 0.2
Cortex					
Expt I	OT	122 ± 46	-	-	ns
Expt G	OT	76 ± 9	134 ± 16	175 ± 25	F=3.148, p < 0.1

results expressed in percentages

OT : one trial per day
MT : 6 trials per day
PP : partial punishment group
CR : continuous reward group

5. Noradrenaline levels

Table 8.5 gives the results of noradrenaline assays in the hippocampus during acquisition and conflict phases. There are no significant between-group differences. Resistance to punishment of the running response after partial punishment training does not seem to depend on raised noradrenaline levels.

TABLE 8.5

NORDRENALINE LEVELS IN HIPPOCAMPUS DURING PARTIAL PUNISHMENT TRAINING.

		Control	CR	PP	Statistic
Acquisition					
Expt G	OT	616 ± 17 (4)	633 + 33 (8)	661 ± 27 (8)	ns
Conflict					
Expt G	OT	568 ± 41 (4)	577 ± 22 (8)	542 ± 15 (8)	ns
Expt I	OT	-	229 ± 20 (6)	301 ± 81 (6)	ns

results expressed in ng NA/g wet weight

DISCUSSION.

The partial punishment experiments described in this section were designed to clarify whether there was any change in noradrenergic neuronal function, as measured by TH activity in synaptosomes and in soluble preparations, during runway training with shock on 50% of trials, or during conflict ,when there was shock on every trial.

The results show no consistent alteration in the synthetic potential of TH in either preparation . It appears that partial punishment training can proceed satisfactorily without such changes in the catalytic activity of TH. Neither was there any evidence of changes in NA levels underlying the behavioural tolerance to conflict in the PP group. Previous results (section 2) suggested that daily footshock at a similar intensity for 7 consecutive days had no effect on total TH activity, although a significant difference in synaptosomal TH was found between handled controls and shocked rats . In the conflict phase of one trial per day partial punishment experiments, CR rats get 7 consecutive days of shock. But synaptosomal TH in these animals is not different from that of handled controls; thus runway training in acquisition may serve to prevent a fall in synaptosomal TH after the repeated shock involved in these experiments.

Other recent evidence from this laboratory stands as additional evidence against a 'necessary and sufficient' role for noradrenergic changes in the locus coeruleus projections in mediating the partial punishment effect. Different findings according to length of training have already been mentioned in connection with drug and lesion work (section 7).

Also , S. Brookes (personal communication, 1981) has now shown in this laboratory that the partial punishment effect can be shown in both OT and MT paradigms after total hippocampectomy; thus the hippocampus, which is one of the principal projection areas of the locus coeruleus, seems to be redundant as far as this particular form of 'tolerance to stress' is concerned.

SECTION 9 :

LONG TERM EFFECTS OF ELECTRICAL STIMULATION OF SEPTAL AREA ON TH ACTIVITY IN THE HIPPOCAMPUS.

Rationale :

The theta rhythm, as recorded in the electroencephalogram of rats and other animals, results from the synchronous firing of hippocampal units. The significance of the theta rhythm has been the subject of some controversy. Recently several investigators have identified frequency bands in which theta appears to be associated with different behaviours.

'Fast' theta, at frequencies of more than 7 or 8 Hz, can be recorded during movement, and the particular frequency is correlated with the speed of movement. 'Slow' theta, with frequencies between 5 and 7.5 Hz, is not related to skeletal movement, and can be recorded in immobile rats (Vanderwolf et al, 1975). It differs in another respect from 'fast' theta activity in that it can be blocked by anticholinergic drugs. Gray and Ball (1970) suggested that theta at 7.5-8.5 Hz occurred in response to frustrative non-reward (as in acquisition on a partial reinforcement schedule, or in extinction after continuous reinforcement; see section 7).

Electrical stimulation of the medial septal area in the brain results in hippocampal 'theta driving' at the stimulation frequency (Stumpf, 1965; Gray and Ball, 1970). This presumably arises from activation of pacemaker cells in the septal area and is transmitted via the large cholinergic projection from medial septal area (MSA) to the hippocampus (Rawlins et al, 1979; Lynch et al, 1978). Gray and Ball (1970) showed that the threshold current intensity for effective theta driving in male rats varied with stimulation frequency over

the range 6-10 Hz, and that the resulting theta-driving curve displayed a minimum level at 7.7 Hz.

Theta driving by stimulation at this optimum frequency during runway training on a schedule of continuous water reward gave rise to effects which resemble the effects of non-reward (Gray,1972). When theta driving was instituted during acquisition of the rewarded behaviour, increased resistance to extinction developed; and when theta driving was given in extinction only, the behavioural response extinguished more rapidly than in unstimulated controls. Glazer (1974) trained rats to produce theta activity at 7.7 Hz on an operant schedule, and found an association with increased resistance to extinction of a bar-pressing task. Gray (1972) suggested that theta rhythm in the frequency range 7.5-8.5 Hz represents a physiological component in the processes underlying behavioural responses to non-reward. In behavioural terms, such responses appear to include inhibition of fixed action patterns and learned behaviour in favour of a search for behaviour appropriate to the new situation.

The theta driving curve was investigated , independently of behavioural correlates, by McNaughton et al (1977). Several drugs which counteract the behavioural effects of frustrative non-reward were found to alter the theta driving curve. Administration of sodium amylobarbitone, chlordiazepoxide and ethanol (all anxiolytics) abolished the minimum at 7.7 Hz in the theta driving curve, which therefore became a more continuous function of stimulation frequency. This provided more evidence for a parallel between the partial reinforcement effect (see section 7, this Chapter) and the theta driving function.

McNaughton et al (1977) also studied the neurochemical basis for the theta driving curve . Drugs which inhibited the synthesis of NA (alpha-methyl

tyrosine , and FLA-63, an inhibitor of dopamine beta hydroxylase) blocked the 7.7 Hz minimum in the curve, as did bilateral injection of the neurotoxin 6OHDA into the dorsal noradrenergic bundle (Gray et al, 1975). Antagonists of cholinergic and dopaminergic neurotransmission did not interfere with the minimum threshold for theta driving ; inhibition of serotonin synthesis by parachlorophenylalanine (PCPA) lowered the threshold current for effective theta driving at frequencies lower than 7.7 Hz. There is thus considerable evidence suggesting that noradrenergic neurones play a part in the two related phenomena discussed here : the lowering of threshold current intensity for effective theta driving from septal electrodes at a particular frequency, 7.7 Hz; and the behavioural response to non-reward . Anti-anxiety drugs block both these phenomena, and so does disruption of noradrenergic function. The results with drugs which act on serotonergic pathways suggest some interaction of noradrenergic and serotonergic systems in the control of neuronal activity in the septo-hippocampal system.

Lee Holt, in this laboratory, is investigating the long term behavioural consequences of a 'course' of theta driving lasting 10 days . Rats are trained on operant Skinner box schedules after theta driving , and their performance in acquisition and extinction phases is compared with that of unstimulated controls. The principal hypothesis under test is that experience of theta driving will (proactively) affect performance in the same way as non-reward during acquisition of a task; thus stimulated rats will show greater resistance to extinction than unstimulated controls (see section 7, this Chapter).

A further hypothesis is that experience of theta driving may affect the relationship between behaviour and aversive consequences of behaviour, including the link with stimuli which have been conditioned to punishment. In a Skinner box paradigm in which an animal has learnt to press a bar for food

reward, pairing of a neutral stimulus (the conditioned stimulus, or CS) with shock in such a way that it comes to predict the shock results eventually in suppression of responding in the presence of the CS alone as well as after footshock. A measure of the degree to which responding is suppressed (compared to a baseline response rate) in the presence of the CS serves as an index of the 'conditioned emotional response', or CER (Estes and Skinner, 1941). The CER paradigm was used in two experiments in this study to investigate the proactive effects of theta driving on behaviour under the control of aversive stimuli.

A series of experiments designed to clarify the involvement of noradrenergic mechanisms in theta driving is described in this section. Synaptosomal and solubilized tyrosine hydroxylase activity was measured in the hippocampus of rats subjected to theta driving , to see whether changes in expressed activity or total activity were apparent at times when behavioural effects of theta driving were evident.

EXPERIMENTAL DESIGN AND METHOD :

Male Sprague Dawley rats were housed individually in plastic cages and introduced to a 23 hour food deprivation schedule. The operations , brain stimulation, and behavioural training were carried out by Lee Holt in this laboratory as described in APPENDIX IV. (description of techniques adapted from Holt, unpublished work). Brain stimulation was delivered to the septal area in such a way as to 'drive' the theta rhythm at 7.7 Hz for periods of 6 seconds at a time.

BRAIN STIMULATION GROUPS are referred to as follows:

'UNCORRELATED' groups obtained food delivery on a random schedule independent of the delivery of bursts of septal stimulation;

'CORRELATED' groups obtained food at the 4-second point in each 6-second burst of septal stimulation.

'THETA-ONLY' groups received septal stimulation at 7.7 Hz with no food delivery during the stimulation period.

An extra stimulation group ('IRREGULAR') was included in experiments D and F to control for pulse frequency; these rats were stimulated via similar septal electrodes so as to give the same number of pulses as to the stimulated group, but at irregular interpulse intervals , so that theta rhythm would not occur in the hippocampus during stimulation.

In Experiments A , B, and C, acquisition training followed a ten day course of theta-driving. Experiment D was an investigation of the long-term effects of brain stimulation on saturated TH activity in the hippocampus , in comparison with a third group of unstimulated controls, regardless of behavioural consequences.

EXTINCTION AFTER FR-5 ACQUISITION TRAINING.

Rats were given training sessions in a Skinner box so that they learnt to press a bar to obtain a food reward. Details are given in APPENDIX IV. Acquisition training on a fixed-ratio schedule lasted 15 days, followed by 12 days of extinction during which no rewards were presented. Behavioural measures were recorded throughout acquisition and extinction; the results are reported in APPENDIX IV. All rats were killed 24 hours after a last extinction session.

In EXPERIMENT A , groups were as follows:

CONTROLS (6 rats); implanted; no brain stimulation and no food in stimulation box. Acquisition on FR-5 (see APPENDIX IV for details), followed by extinction.

UNCORRELATED (6): theta driving by septal stimulation with independent delivery of food pellets. Acquisition on FR-5, followed by extinction .

CORRELATED (6): theta driving followed by delivery of food pellets. Acquisition on FR-5, followed by extinction.

Interval between end of stimulation and death; approx 30 days.

EXPERIMENT B :

CONTROLS (6): implanted; no stimulation, no food during stimulation phase. Acquisition on FR-5, followed by extinction.

FOOD CONTROLS (6): implanted; no stimulation, but food delivery in stimulation box.Acquisition on FR-5, followed by extinction.

UNCORRELATED (6) : as above.

THETA ONLY (6): theta driving stimulation without food; acquisition on FR-5, followed by extinction.

Interval between end of stimulation and death: 30 days.

EXPERIMENT C :

FOOD CONTROLS (10) : as above .

THETA ONLY (9) : as above.

Interval between end of stimulation and death : 44 days.

RESULTS:

1. Behavioural results of the experiments described here will be discussed in detail elsewhere (Holt, unpublished material in preparation). A summary is given in APPENDIX IV. Briefly, it was shown that increased resistance to extinction emerged in the stimulated groups of Experiments A and B , after 8-9 days of extinction. In Experiment C, however, no increase in resistance to extinction was found in the stimulated group. It was felt that the increased interval between stimulation and death was the main point of difference between this experiment and Experiments A and B, and that this might in some way interfere with the relationship between theta driving and resistance to extinction.

2. Synaptosomal TH activity after theta driving.

EXTINCTION: The results of the synaptosomal TH assay on hippocampus in Experiment A are as follows: (groups designated as Controls, Uncorrelated or Correlated as described under 'methods').

Control	Uncorr.	Corr.
207 ± 29	223 ± 36	229 ± 34
(6)	(6)	(6)

fmol/min/mg P_2 protein.

There are no significant differences between groups at this time, 24 hours after the last extinction trial. Theta stimulation does not effect a long-lasting change in synaptosomal TH at 20 μM tyrosine and pH 7.4.

3. Saturated TH activity in hippocampus after theta driving:

For all experiments incorporating 3 or more treatment groups, results were subjected to one way ANOVA and appropriate t tests based on the ANOVA error variance. Log_{10} transformation of the data was used where necessary. For experiments containing only 2 treatment groups, results were analysed by t test (unpaired). Results of assays are shown in Table 9.2 .

EXTINCTION :

Experiment A

As shown in Fig 9b (see APPENDIX IV), rats given theta stimulation and food pellets independently (UNCORRELATED group) during the 10-day stimulation period showed increased resistance to extinction, in that in late extinction the groups separated and the UNCORRELATED group continued to bar-press faster than other groups. The results of saturated assays of TH activity are shown in Tables 9.1 and 9.2. One way ANOVA of the saturated TH activity in the hippocampus showed significant variation between groups (F=6.13, df 2,15, p 0.025) and a t test showed that the 19% increase in the mean activity in the UNCORRELATED group over that in CONTROLS was significant (t=2,85,df 15, p 0.02). The CORRELATED group SATURATED TH activity was not significantly different from CONTROL activity.

Experiment B

This experiment served as a replication and extension of Experiment A. Unstimulated controls were divided into 2 groups, one of which had food during the 'stimulation' period and one of which had no food delivery into the 'stimulation' box. A third group corresponded to the UNCORRELATED group in Experiment A, and a fourth group had theta stimulation but no food in the stimulation box. The behavioural results (Holt, unpublished data) suggested

increased resistance to extinction in both stimulated groups , this effect again emerging late in extinction. Saturated TH activity in the hippocampus was assayed (see Table 9.1). One way ANOVA showed no significant differences associated with TREATMENT (F=1.44, df 2,21); although Table 9.2 shows that both stimulated groups had somewhat higher mean TH activity than unstimulated groups, these differences did not reach significance. The two control groups had very similar values; food delivery in the absence of stimulation clearly did not affect TH activity.

TABLE 9.1

EFFECT OF PREVIOUS SEPTAL STIMULATION ON SATURATED TH ACTIVITY DURING EXTINCTION IN SKINNER BOX.

EXPERIMENT	Control	Theta-only	Uncorr.	Corr.
A	1230 ± 104	-	1866 ± 184	1156 ± 173
B	1324 ± 172	1554 ± 173	1646 ± 205	-
	1329 ± 119*			
C	1782 ± 206	2125 ± 387	-	-

	Control	TO	IRREG	
D	974 ± 234	1305 ± 221	1331 ± 197	-

results expressed as fmol/min/mg P_2 protein

*food controls

A two way ANOVA for the results of experiments A and B using treatment group and replication as factors showed a significant effect of septal stimulation ($F=7.90$, df 1,20, $p < 0.025$); neither the replication main effect nor the interaction term reached significance ($F=0.13$ and $F=0.85$ respectively). In addition to the increased resistance to extinction associated with previous theta driving, these experiments suggest a long-term increase in maximum NA synthesis potential by virtue of an increase in maximal TH activity. This is manifested a month after the last stimulation session, after behavioural differences between groups appear to have passed the maximum differentiation. It is not clear from these results whether the TH changes are attributable to behavioural performance per se or to after effects of brain stimulation, or both.

Experiment C

In this experiment the stimulated animals did not show resistance to extinction in this experiment compared to the controls. This may be due to the increase in duration of acquisition and extinction in the behavioural training phase, resulting in a greater interval between the end of the stimulation phase and death. Saturated TH showed a small increase in the stimulated group (Table 9.1) but this did not reach significance.

However, two way analysis of variance of the results from Experiments A,B and C with STIMULATION and REPLICATION as factors showed a significant main effect of STIMULATION in the $\log_{10}$ transformed data ($F=5.96$, df 1/49, $p < 0.025$). The main effect of REPLICATIONS was not significant ($F=2.91$, df 2/49, $p < 0.1$); and there was no significant interaction term.

Experiment D

In order to assess the effects of septal stimulation in the absence of behaviour-shaping procedures, three groups of animals were implanted with electrodes , and either subjected to septal stimulation at 7.7 Hz, or to septal stimulation at irregular interpulse intervals, or left unstimulated. After the stimulation period , the rats were left undisturbed for 15 days.

One way ANOVA of the saturated TH activity in the different groups did not show a significant main effect of treatment ($F = 0.79$, df 2,15); although Table 9.2 shows a rise in the TH activity of the stimulated groups with respect to controls, this does not reach significance, and the theta stimulation group shows no difference from the IRREGULAR (theta-blockade) group.

Another two way ANOVA incorporating results from all control and all 7.7 Hz stimulation groups in the above 4 experiments was performed; the main effect of STIMULATION was significant ($\log_{10}$ transform; $F=5.92$, df 1/60, $p < 0.025$) . Replication was also significant as a main effect ($F=6.12$,df 3/60, p 0.025); but the interaction term was not significant ($F=0.43$). Thus there is evidence that the long term effects of septal driving of theta activity in the hippocampus at 7,7 Hz include an increase in NA synthetic potential in the hippocampus, i.e. in saturated TH activity, although there is no change in synaptosomal TH activity in the same region.

TABLE 9.2

SUMMARY OF SEPTAL STIMULATION EXPERIMENTS:

SATURATING HIPPOCAMPAL SOLUBLE TH OF STIMULATED GROUPS

EXPRESSED AS PERCENTAGE OF UNSTIMULATED GROUPS.

EXPT.	Control	Theta-only	Uncorr.	Corr.
A	100 ± 8	-	152 ± 15	94 ± 14
B	100 ± 17	117 ± 13	124 ± 15	-
C	100 ± 12	119 ± 22	-	-

	Control	Theta-only	IRREG
D	100 ± 24	134 ± 23	137 ± 20
E	100 ± 17	92 ± 14	-
F *	100 ± 7	96 ± 6	103 ± 5
F(CER)	100 ± 8	104 ± 6	95 ± 9

results expressed as percentage of control ± S.E.M.

* Pseudo-CER groups (no shock - see APPENDIX IV)

CONDITIONED SUPPRESSION EXPERIMENTS.

In Experiments E and F the behavioural design employed was a conditioned suppression paradigm . The acquisition of the conditioned emotional response (CER) is described in APPENDIX IV. The bar-pressing response is shaped on a random-interval schedule, and later in training a tone+shock pairing is presented repeatedly. Bar pressing comes to be suppressed in the presence of the CS alone as well as during footshock ; the tone thus seems to take on aversive stimulus properties through association with the shock (CS-UCS pairing). The ratio of the rate of bar-pressing in the CS period to the sum of the preceding baseline and the CS rates (duration of CS and pre-CS periods is 20 sec) is designated the suppression ratio and is used as an index of the CER (see above). 24 hours after the last session, rats were killed by a blow to the head and immediate decapitation.

EXPERIMENT E : groups were as follows
(all animals were subjected to footshock):

SHOCK-CONTROL (5 rats): no septal stimulation .
CER training with shock current intensity 0.16 mA on day 1 of acquisition, 0.2 mA on day 2, 0.3 mA on all subsequent days.

SHOCK + THETA (7): theta driving at 7.7 Hz for 10 days,as above. Then CER training as above.

EXPERIMENT F :
(including non-shocked controls for each of the stimulation conditions)

CS-CONTROL (4): no stimulation.
Pseudo-CER training, i.e. presentation of CS without shock.

CS-THETA (4) : theta driving at 7.7 Hz.
Pseudo-CER training as above.

CS-IRREGULAR (4): irregular septal stimulation, not inducing theta. Pseudo-CER training as above.

CER-CONTROL (4): no stimulation.
CER training with CS and shock presentation.

CER-THETA (4); theta driving at 7.7 Hz.
CER training as above.

CER-IRREGULAR (4): irregular stimulation.
CER training as above.

RESULTS:(CONDITIONED SUPPRESSION EXPERIMENTS) :

4. Behavioural results : In Experiment E, theta-driven rats showed slower development of the suppression of responding than controls. In Experiment F, theta driving can be shown to affect the CER if the stimulation-related effects on baseline response rate are dissected out by covariance analysis.
See APPENDIX IV for fuller description of results.

5. Synaptosomal TH :

. CONDITIONED SUPPRESSION: The results of hippocampal and hypothalamic synaptosomal TH after 12 days of conditioned suppression training at 6 shocks per day , in Experiment E, are shown below. Note that all animals have been shocked ; the difference between the groups lies in experience of previous theta driving rather than in experience of shock.

	Shock-control	Shock + theta
Hippocampus	185 ± 21	172 ± 12
	(5)	(7)
Hypothalamus	496 ± 35	457 ± 31
	(5)	(7)

fmol/min/mg P_2 protein.

Again, there were no statistically significant effects of septal stimulation on synaptosomal TH in either region at this interval (30 days between the end of the stimulation period and assay). Both groups in this experiment have had a total of 72 shocks at 0.16-0.25 mA over the 12 preceding days.

6. Saturated TH activity in hippocampus after theta driving:

Experiment E:

This experiment showed that previous theta driving experience was associated with reduced suppression of bar-pressing in the presence of a CS for footshock. This form of 'resistance' to stress-induced behaviour change may be seen as a parallel to the resistance to extinction seen in in experiments A and B. However, there was no difference in saturated TH activity in the hippocampus of the two groups (Table 9.3; and see also synaptosomal results above). Nor was there any significant linear correlation between suppression ratios on the last day of CER training and TH activity in the synaptosomal or saturating solubilised assays of TH activity (r=0.30, r=0.10 respectively). The fact that both groups had been subjected to footshock and there was no control, non-shocked group, however, meant that the effects of shock could not be differentiated from those of brain stimulation.

Experiment F:

Non-shocked controls were incorporated in the design of this experiment , which used a similar behavioural procedure to that of Experiment E. However, there were only 4 rats in each group, which is less than the number generally required for biochemical assay. The results of all the 6 groups in this experiment (see above for enumeration of groups) were subjected to two way ANOVA with SHOCK and TREATMENT as factors; neither main effect proved significant, although the effect of SHOCK in reducing mean saturated TH activity was near significance (F= 2.49, df 1,18, p 0.2). There are, therefore, grounds for believing that the effect of recent repeated shock per se may interfere with a possible long term effect of theta driving on TH levels in the hippocampus.

TABLE 9.3

EFFECT OF PREVIOUS SEPTAL STIMULATION ON SATURATED TH ACTIVITY IN HIPPOCAMPUS DURING CONDITIONED SUPPRESSION.

EXPERIMENT	Control	Theta-only	IRREG
E	1285 ± 214	1185 ± 186	-
F (CER)	2089 ± 174	2173 ± 132	1987 ± 184
F (Pseudo CER)	2281 ± 150	2180 ± 136	2340 ± 12
	fmol/min/mg P_2 protein Numbers in each group are specified in text		

DISCUSSION:

Two conclusions seem to emerge from this series of experiments. First, synaptosomal TH in the hippocampus , assayed 24 hours after the last extinction trial, did not reflect between-group differences in extinction performance in the Skinner box (Experiment A) nor in CER performance (Experiment E). Nor was there any correspondence with the finding of raised saturated TH in Experiment A. This dissociation between synaptosomal and solubilized saturating TH responses gives further support to the arguments previously put forward about the many different mechanisms which may contribute to the long term and short term control of NA synthesis (see Chapter 3, and sections 3-5 of this Chapter).

Second, changes in saturated solubilized TH have been found in rats killed a month after the last of 10 theta driving sessions. As in previous experiments using solubilized tissue assayed on different days, there was considerable variation in control TH activity from unstimulated animals which may represent factors in the assay, or variations in tissue enzyme activity after storage. Overall, in the experiments performed in the extinction phase after FR-5 acquisition, the stimulated animals had higher saturating TH levels than unstimulated animals.

No significant biochemical differences between groups in the CER conditioned suppression paradigm were found. The 72 low-intensity footshocks (spread over 12 days) did not produce a significant change in the saturated TH in the hippocampus of unstimulated controls in Experiment F; this is not surprising, perhaps, in view of the similar findings (albeit with fewer shocks) in sections 2 and 8 of this Chapter. However, there was some suggestion that shock might interfere with the expression of the long-term effects of theta driving on TH.

In summary, the results of this section,though providing some evidence for a long term effect of septal stimulation on maximal TH activity in the hippocampus , do not substantially improve the evidence for a role for noradrenergic changes underlying the development of resistance to extinction of a previously rewarded bar-pressing response; nor do they suggest a noradrenergic basis for the reduction of the suppression ratio in the CER experiment in rats previously subjected to theta driving. An analogy between these results and those of experiments on the effect of dorsal bundle lesions and drugs on the partial reinforcement extinction effect can be drawn; in all these examples there is , under certain circumstances , evidence that noradrenergic changes are associated with the behavioural ones. However, when training is given with an increased number of acquisition trials, the evidence for noradrenergic mechanisms in maintaining behaviour in the extinction phase becomes less compelling.

The biochemical assay employed to measure maximal enzyme activity for the purposes of finding evidence of induction or 'delayed activation' of tyrosine hydroxylase has shown poor inter-assay reliability, which would tend to obscure between-group differences. However, individual experiments have provided enough evidence to indicate that theta driving may activate noradrenergic TH under certain circumstances. More work with careful selection of control groups is required to determine the time course of this effect, its anatomical basis, and its relation to behavioural consequences of theta driving.

CHAPTER 6

EVALUATION :

TYROSINE HYDROXYLASE ACTIVITY AS AN INDEX OF NORADRENERGIC FUNCTION.

A . LIMITATIONS OF METHODOLOGY .

B . TH ACTIVITY AS AN INDICATOR OF RAPID CHANGES IN NORADRENERGIC NEURONES.

C . TH ACTIVITY AS AN INDICATOR OF SLOW-ONSET CHANGES IN NORADRENERGIC NEURONES.

D . NORADRENALINE SYNTHESIS AND STRESS TOLERANCE.

E . NORADRENALINE , ANXIETY AND DEPRESSION.

A. LIMITATIONS OF METHODOLOGY.

It was pointed out in the introductory chapter that an understanding of the noradrenergic pathways in the brain would probably require investigations which took into account the characteristic differences between the properties of these neurones and those, for example, of the ascending sensory pathways or the cholinergic innervation of skeletal muscle (Fillenz, 1977). Clark (1979) ends his review of the locus coeruleus and its role in behaviour regulation with a plea for

> ' research emphasising more subtle behavioural measures, an expanded time range of observations, measurement of environmental and biochemical systemic stress effects, and in vivo, kinetic, microchemical analysis.'

For reasons outlined in Chapters 1 , 2, and 3, the measurement of tyrosine hydroxylase activity was chosen as the index measure of neuronal activity for the experiments presented in Chapter 5. Briefly, it was assumed that since neuronal activity in noradrenergic neurones results in the release of noradrenaline, and since noradrenaline levels can often be maintained at normal levels in spite of increased neuronal activity, the synthesis rate of NA might be a sensitive index of neuronal activity.

The measurement of NA synthesis in vivo is subject to various problems which were outlined in Chapter 3 and in the references cited there. Since tyrosine hydroxylase catalyses the rate-limiting step in NA synthesis, direct measurement of the activity of this enzyme in fresh rat brain tissue in vitro was undertaken. An adaptation of an existing assay of TH activity in synaptosomes (Boarder and Fillenz, 1978) facilitated the use of this method at a physiological pH in noradrenergic brain regions which had previously been found to yield insufficient activity for the analysis of differences in activity

generated by in vitro treatments (Boarder and Fillenz,1978). The synaptosomal TH assay in different noradrenergic brain regions was then used to investigate the effects of various treatments, using the post-mortem TH activity in freshly-prepared synaptosomes as an indicator of NA synthesis rate ante mortem. TH activity was also assayed, in some experiments, in lysed synaptosomes, or in homogenates of brain regions, at saturating concentrations of substrate and cofactor, to give an index of total potential NA synthetic activity; and at subsaturating concentrations, to get an indication of kinetic changes in the enzyme.

Before summing up the results and implications of the different treatments undergone by rats in the experiments described in Chapter 5, we now have to discuss whether assays of TH activity in synaptosomes or in solubilised preparations are valid indicators of noradrenergic activity in the rat brain, and what relationship they bear to other measures . Except in a few experiments, in which NA levels or storage capacity were measured in the same tissues as TH activity, direct comparisons with other measures of NA function were not available. Indirect comparisons were possible when the experimental conditions were carefully matched to reports of work in other laboratories using other measures; but these manipulations provide only a rough guide.

The usefulness of indicators depends on their sensitivity and specificity. Specificity denotes the extent to which the indicator reflects exclusively the functioning of the system under investigation , in this case the noradrenergic neurones, to the exclusion of interference by changes in other systems . Sensitivity is associated with the extent to which true changes in the system under investigation are detected and reflected as changes in the value of the indicator . One source of information which is pertinent here is the pool of results on control, untreated rats in the present experiments.

Variation between experiments.

An ideal measure of biochemical or physiological function shows minimal variation between different subjects around a control value established under standard experimental conditions. Likewise, control variation between experiments should ideally be kept to a minimum if a given measure is to provide a sensitive index of change due to some independent experimental variable. It is clear from the analysis of the results of sections 1 and 2 of Chapter 5 that there was significant variation in synaptosomal and soluble TH activity in both hippocampus and hypothalamus which was attributable to between-experiment variation in assays performed on control animals. Within-group variation in the individual experiments (replications) remained within acceptable limits (e.g. standard error of mean was usually 10 % of mean value or less). The range of variation was wider for the soluble assay than for the synaptosomal assay. Although the sources of this variation have not been satisfactorily located (see last section of Chapter 4), the fact that the soluble assay involves the use of a complicated incubation 'cocktail ' which is freshly prepared from a large number of ingredients individually added to the tissue samples just before incubation may account for the excess variation between controls in this type of assay. This problem is shared by other laboratories using similar assays (Salzman and Roth, 1980a; Weiner, 1979).

The discovery of the extent of between-experiment variation in control TH activity ruled out the possibility of using the same pool of controls for comparison with treated animals in successive experiments. Each single experiment required a control group, and where matching of control and treated rats in pairs was possible, this sometimes facilitated the detection of treatment effects. Analysis of variance including experimental replications as a factor was used whenever appropriate, in order to distinguish between-experiment variation from the genuine effects of treatment.

Many experiments reported elsewhere using the in vitro assay of solubilised TH activity have employed pooled tissue from different brain regions and from different animals; and , as was emphasised in earlier chapters, most of the formative work on TH was performed with striatal enzyme rather than noradrenergic tissue . More recently, other workers have looked for between-animal differences and important differences between noradrenergic and dopaminergic TH have been found. New sources of variation uncovered recently include a possible diurnal variation in the amount of protein in the locus coeruleus , and a multiphasic diurnal variation in the saturated activity of TH (Natali et al, 1980). The pteridine cofactor synthesis and reduction reactions are also controlled by enzymes whose activity may show diurnal variations . This is newly charted territory; further work will probably reveal plausible sources for variation which cannot at present be accounted for.

One major disadvantage of the between-experiment variation in measures of control TH activity in the present study was that it undermined the anticipated value of expressing synaptosomal TH activity as a percentage of saturated soluble TH activity in the same animals. There were few examples of significant correlations between synaptosomal and soluble TH activity within groups, and fewer still when the correlation included groups from several experiments. Overall, the synaptosomal activity generally 'expresses' around 10 % of the saturated soluble activity in the hippocampus and cerebellum, and about 2 % in the hypothalamus. The regulatory mechanisms controlling the expressed and the total enzyme activity were discussed in Chapter 3; the results obtained in Chapter 5 provide further evidence of the independence of these measures in individual animals.

Variation between regions.

It was anticipated that synaptosomal TH activity would provide an index of neuronal activity in noradrenergic neurones. From the discussion of the anatomy of these neurones in Chapter 1 it is clear that neurones in the LC provide all the noradrenergic innervation of hippocampus and cerebellum, and part of the noradrenergic innervation of the hypothalamus. If synaptosomal TH activity were to reflect rate of firing of LC cells, and if the LC cells projected uniformly to all regions with considerable contribution from collateral networks, a close relationship between TH activity in synaptosomes of hippocampus and cerebellum would be expected. This expectation was undermined : first, by recent work (Mason and Fibiger, 1979) suggesting that the LC is by no means a homogeneous nucleus and that different subdivisions of it appear to project to different brain regions; second, by results in control rats which failed to show a closer correlation between TH activity in hippocampus and cerebellum than between hippocampus and hypothalamus, despite the non-LC noradrenergic and dopaminergic TH contributions to the measured TH activity in the hypothalamus.

Regional differences in density of noradrenergic innervation and in the morphological characteristics of NA terminals in different regions may also be important determinants of total or expressed TH activity (see Bacopoulos and Bhatnagar,1977). There is still a lack of agreement in the literature as to which regions to choose for biochemical analysis to demonstrate changes in noradrenergic mechanisms(see Salzman and Roth, 1979, for a caveat on the results obtained in cerebral cortex in various studies); and the technical difficulties of single-unit recording have not permitted simultaneous recording from different noradrenergic projections. The extent to which there is coordination of neuronal activity in noradrenergic pathways , and particularly in the ascending projections of the LC , is an

interesting and as yet unresolved question, especially in the light of Segal's suggestion (1980) that the different characteristics of noradrenergic projections may allow for different functions of these pathways. (Segal cites control over the transfer of information as a possible function of the 'closed loop' projections from the LC, such as the projection to the spinal cord; but suggests that the 'open loop' system from the LC to the hippocampus, which lacks a direct pathway back to the LC, may serve instead to facilitate certain types of information processing in the projection area, for example altering thresholds for differentiation between incoming stimuli.)

From the studies reported here, it would appear that synaptosomal TH activity is probably a measure of something other than firing rate or accumulation of metabolic products; it seems to be under the control of regional factors about which little is known. Solubilised TH activity at subsaturating concentrations of cofactor and substrate also failed to follow anticipated changes in firing rate and synthesis of NA (see APPENDIX I).

Jacobowitz (1979) interprets the evidence for a heterogeneous regional response of NA levels to stimuli such as immobilization, cold, and isolation in a similar way, and makes the almost heretical suggestion that the primary responses to stress in the brain may not be mediated by the firing of the noradrenergic neurones. He suggests instead that local changes around noradrenergic terminals (release of other substances, changes in pH or oxygen tension, etc) involving the 'receptive' (postsynaptic) neurone may in fact

> 'influence the (noradrenergic) terminal nerve membrane in such a way as to modulate the release of NA, which, in turn, acts upon the adrenoceptor.... this may be a physiological mechanism whereby moment to moment local control can occur without involving impulse discharge from cell body groups.'

This mechanism may also bring about localised changes in synthesis rate and enzyme activity.

Some of the treatment-related changes in TH activity in the experiments reported in Chapter 5 will now be discussed, in an attempt to assess the significance of the results in terms of possible modes of action of noradrenergic neurones.

B. TH ACTIVITY AS AN INDICATOR OF RAPID CHANGES IN NORADRENERGIC NEURONES.

In chapter 3 , it was shown (section 8) that synaptosomal TH activity was increased by the in vitro addition of cyclic AMP or depolarizing agents to the incubation medium; addition of NA reduced the synaptosomal conversion of tyrosine to DOPA. These in vitro manipulations showed the synaptosomal preparation to be comparable to preparations using brain slices . The results with hippocampal and cerebellar synaptosomes were similar, whereas the hypothalamic synaptosomes showed a different pattern of response, probably because of the presence of mixed dopaminergic and noradrenergic terminals. It was also shown that the effects of depolarization on enzyme activation are to some extent independent of NA release, in that depolarization causes activation of the synaptosomal enzyme even in the absence of extracellular calcium ,which is essential for NA release. Other workers have also recently produced data which support the notion that the processes which control NA synthesis and release can be separated (Salzman and Roth, 1980a).

In the studies reported in Chapter 5, which were performed concurrently with the in vitro experiments indicated above, the synaptosomal method was used after in vivo treatments which were thought likely to affect noradrenergic neuronal activity. After a single mild footshock, a small activation of synaptosomal TH activity was often found in both hippocampus and hypothalamus; but the activation was only evident , in pairs of rats , when

the TH activity in the control rat was below a threshold figure of about 170 fmol/min/mg P_2 protein. Another effect of the footshock was discovered when the between-replications variance of shocked rats was found to be less significant than that of control rats. This suggested a mechanism such as a ceiling effect or the induction of some post-arousal state characterised by a particular level of enzyme activity. In the discussion of these data (Chapter 5, section 1), an analogy was drawn with Weiner's model for conversion of TH from a low-affinity to a high-affinity form, and the accentuation of the effects of 'activating' stimuli when preincubation is instituted as a routine , since the enzyme reverts during preincubation to the low-affinity form. A further comparison may be made with Herman's study (1970) of the behavioural effects of intracerebral NA injections: the effects of the injections turned out to be dependent on the baseline behavioural arousal level of the subjects, especially when low doses of NA were used. A further example of the dependence of noradrenergic responses on the baseline level is found in an electrophysiological study by Szabadi et al (1977), in which the direction of the change in firing rate of NA neurones was associated with the rate of firing of the resting neurones.

If the responses of noradrenergic neurones to stimulation are dependent on antecedent factors to the extent suggested by the above results, then the most plausible experimental paradigm for investigating activation of neuronal activity would be a within-animal study with subjects acting as their own controls. Electrophysiological studies on single units fall into this category; but because of the technical difficulties involved , studies on noradrenergic neurones in unanaesthetized animals have only been attempted in a few laboratories . Biochemical studies of the type reported in this thesis are relatively insensitive to such changes, since each subject is examined once only, either after random allocation to a control or a treatment group, or, at best, as a member of a matched pair.

The synaptosomal TH assay , then, gave a crude indication of biochemical processes involved in the response to a mild and short-lasting noxious stimulus, namely footshock at 0.3 mA intensity. Other probable features of this type of noradrenergic response , judging from work in other laboratories, include a rapidly-damped burst of electrical activity in cells of the the LC, a possible inhibition of spontaneous activity in post-synaptic neurones in LC projection areas, and a rapid release of corticosteroids from the adrenal cortex. Although not optimally sensitive to the transient changes involved in this response , the synaptosomal method evidently has some advantages over the in vivo measures of NA synthesis documented in Chapter 3 (section 11) which involve the accumulation or collection of metabolites or transmitters over periods of 30 to 120 minutes , and are less likely to be able to reflect transient activation of activity against a varying baseline. Saturated solubilized TH activity , on the other hand, was unchanged after a single footshock. This assay presumably represents the maximal potential activity of the solubilised enzyme and was not expected to change in response to a short-lasting burst of neuronal activity.

The time courses of noradrenergic responses to administration of single doses of the drugs used in sections 3 and 4 of Chapter 5 are not comprehensively documented in biochemical or electrophysiological studies. Presumably the duration of action is related to the distribution and solubility of the drug as well as to neuronal responses to the particular pharmacological effects of the drug. Certainly the effects are likely to be of a more sustained nature than the effects of a single footshock, and in most cases probably last for several hours. Evidence summarised in Tables 3.1, 3.2, and 4.1 showed that the biochemical and electrophysiological effects of a number of drugs tallied with each other with respect to the direction of the effect. An exception was made for morphine, which, whilst causing a profound inhibition

of LC neuronal activity, is not associated with a sustained increase or decrease of NA synthesis as measured by in vivo turnover studies.

Even when distinct changes in synaptosomal tyrosine hydroxylation rate were detected after administration of drugs in these experiments, in which the timing of the injections were carefully selected , they did not always follow the pattern predicted from the electrophysiological or previous biochemical studies. In the case of morphine, a single dose was found to reduce hippocampal TH activity significantly with respect to controls; after naloxone-precipitated withdrawal from morphine, an increase was found. In this case, the TH follows the electrophysiological responses but not the biochemical ones quoted in Table 3.2.

Administration of parachloramphetamine was also associated with a rise in TH activity, probably arising from the reduction in inhibitory input from raphe neurones on the LC. Although the evidence for a reciprocal effect of 5HT and NA neurons was thus strengthened,the biphasic effect predicted by extrapolation from the behavioural and electrophysiological studies reported in section 6 of Chapter 5 was not demonstrated .

Neither chlordiazepoxide nor desipramine, which reduce firing rate in the LC, produced any significant change in synaptosomal TH activity. Administration of clonidine, which reduces LC firing rate by a mechanism probably similar to that of desipramine in the LC, was followed by a rise in synaptosomal TH activity. Since yohimbine had the opposite effect to clonidine, it seemed likely that an action at presynaptic receptors on NA terminals was involved; but the mechanism remains unclear. There is as yet no other evidence from in vivo studies to suggest that a drug which inhibits release of NA should also increase the synthesis of NA in the short term (but see Tissari et al, 1979 for a report of anomalous receptor-mediated effects on synthesis in dopaminergic neurones).

Further work is needed to extend these new findings of receptor-mediated changes at the rate-limiting step in NA synthesis. Further acquaintance with the distribution of adrenergic receptors in different brain regions and the definition of structure-function relationships with respect to impulse traffic in the patterns of innervation of the regions will help. The effects of clonidine and yohimbine in the experiments described here were confined to the hippocampus; this may reflect the possible differences in the function and structure of LC projections alluded to above, and in Chapter 1; but more data are needed to back up such speculation.

With respect to short term changes in noradrenergic activity, then, there is evidence for at least 4 different types of response which can be measured post mortem in freshly prepared hippocampal synaptosomes:

1. activation of TH activity in rats killed 10-15 seconds after a single mild but noxious stimulus. The extent of the activation is probably inversely related to the resting enzyme activity.

2. activation of TH activity 1-3 hours after administration of parachloramphetamine. This activation may be due to the loss of inhibitory effects of serotonergic release either near the locus coeruleus noradrenergic cell bodies, or near noradrenergic terminals in the hippocampus.

3. reduction of TH activity 2 hours after morphine injection, and increased TH activity shortly after precipitation of opiate withdrawal in dependent rats. This may be related to effects on opiate receptors on the LC.

4. an apparently paradoxical effect after administration of alpha$_2$ adrenergic receptor agonist and antagonist drugs (clonidine and yohimbine); the alteration in hydroxylation rate was in the opposite direction to that found with in vivo studies.

Synaptosomal TH activity seems to be more sensitive than other biochemical methods for detecting short-term changes which may affect NA synthesis rate; but the different mechanisms of local activation or inhibition of the enzyme in synaptosomes are in need of clarification with further work. The question of the specificity of TH activity as a measure of NA function calls for simultaneous monitoring of many aspects of noradrenergic neurones, including tyrosine hydroxylation. At present it seems that NA synthesis is probably under the control of factors other than firing rate, some of which operate at a local, regional level (Jacobowitz, 1979). It is also clear that NA synthesis and release are to some extent independently controlled. Synthesis, as we have seen, can be raised whilst release is probably inhibited in the presence of clonidine, and vice versa for the effect of yohimbine .

This tallies with evidence from Salzman and Roth (1980b) and Nicholass and Fillenz (in press) who showed that in vitro TH activity could be accelerated in the absence of calcium ions, which are essential for NA release.

In some of the drug experiments in Chapter 5, an attempt was made to measure solubilized TH activity under subsaturating conditions (see APPENDIX I). The results are occasionally tantalizing, but generally inconclusive ; and where a significant effect was found (eg with a single dose of desipramine) it did not agree with the synaptosomal results. Further work is needed to optimise the sensitivity and reliability of this assay for noradrenergic regions, with special attention to the concentration of tyrosine , and the use , where possible, of the natural pteridine cofactor. It may also be possible to improve sensitivity to enzyme activation by using a pH of 7 or more in the incubation (Bahkit and Gibb, 1979). The proliferation of different assay methods for measuring TH activity in vitro , as discussed in Chapter 2, bears witness to the difficulties encountered by many workers in standardising this assay satisfactorily.

C. TH ACTIVITY AS AN INDICATOR OF SLOW-ONSET CHANGES IN NORADRENERGIC NEURONES.

Alterations in synaptosomal TH after repeated stimulation are open to several interpretations. When repetition of a stimulus ceases to elicit the same behavioural response as on first presentation, one explanation is that habituation has occurred. This denotes a response decrement ; but the mechanism may not be specified in physiological terms. A different interpretation, used with reference to pharmacological responses, is that tolerance has occurred. The development of tolerance implies a change in the neurone associated with a raised threshold for the response to such stimuli , such that increased intensity of the stimulus is required to elicit the response at the original strength. A third possibility is that the response decrement is due to the recruitment of some new mechanism which actively suppresses the original type of response to the stimulus.

The responses to repeated clonidine administration are those expected when tolerance develops. Acute administration of clonidine increases TH activity in synaptosomes. But two hours after a last exposure to clonidine, either in drinking water or by injection, there was no difference in synaptosomal hydroxylation rate between clonidine-treated and control rats.

However, the responses to repeated shock and handling cannot be explained in this way. The finding of a raised TH activity in handled , unshocked controls and the non-significant response to a seventh daily footshock suggested that repeated handling over a week or two resulted in some neuronal activation. This is not accompanied by obvious behavioural activation; on the contrary, handled rats become noticeably more tame , and defecate less in a given period of handling , the sharpest decrement being over the first 3-5 days. There was no difference between the synaptosomal TH

activity of unhandled controls and repeatedly shocked rats. The conclusion drawn was therefore that the effect of repeated shock was to suppress the effect of handling on TH activity, possibly by recruitment of an unknown mechanism which takes some time to become effective. If the handled controls had not been assayed, the assumption that repetition of shock simply led to habituation, or to the raising of some threshold to shock , would have been tempting; but the mechanism of simple habituation or tolerance will not account for the results as they stand. There is clearly an interaction between handling and footshock with respect to synaptosomal TH activity.

Although the handling effect appears to comprise a long-lasting and stable elevation of synaptosomal TH activity, it is confined to the hippocampus; and it is not accompanied by any evidence of induction of the enzyme, since the saturating TH activity is unchanged. Animals which are subjected to handling and also to runway training with or without footshock do not have a lower synaptosomal TH activity than handled controls (Chapter 5, section 7). Thus the interaction between footshock and handling may be susceptible to further interference by runway experience. Further investigation of enzyme activity in handled animals at different substrate and cofactor concentrations will be needed to discover whether the synaptosomal activation is related to any change in the kinetic properties of the enzyme; and the effect of a history of handling on the synaptosomal response to acute mild footshock (acute-on-chronic stimulation) also calls for investigation.

Saturated TH activity was not altered by repeated handling, shock, runway experience, or repeated administration of clonidine. But repeated administration of desipramine and morphine, withdrawal from clonidine treatment and electrical stimulation of the septal area at a frequency of 7.7 Hz were all associated with changes in this measure of maximal NA synthesis potential . (No changes were found in synaptosomal TH activity in these

circumstances.) Without using techniques such as immunoprecipitation to demonstrate changes in the quantity of enzyme protein similar to those found in the well-documented case of reserpine treatment , these changes in soluble activity of the enzyme cannot be imputed to enzyme induction or to negative induction. They have been found in the LC, in the hippocampus, and in the hypothalamus, and may be related to other types of long term, possibly trans - synaptic activation such as that found with oxotremorine (Lewander et al, 1975).

As pointed out in Chapter 3, these long term changes in total NA synthetic capacity are not necessarily manifested in the form of increased NA synthesis after the precipitating stimulus. They appear to provide a mechanism for adaptation, as if in anticipation of the need for an expansion of synthesising capacity, in the case of induction. The increase in TH total activity occurs regardless of whether the anticipated threat materialises and indeed regardless of whether the increase in synthesis actually comes about. This is thus a relatively uneconomic system, with inertia at both initiating and offset points.

Mandell's discussion (Mandell, 1978) of redundancy in the control of tyrosine hydroxylase activity has already been cited. The results summarized here provide further evidence of these various mechanisms, and point to different circumstances in which particular mechanisms may come into play or predominate over others. We have still not reached the stage of being able to predict the necessary and sufficient conditions under which changes in synaptosomal synthesis rate or in maximal activity of the enzyme will occur. Nor is the physiological significance of these changes clear in terms of noradrenergic function in vivo.

The results of the work reported here suggest that experimental demonstrations of alterations in average firing rate in noradrenergic neurones after administration of drugs do not provide a sufficient basis for prediction of changes in post mortem synaptosomal TH activity. On the other hand, the alterations found in saturated TH activity do correspond with the notion of suppression of noradrenergic functional capacity during long term antidepressant or morphine treatment. In addition, there is evidence for a sustained increase in noradrenergic potential activity after withdrawal of clonidine.

D . NORADRENALINE SYNTHESIS AND STRESS TOLERANCE.

The involvement of NA neurones in responses to acute noxious stimulation was discussed earlier. There is a rapid response of synaptosomal TH activity to a single mild footshock, although this is by no means an ideal indicator of firing rate in noradrenergic neurones.

The behavioural studies of the partial punishment effect showed that occasional exposure to punishment (mild footshock) as well as reward during acquisition of a running response in a straight alley results in persistence of responding during the conflict phase when every trial is punished. This phenomenon is a manifestation of tolerance to stress, since during acquisition the probability of a response is reduced by presentation of shock. A similar pattern is found with the behavioural resistance to extinction described earlier with respect to the partial reward extinction effect (see sections 7 & 8, Chapter 5).

Given that synaptosomal TH served as an indicator of a mild stress response, and that repeated handling and shock also affect synaptosomal TH activity, the behavioural experiments in sections 7 and 8 of Chapter 5 were performed to test the hypothesis that TH activity would be affected by the reinforcement schedule under which rats were performing during acquisition and/or extinction of the running response . Thus, although the rats were never subjected to severe stress as in the experiments of Weiss et al (1975), it was suggested that behavioural tolerance of mild stress would be accompanied by an increase in TH activity. This increase, which might arise from increased activity of the noradrenergic neurons, might indeed be responsible for the behavioural changes (Gray et al, in press). Previous results with lesions of

ascending noradrenergic fibres had suggested that under certain circumstances, such manifestations of tolerance to stress were dependent on neuronal activity in these pathways.

Pursuing this line in the light of pharmacological experiments, it was tempting to think of the noradrenergic system of neurones as capable of responding to repetition of stressful stimulation in such a way as to anticipate further demands on the system. Rather than merely releasing more noradrenaline each time a stimulus occurred , these neurones could perhaps also set in motion an increase in the total synthetic potential . Increased capacity takes time to develop, and may not be evident until after the precipitating stimulus has ceased. It would not necessarily be accompanied by increased release or synthesis of NA in terminals under physiological conditions. In fact additional mechanisms such as habituation or tolerance might be operating simultaneously on synaptosomal noradrenergic function.

The results of the biochemical assays of TH activity performed during the behavioural experiments did not show a reliable activation of synaptosomal synthesis of NA during the development of tolerance to stress in either paradigm. Nor was there any evidence for an increase in Vmax, i.e. an expansion of the maximal NA synthesis potential. To the extent that TH activity can be seen as an indicator of noradrenergic function, as discussed in the earlier sections of this evaluation, these results stand against the hypothesis of a noradrenergic basis for the development of behavioural tolerance to mild stress. There was some suggestion of differences between runway-trained animals and handled controls, but there was no clear separation on biochemical grounds between different reinforcement conditions. However, there was an increase in saturating TH activity in the hippocampus of the theta-driving groups in section 9 compared to unstimulated controls; and these groups also showed resistance to extinction of a bar-

pressing response, in two out of three experiments.

There is other evidence which detracts from the view of noradrenergic neurones as responsible for tolerance to stress. Although Weiss et al (1975) showed that repeated shock and repeated cold swim produced resistance to stress, cold swim was not associated with increased TH activity. Also the failure of dorsal bundle lesions to affect resistance to extinction after a large number of acquisition trials has been pointed out, as has the persistence of the partial punishment effect after total hippocampectomy.

In summary, resistance to extinction does appear to be accompanied by changes in noradrenergic pathways, under certain circumstances; but there is mounting evidence that similar behavioural phenomena can occur without any alteration of noradrenergic function.

E. NORADRENALINE , ANXIETY AND DEPRESSION .

The question of noradrenergic involvement in human emotional states was introduced in Chapter 1. A short summary of the current status of the noradrenergic hypotheses as they relate to anxiety and depression is presented here to conclude this discussion.

It is not easy to define anxiety in behavioural or phenomenological terms. Redmond (1979) suggests that 'fear' and 'anxiety' can be used interchangeably as descriptions of a normal human emotion, and distinguishes 'pathological anxiety'. Human anxiety can resemble an 'alarm reaction', as in a panic attack, or it can develop as a chronic , pervasive state . Anxiety can be triggered off by specific objects and situations, in which case it is referred to as phobic anxiety; or, again, it can be a non-specific response or state ('free-floating anxiety'). In animal experiments, fear is seen as that part of the response to 'punishment' (aversive stimulation) which can be conditioned to stimuli associated with punishment. When this has occurred, the emotional response can be elicited by presentation of the conditioned stimulus (CS) alone. Fear can thus be empirically differentiated from 'pain', which only occurs if the aversive stimulus is actually presented and felt.

There is plenty of evidence for a noradrenergic component in rapid alarm reactions to noxious or intense or novel stimuli (see Chapter 1, and section 1 of Chapter 5). Redmond has investigated the activity of the locus coeruleus in monkeys and has shown that behaviour seen when the animals are presented with 'threatening stimuli' accompanies LC stimulation. These behaviours are reduced by administration of substances which slow LC firing

rate (Redmond, 1979).

Some authors have demonstrated noradrenergic changes during behaviour which is under the control of conditioned stimuli associated with aversive events, or changes in such behavioural patterns when noradrenergic pathways are interfered with by lesions or drugs (e.g. Tilson et al. 1975; Cassens et al, 1980; Davis, M., 1979). In sections 7 and 8 of Chapter 5, some of the evidence for a noradrenergic component to behaviour associated with anticipation of non-reward and punishment was discussed; the evidence is as yet equivocal. McNaughton and Mason (1980) conclude in their review that the dorsal noradrenergic bundle , although it may play a role in responses to novel stimuli, is not critically involved in fear; for example there is no consensus as to a clearcut role for the dorsal bundle in passive avoidance learning or performance.

Although these experiments do not contribute greatly to an understanding of 'pathological anxiety' in humans, another approach to an understanding of anxiety has been the investigation of anxiolytic drugs; for instance, a case could be made for a noradrenergic basis for anxiety if all drugs with specific anxiolytic properties were shown to affect noradrenergic function in a similar way (Gray, 1977). The problem of finding an animal model remains, despite the focus on responses to anxiolytics. So far , one of the most satisfactory models for investigating the anxiolytic effects of drugs is the Geller-Seifter conflict test (Geller and Seifter, 1960), and another is the conditioned suppression test (as described in APPENDIX IV). These have been used to discriminate between the sedative and anxiolytic effects of drugs such as benzodiazepines and barbiturates (see Davis, N., 1979, for review) and , more recently, clonidine (Bullock et al, 1978). However, although these are valuable tests, they have not demonstrated an exclusively noradrenergic basis for the effects of anxiolytic drugs.

Another indication that 'anxiolytic ' activity is not synonymous with a reduction in noradrenergic function comes from studies of the opiates; morphine is an effective inhibitor of LC neurons, but is unlike the minor tranquillizers in animal studies. The effect of clonidine in alleviating opiate withdrawal symptoms has led Redmond (1979) to claim this too as evidence for the role of the LC in anxiety; but it may be that the phenomenon of LC activation in opiate abstinence is another sign of noradrenergic activation accompanying, rather than causing, behavioural changes. In fact it now seems most unlikely that changes in one neuroregulator substance will be found to account for the complex changes observed in anxiety and depression. Although simultaneous monitoring of different neurochemical systems is technically very difficult, it is clear that noradrenergic neurones are not functioning in isolation from other systems (see, e.g. Antelman and Caggiula, 1977; Fechter, 1974; Asin et al, 1977; Carlton, 1963) even in relation to conditioned responses to stimuli.

Redmond's locus coeruleus model of anxiety does not account for chronic human anxiety; nor does it help in the differentiation of sedative from anxiolytic drug effects, or indeed the differentiation of anxiolytic from analgesic properties.

The noradrenergic hypothesis of depression, in its original formulation, amounted to an assertion that depression was caused by a lack of noradrenaline at postsynaptic receptor sites. Conversely, excess of noradrenaline was thought to cause mania. Several of the antidepressants do cause an increase in noradrenergic effect, at any rate in the short term. But the antidepressant effect may well be mediated by a change in the postsynaptic receptors, as discussed in Chapter 5, section 5 . Again, other transmitters and receptor sites are also likely to be involved (see Green and

Grahame-Smith, 1978; and Maas, 1978). Attempts have been made to subdivide depression into different clinical and chemical subtypes; as with anxiety, problems remain with respect to animal models of a human condition.

The characteristics of the 'endogenous ' type of depression have led some workers towards a model of alterations in the hypothalamus during depression. Gray 's (1981) work goes some way towards encompassing models for both anxiety and depression within one framework; he suggests that the normal animal responds to stress with an increase in noradrenergic activity in the forebrain, but that the animal which is subjected to prolonged, severe, inescapable or uncontrollable stress may eventually suffer depletion of noradenaline stores in the hypothalamus and display signs of 'helplessness ' in the face of stress. The 'helpless' syndrome is therefore seen as a model of depression. The implication from Gray's latest work is that stress begins by causing anxiety and arousal, and may continue on until depression sets in ; i.e., depression is one end result of chronic anxiety. The mechanism for the proposed anatomical shift between the forebrain and the hypothalamus is not clear; but some of the results in this thesis suggest that the noradrenergic system in the hippocampus is , in terms of rapidly-induced changes in synaptosomal TH activity, more labile than the hypothalamus. Further work will be needed to unravel the transition proposed by Gray from forebrain noradrenergic overactivity to hypothalamic exhaustion, taking account of the wide range of adaptive mechanisms which control noradrenergic activity in the brain.

APPENDIX I

ASSAYS OF SOLUBLE TYROSINE HYDROXYLASE ACTIVITY AT SUBSATURATING CONCENTRATIONS OF COFACTOR AND SUBSTRATE.

In some of the drug experiments in sections 3, 4, and 5 of Chapter 5, solubilized TH activity was assayed under subsaturating conditions to assess whether changes in synaptosomal hydroxylation rates or in saturating solubilised preparations were accompanied by changes in kinetic properties of the solubilised enzyme. The rationale for the use of subsaturating conditions was discussed in Chapter 2. Since the substrate and cofactor concentration which obtain for in vivo tyrosine hydroxylation are unknown,any assay of this kind is necessarily performed under somewhat arbitrary conditions. (See Chapter 2, TABLE 2.3 for examples of the different assays used by different research groups).

In the present study, the subsaturating assay was performed at a tyrosine concentration of 40 µM as substrate, with DMPH4 at 0.5 mM. These conditions were chosen in order to obtain enough activity in preparations of noradrenergic brain regions (dopaminergic regions such as the striatum contain at least 10 times as much activity as noradrenergic terminal regions such as the hippocampus) , and were based on a Km of 75 µM for the solubilised enzyme (reported by Lovenberg, 1978) and on a study by Kane and Johnson (1978). The assays of Salzman and Roth (1980a) used 10 µM tyrosine for measurement of the activity of TH in the hippocampus, but this concentration was felt to be too low to yield results which would be interpretable, given the sensitivity of the assay as developed in this laboratory. The assay was performed at pH 6.5 as well as at pH 6.1 in a pilot experiment, in case it was possible to perceive activation of TH by virtue of a shift in the pH-dependency

curve, as discussed in Chapter 2; but there was no sign of activation at the higher pH, and the difference in activity of control tissue incubated simultaneously at the different pH's was negligible. pH was therefore maintained close to the optimum, at around 6.0 .

The results of the subsaturating solubilised assays of TH activity are shown in the following tables.

A. (see CHAPTER 5, section 3)

SOLUBILIZED TYROSINE HYDROXYLASE ACTIVITY
IN SUBSATURATING ASSAY CONDITIONS
AFTER DRUGS WHICH REDUCE FIRING RATE IN NA NEURONS.

	CONTROL	DRUG	DRUG/CONTROL%
HIPPOCAMPUS			
DESIPRAMINE	452 ±51 (9)	644 ±28 (9)	142 ±6 % *
MORPHINE	336 ±51 (5)	273 ±53 (4)	81 ±16 % ns
HYPOTHALAMUS			
DESIPRAMINE	1427 ±93 (11)	1310 ±131 (11)	92 ±9 % ns
MORPHINE	2922 ±453 (5)	2508 ±667 (4)	86 ±23 % ns

fmol/min/mg P2 protein. * p 0.02,paired,
(numbers of rats in each group are shown in brackets)

Solubilized TH activity ,with 40 µM 3H-tyrosine and 0.5 mM DMPH4, showed a significant increase of 42% in the hippocampus of DESIPRAMINE-TREATED rats compared with those of controls . This increase was significant , as shown in a two way ANOVA; the DRUG effect was significant (F=17.97, df 1,14, p 0.001) as was the REPLICATION effect (F=8.54, df 1,14, p 0.025); the interaction term did not reach significance (F= 3.86,df 1,14). Paired comparisons also attested to the significance of the rise in desipramine-treated rats (paired t= 3.21, df 8, p 0.02). Hypothalamic samples, however, did not show any change in the solubilized subsaturating assay; the mean value for desipramine-treated rats was 92% of the control mean , and the main effect of DRUG in the ANOVA was not significant (F=0.5,df 1,18).

Solubilized TH activity under subsaturating conditions was assayed in samples from one of the 3 MORPHINE experiments . Hippocampal samples showed a reduction in the morphine group to 81% of the saline control hydroxylation rate, but this did not reach significance. Hypothalamic samples also showed a reduction in the morphine group which did not reach significance.

B. (see CHAPTER 5, section 4)

EFFECT OF 2-DEOXYGLUCOSE ON TYROSINE HYDROXYLASE ACTIVITY UNDER SUBSATURATING CONDITIONS

	CONTROL	2DEOXYGLUCOSE (500mg/kg ip)	DRUG/CONTROL%
HIPPOCAMPUS	1162 ±263 (5)	1676 ±598 (5)	144 ± 51 % ns
HYPOTHALAMUS	1436 ±152 (5)	1220 ±211 (5)	85 ± 15 % ns

fmols/min/mg P2 protein.
(numbers of rats in each group are shown in brackets)

There is an apparent increase (+44%) in TH activity in the hippocampus of the deoxyglucose group,but this is associated with a particularly large scatter, and the difference is not statistically significant (paired t=1.07, df 4). The hypothalamus too fails to show any activation of the hydroxylation rate under standard subsaturating conditions.

C. (see CHAPTER 5, section 5)

EFFECT OF 20 DAYS DESIPRAMINE TREATMENT

ON TH ACTIVITY IN RAT BRAIN.

SYNAPTOSOMAL, SATURATING AND SUBSATURATING ASSAYS.

	SYNAPTO. TH	SUBSAT. SOL. TH	SATURATING TH
HIPPOCAMPUS			
SALINE (n=5)	211 ± 14	919 ± 63	2354 ± 218
DMI (n=5)	196 ± 12	922 ± 38	1775 ± 206
DRUG/CONTROL %	93 ± 6 %	100 ± 4 %	75 ± 9 %
HYPOTHALAMUS			
SALINE (n=5)	644 ± 12	1648 ± 56	16,350 ± 920
DMI (n=5)	658 ± 45	1704 ± 63	13,260 ± 1150
DRUG/CONTROL %	102 ± 7 %	103 ± 4 %	81 ± 7 %
LOCUS COERULEUS			
SALINE (n=5)	-	2263 ± 170	5381 ± 410
DMI (n=5)	-	2241 ± 311	4826 ± 595
DRUG/CONTROL %		99 ± 14 %	90 ± 11 %

fmol/min/mg P_2 protein

The results for hippocampus and hypothalamus in 3 different assays (synaptosomal, subsaturating TH and soluble saturating TH) are shown in Table C. TH rates are recorded as fmol/min/mg P2 protein, and the results are also expressed as a percentage of the appropriate control value ± S.E.M.

TH activity in the triton-treated, solubilised enzyme preparation at subsaturating cofactor and substrate concentrations was not altered in DMI-treated animals in any region (cf saturating assays discussed in Chapter 5, section 5).

D. SOLUBILISED TH ACTIVITY AFTER REPEATED 2-DG ADMINISTRATION.

SUBSATURATING TH ACTIVITY

	CONTROL	2-DG	2-DG/CONTROL %
HIPPOCAMPUS			
Expt I	969 ± 93	681 ± 57	70 ± 6 % **
Expt II	943 ± 141	894 ± 95	95 ± 10 %
Combined	956 ± 80	787 ± 63	82 ± 7 %
HYPOTHALAMUS			
Expt II	1991 ± 173	1737 ± 75	87 ± 4 %

fmol/min/mg P_2 protein

** $p < 0.02$ (paired)

Results of subsaturating assays performed on solubilized preparations of hippocampus and hypothalamus after two weeks treatment with saline or 2-DG injections on alternate days are shown in the table. The hippocampus was assayed in both experiments, but a two way analysis of variance with replications as a factor showed no overall significant main effects of TREATMENT or REPLICATIONS ($F=2.773$ and $F=0.855$ respectively) in the subsaturating assay. However, a paired t test showed a significant reduction of hippocampal subsaturating TH activity to 70% of the control mean in the first experiment ($p < 0.02$) whereas there was no difference between groups in the second experiment ($p < 0.2$). There was no difference between 2-DG-treated and control rats in hypothalamic subsaturating solubilized TH activity (data from one experiment only).

DISCUSSION: SUBSATURATING TH ASSAYS.

The subsaturating assay results reported here do not allow any firm conclusions to be drawn about kinetic changes in tyrosine hydroxylase during treatment with any of the drugs used in 'acute' or 'chronic' experiments. The only significant drug-related changes in TH activity in this assay were found in the hippocampus: a significant rise associated with a single dose of desipramine; and a significant fall after 2 weeks treatment with 2-deoxyglucose, although this effect was not replicated. These two results stand in contrast to the effects on the synaptosomal assay, in the case of a single dose of DMI; and to the effects on the saturating soluble TH assay, in the case of the repeated 2-DG injections. No other significant effects of treatment were found, and the results were poorly correlated with either saturating or synaptosomal activity in the same animals and brain region.

Pearson's 'r' was calculated for the relationship between synaptosomal and subsaturating soluble TH activity . The correlation coefficient, r, was -0.28 for a group of 16 control rats taken from 3 different experiments, suggesting that there was no constant linear relationship between the results of the two assays . Similarly, correlation coefficients based on the results from the treated animals in various drug experiments yielded no significant correlations between synaptosomal and subsaturating TH activity.

Correlations between subsaturating and saturating TH activity were also investigated, but no significant relationship was found, even when the assays were performed on the same day to eliminate some of the factors which might be contributing to the variation in baseline counts per assay. It appears that the assay at 40 μM tyrosine and 0.5 mM DMPH4 measures a different aspect of noradrenergic function than that measured by the saturating assay,

as indeed it was intended to; on the other hand, this subsaturating assay does not show up activation in the tissues which showed an elevated synaptosomal tyrosine hydroxylation rate. The subsaturating assay needs further standardization with the use of a range of substrate concentrations; and it would probably be advantageous to use BH4 or 6MPH4 as cofactor, rather than DMPH4 (see discussion in Chapter 2).

APPENDIX II

(see CHAPTER 5, SECTION 7.)

PARTIAL REINFORCEMENT ACQUISITION TRAINING:

DETAILS OF BEHAVIOURAL TRAINING AND BEHAVIOURAL RESULTS.

EXPERIMENTAL METHOD AND DESIGN.

EXPERIMENT A :

This is the published experiment referred to at the beginning of section 7 (Boarder et al,1979). Data including and additional to the published figures are given below.

EXPERIMENT B .

This experiment was set up to replicate the Boarder et al (1979) experiment and to look for any long-lasting effects of acquisition training in continuously rewarded (CR) or partially rewarded (PR) animals. A total of 80 male Sprague-Dawley rats were handled for 21 days and gradually introduced to a 23-hour food deprivation schedule in preparation for runway training for food reward. Rats were randomly allocated to one of 4 main groups (20 rats in each), each of which was subdivided into three sub-groups, namely handled controls (HC; n=8), continuous reward (CR , n=6), and partial reward (PR , n=6). Handled controls were handled daily but were never placed in the runway.

Runway dimensions: The start box was 20 cm square; the runway was 110 cm long and 16 cm wide; the goal box was 30 cm square with a grid floor. The walls of the entire apparatus were made from aluminium sheets 32 cm in height.

CR and PR animals were given 2 days pretraining in the runway with food available, and allowed to habituate to the apparatus . Acquisition training was started the next day. Each rat was placed in the start box with the door closed. The door was then opened electronically , activating an electronic timer (Advance instruments SC3), and the times at which the rat broke 3 successive photobeams in the runway and the goalbox were recorded as 'Start', 'Run', and 'Goal' times. On rewarded trials, 20 food pellets (Campden Instruments) were available in a food cup in the goal box; on non-rewarded trials, there was no food available. After 1 minute, or when the rat had consumed the reward, it was removed from the apparatus and replaced in a waiting box. Of the 4 main groups of 20 rats, two (one trial, OT groups) were put through acquisition at one trial per day. CR animals had reward on every trial. PR animals had non-rewarded trials on days 4,5,7,8,10 and 11. The other 2 main groups (multi-trial, MT groups) had 8 days of training at 6 trials per day with an inter-trial interval of 5-7 minutes. Half the trials on each day o a random schedule were non-rewarded for the PR groups. All groups started acquisition training on the same day. 24 hours after the last acquisition trial, half the rats were killed (MT group on day 9; OT groups on day 13) ; the survivors were left in the home cages without handling or further exposure to the runway for another week. Handled controls were killed along with the runway animals, either on the day after the end of acquisition or after the 7 day lapse .

ASSAYS:

Hippocampus, cerebral cortex, and hypothalamus were dissected out. Hippocampal synaptosomes were prepared from one hippocampus in this experiment by Doreen West. Synaptosomal pellets were resuspended in incubation medium at pH 6.2 and assayed by incubation with 3H-tyrosine at 10 µM. TH in the other hippocampus,cortex and hypothalamus was solubilised with triton X-100 in phosphate buffer 5 mM at pH 6.5 by homogenising; saturated TH was assayed with 1 mM BH4 and 3H-tyrosine at 110 µM. NA measurement in these samples was by the radioenzymic method described in Chapter 4 .

This experiment provided the opportunity for a full replication of Experiment A, with additional animals subjected to the same acquisition training but not assayed till 7 days later. Also a full range of saturating TH assays was carried out in all three regions dissected, enlarging the search for evidence of enzyme induction.

EXPERIMENT C.

This experiment was run by Cliff Preston. It constituted a third replication of acquisition training effects in the OT paradigm. After initial handling and food deprivation , forty rats were allocated to either HC or CR or PR conditions. There were 13 days of acquisition training, and PR rats had 6 nonrewarded trials during this period. Half the rats (4 HC rats, 8 PR and 8 CR rats) were killed 24 hours after the last acquisition trial, and the other PR and CR groups were given 7 days of extinction in the runway at one trial per day with no reward for either group. On the day afer the last trial, rats were killed and brains dissected as above. Synaptosomal TH activity was assayed in the hippocampus but at a higher pH , 7.4, than in Experiments A and B, and with a tyrosine concentration of 20 µM.

EXPERIMENT D.

This experiment was run with a similar protocol to that given above for one trial per day acquisition training. A total of 30 rats were used. These included a group of rats which were shocked in the runway (see 'partial punishment' experiments, described in section 8 of Chapter 5) ; this is mentioned here as it is conceivable that this could affect the associations made by non-shocked animals to the apparatus (e.g. interference by olfactory cues). The other difference with respect to experiments A and B was that in this experiment there was no change of handler on the last day, i.e. the rats were treated in the same way as on all previous days up to the moment of death. This seemed important in view of the emphasis on anticipatory emotional state for the theoretical account of the PREE at long inter-trial intervals; in this experiment we could presume that the anticipatory state and immediate pre-mortem experience of the rats , whether or not this were to be reflected in synaptosomal TH activity, was similar to that on the last day of acquisition.

After 13 days of handling and food deprivation , CR and PR rats were exposed in groups of 4, and then in pairs, to the runway for pretraining. Acquisition training proper consisted of 12 days at one trial per day, with daily rewarded trials for CR animals . The nonrewarded trials for PR rats on fell on days 2,4,5,8,10 and 11. Twenty-four hours after the last acquisition trial,all rats were killed . Synaptosomal preparations were made with the help of Jane Nicholass, and synaptosomal activity of TH was assayed in both cortex and hippocampus. The assay was at pH 7.4 and 20 μM tyrosine .

EXPERIMENT E.

This experiment was also run by the author. The apparatus was the same as for Experiment D. A total of 29 rats in 4 groups were used (again including a shocked group which is discussed in section 8, Chapter 5). The number of trials at one trial per day was increased to 20; CR rats received reward on all days (except day 10 when no trials were run because of technical difficulties with the apparatus). PR rats had non-reward trials on days 2,4,5,8,11,13,14,16,18 and 19. All rats were killed 24 hours after the last acquisition trial. Hippocampus and locus coeruleus were dissected. Synaptosomal pellets were prepared for hippocampus and assayed at pH 7.4 with 20 μM tyrosine. The hippocampal P_2 resuspension was solubilized with phosphate buffer containing triton for saturating TH assay; and the locus coeruleus was also assayed at saturating concentrations of cofactor and tyrosine after homogenizing with the same buffer.

FIG. 7a Experiment B: Running speeds in acquisition at one trial per day.

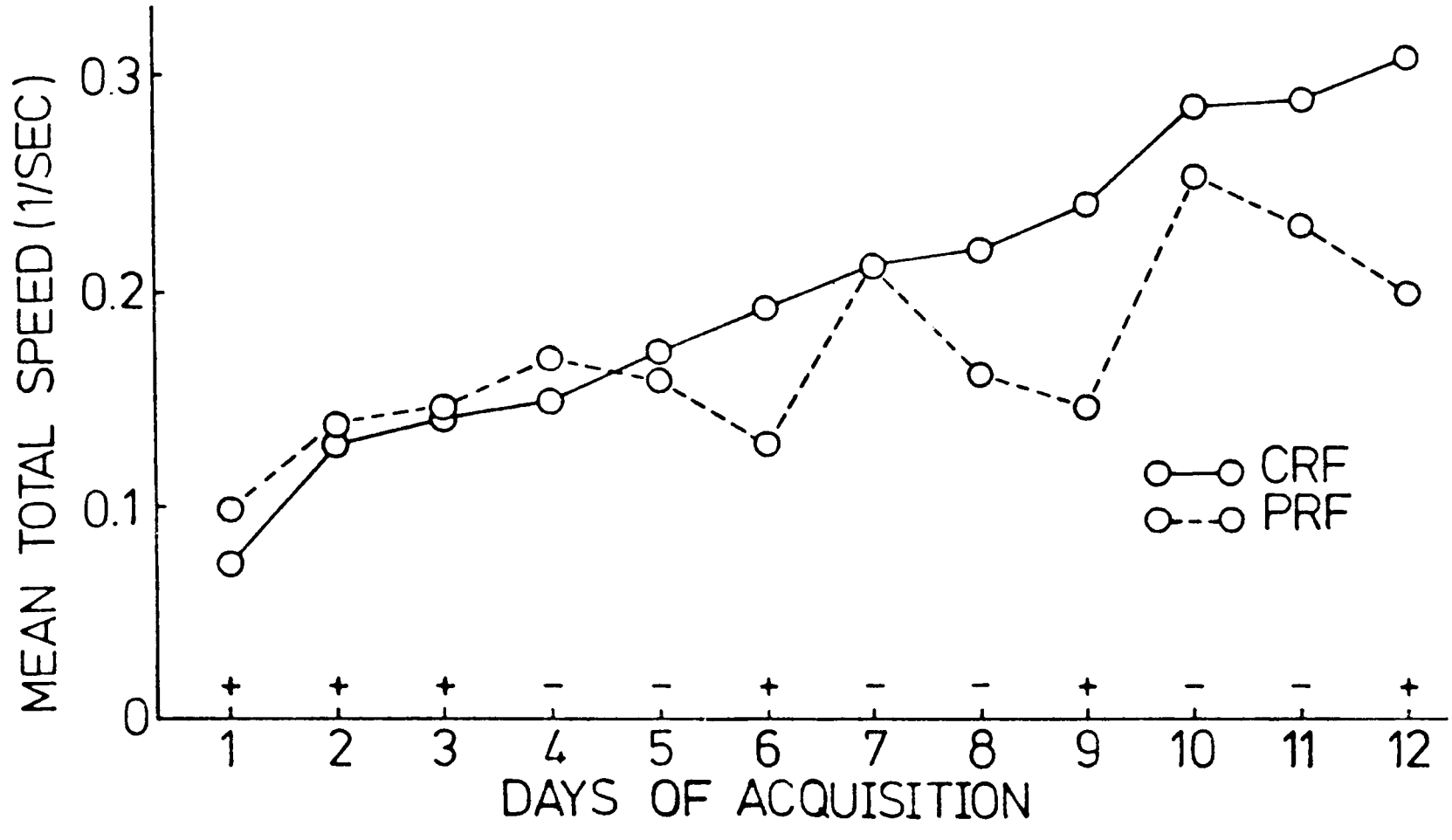
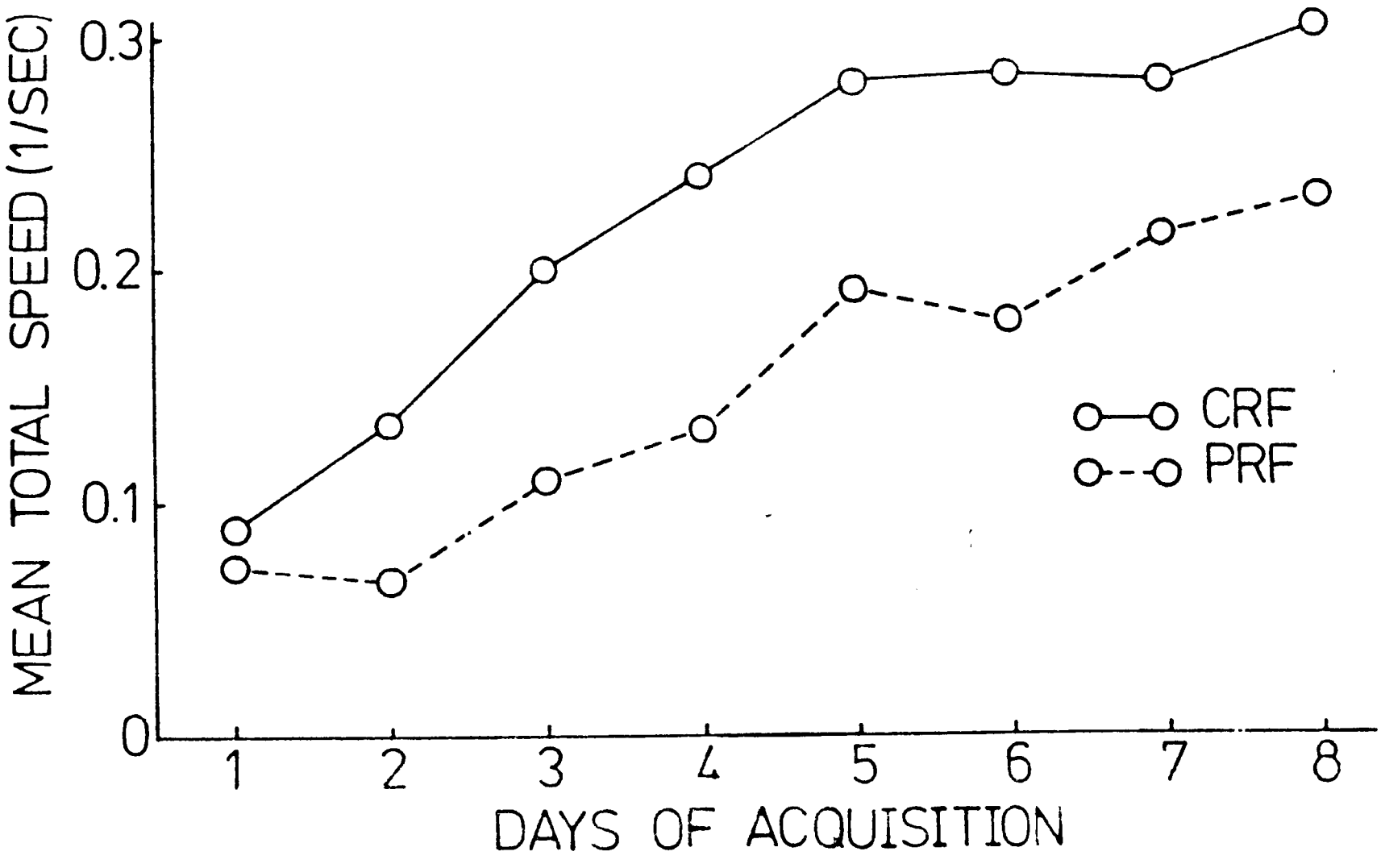


FIG. 7b Experiment B: Running speeds in acquisition at 6 trial per day.



RESULTS :

1. Behavioural measures:

Start, run and goal times were recorded during acquisition (and extinction, in experiment C) for all CR and PR rats. The data for experiments B and C were converted to mean total speed, and the results are shown in FIGS 7a-7c. FIGS 7d and 7e show the original data from experiments D and E as mean total times . There is some slowing of the increase in running speed in PR rats when non-rewarded trials commence. However, in experiments D and E, acquisition running speeds are clearly very similar for PR and CR rats by the end of training. Asymptotic running speeds are usually fairly similar in PR and CR animals, whereas slowing in response to non reward depends on the amount of previous habituation to the apparatus and the handler, and on other factors which contribute to the counter-conditioning process.

Analysis of variance of the results from Experiment A (not shown in graphic form) showed an increase in running speed over days, denoting acquisition of the response, in both OT and MT paradigms . In addition to this finding, which was present in all acquisition results, there was a significant main effect of REINFORCEMENT ($F=18.6$, df 11/154, $p < 0.001$) and a significant DAYS X REINFORCEMENT interaction ($F= 2.4$, df 11/154, $p < 0.01$) in the OT paradigm; this reflected the faster increase in running speeds in the CRF group than in the PR group. In the MT paradigm, this difference was not seen (main effect of REINFORCEMENT, $F=1.2$, ns; and DAYS X REINFORCEMENT, $F = 0.8$, ns).

For Experiment B (see FIGS 7a and 7b), analysis of variance showed a significant interaction of DAYS X REINFORCEMENT in the OT paradigm ($F= 6.2$, df 11/220, $p < 0.004$), suggesting that CR animals acquired the running response faster than PR animals ,as in Experiment A. This was also true in the

MT paradigm; the main effect of REINFORCEMENT was significant ($F = 6.5$, df 1/20, $p < 0.05$), although the interaction term was not significant.

The running speeds for Experiment C (OT) are shown in FIG 7c. Analysis of variance was performed on the extinction data, and showed a significant difference between the linear trends across days for CR and PR groups; the CR group extinguished faster ($F = 10.9$, df 1/84, $p < 0.01$), confirming the presence of the partial reinforcement extinction effect.

The behavioural data from Experiments D and E are shown in FIGS 7d and 7e, but were not subjected to analysis of variance.

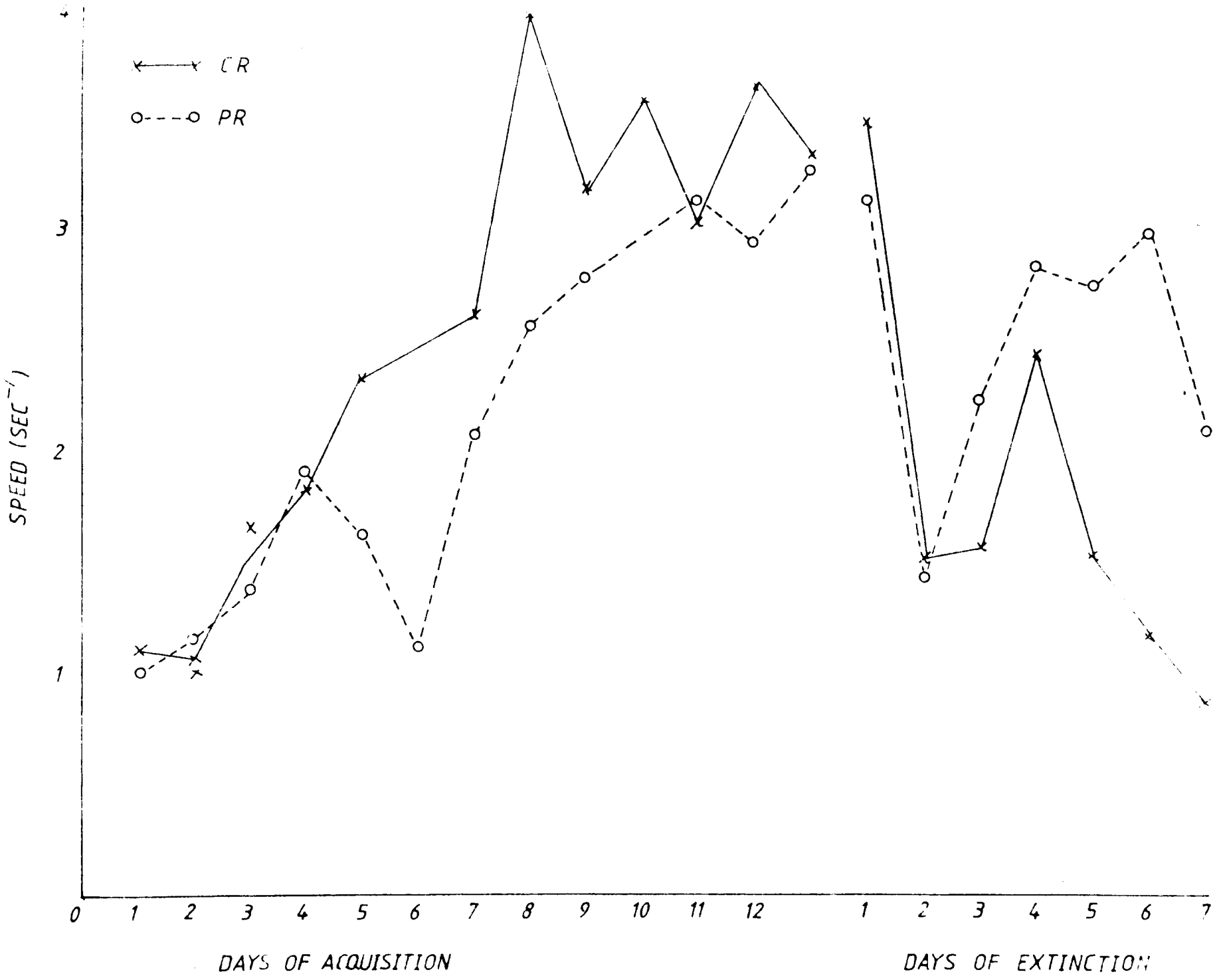


FIG. 7c Experiment C : Running speeds in acquisition and extinction, showing the partial reinforcement extinction effect at one trial per day (Preston, unpublished data).

FIG. 7d Experiment D: Runway times in acquisition at one trial per day; partial reward and partial punishment.

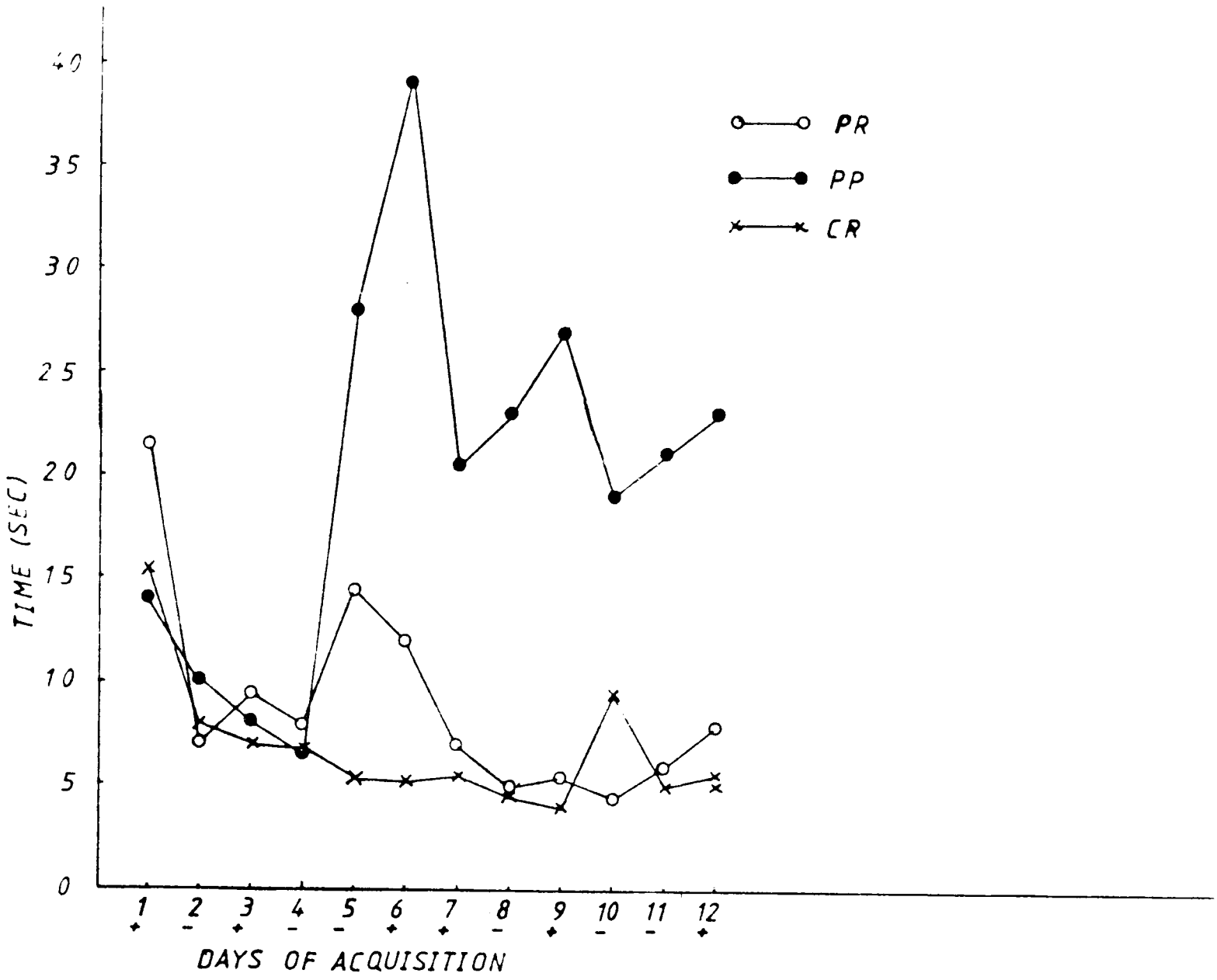
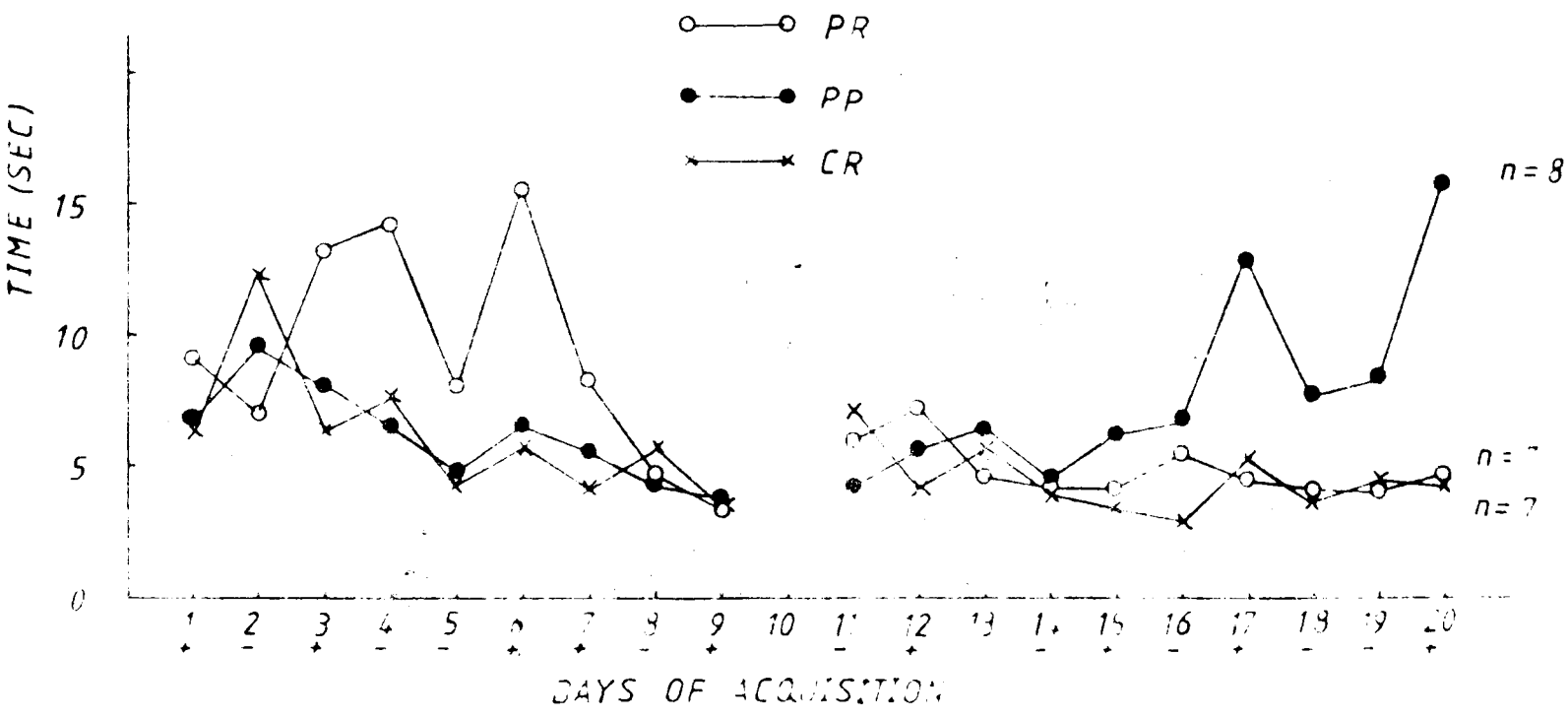


FIG. 7e Experiment E: Runway times in acquisition ; partial reward and partial punishment. (No trial on day 10).



APPENDIX III

(see CHAPTER 5, SECTION 8)

PARTIAL PUNISHMENT ACQUISITION AND CONFLICT:

BEHAVIOURAL PROCEDURES AND RESULTS.

EXPERIMENTAL METHOD AND DESIGN:

EXPERIMENT D :

The runway apparatus was as described in APPENDIX II; but a Campden Instruments shocker and scrambler were connected to a power supply and to the grid floor of the goal box so that scrambled shock was delivered for 0.5 sec across the bars when the photobeam in the goalbox was interrupted on a 'punished' trial. At other times the shocker was switched off. Current was measured as the root mean square intensity.

As described in APPENDIX II, this experiment used 30 male rats. All rats were handled and gradually introduced to a food deprivation schedule over the first 2 weeks. Water was available ad lib in the home cages. Rats were allocated to one of 3 or 4 experimental groups; handled controls, which had no runway experience; CR training with continuous reward; partial punishment (PP), with reward on every trial but shock on half the trials; and (in experiments D and E only) a PR group. Two days of pretraining were given, the appropriate groups of rats being put into the runway with food in all sections on the first day, and with food confined to the goal box on the second day.

During acquisition, at one trial per day, rats were individually placed in the start box; each trial was run as described in APPENDIX II. All experimental sessions were run at approximately the same time each day and

the rats were run in squads containing one member of each experimental group. Twenty Campden Instruments food pellets were placed in the goal box food cup before every trial for CR and PP groups. Punished trials for the PP group occurred on days 2,4,5,8,10 and 11. The current intensity was 0.2 mA on days 2 and 4, 0.25 on day 5, and 0.3 mA on all subsequent days. Start, run and goal times were recorded for each trial throughout acquisition.

24 hours after the last acquisition trial, rats were brought to the runway room as if for a further session; each rat, including handled controls, was stunned and decapitated. Synaptosomal pellets for hippocampus and cortex were prepared in this experiment, and synaptosomal TH was assayed the same day at pH 7.4 and tyrosine at 20 μM. This experiment was performed in order to compare TH activity from synaptosomes of PP rats with that of PR ,CR and HC rats . Two NA terminal regions, hippocampus and cortex,were assayed.

EXPERIMENT E.

This experiment was also described in APPENDIX II. A total of 29 rats were used, allocated as follows; HC (7); CR (7): PR (7): PP (8). Until day 9 of acquisition the procedure was identical to that of experiment D. But the shocker was not working reliably and on day 10 the apparatus was dismantled and faulty connections were found in the goal box . Trials were recommenced on day 11 and PR and PP groups then received non-reward or shock respectively on days 11,13,14,16,18 and 19. Acquisition lasted for 20 days in all, instead of the 12 days in other OT experiments. 24 hours after the last trial, animals were killed and hippocampus and locus coeruleus were dissected. Synaptosomal TH was assayed the same day, and the remaining P_2 resuspension was stored for later assay of total TH . Triton-homogenates of locus coeruleus were also stored.

This experiment had a long acquisition period for PR and CR rats, and for this reason it was expected that induction, if occurring under these conditions, would show up in this experiment.

EXPERIMENT F.

This experiment was run by Sorrel Brookes. There were 4 groups of rats, all of which were runway-trained. There were no handled controls. This experiment was intended to test the effect of chlordiazepoxide on the multitrial partial punishment effect. The 4 groups were: CR (saline), CR (drug), PP (saline) and PP (drug). Ten minutes before the experimental session, each squad was injected intraperitoneally with chlordiazepoxide (CDP) at 5 mg/kg or equivalent volume of saline. Acquisition trials were run at 6 trials per day and PP rats were shocked on half the trials each day. Possibly because a new and slightly longer goal box was used in this experiment, the shocks used had more impact than in other experiments on the rats' behaviour, and the running response was not readily learnt by the PP groups. The last two days of the experiment were run at one trial per day and with no further drug or saline injections. Acquisition thus lasted 8 days. 24 hours after the last trial, the rats were stunned and decapitated, and sample preparation was carried out as usual; synaptosomal TH was assayed in hippocampus (pH 7.4, tyrosine concentration 20 μM) , and total TH in hippocampus and hypothalamus P_2 resuspensions after treatment with triton. Although not satisfactory for behavioural analysis, this experiment was regarded as a pilot investigation of the possible long-term effects of shock and a multitrial partial punishment schedule, interacting with those of CDP.

EXPERIMENT G.

This experiment was run by Nicola Davis as one of a series of experiments on the partial punishment effect (Davis, 1979). It was similar in most respects to the one trial per day experiments above (Experiments D & E)

except that no PR group was included, and the reward was in the form of wet mash rather than dry pellets. 40 rats were included; 32 had acquisition training at one trial per day for 12 days. Twenty four hours after the last trial of acquisition, a group of 4 handled controls, together with 8 CR and 8 PP rats, were killed . Solubilized TH was assayed at saturating conditions in hippocampus, hypothalamus and cortex, and NA levels were assayed in hippocampus.

Another 8 CR and 8 PP rats were given 7 days in the conflict phase, with reward and shock on every trial. The day after the last trial, these were killed together with another 4 handled controls; hippocampal synaptosomal TH was assayed, this time at pH 6.2, tyrosine concentration 10 µM. Homogenates of hippocampus, hypothalamus and cortex were also prepared with triton-containing buffer, and assayed at 110 µM tyrosine with 1.27 mM BH4 as cofactor.

Experiment G was the only experiment in this series in which data were available from both acquisition and conflict phases.

EXPERIMENT H.

This experiment was run jointly by Sorrel Brookes and Nicola Davis as part of an investigation of drug effects on behaviour in the partial punishment effect. The rats chosen for TH assay, however , were those used as controls in the drug experiment, and had saline injections at 1 ml/day throughout acquisition and conflict stages. There were 7 CR, 7 PP, and 5 'control' rats; 'controls', however, had had 1-3 days of runway experience in addition to handling, and were not behaviourally naive as in the other experiments described above.

The experimental groups had 8 days of acquisition at 6 trials per day, then 2 days of rewarded trials, to achieve similarity of asymptotic running speed, and then 7 days of reward and shock on every trial (0.25 mA) in the conflict phase. Twenty four hours after the last trial, all rats were killed . Hippocampal synaptosomes were prepared and synaptosomal TH was assayed at pH 7.4, tyrosine concentration 20 μM. Solubilized TH was prepared from the lysed synaptosomes and assayed at 110 μM tyrosine, 1.27 mM BH4. This experiment served as an investigation of the effects of the conflict phase after acquisition training on the multitrial schedule on hippocampal TH.

EXPERIMENT I.

This experiment was run by Nicola Davis as part of the drug investigation mentioned above. Saline injected animals were taken for assay; 6 from the PP group and 6 from the CR group. There were no behaviourally naive controls. The behavioural training, on the one trial per day schedule, was run as previously described through acquisition and extinction. The day after the last conflict trial, all rats were killed and hippocampus,hypothalamus and cortex were dissected and homogenised in triton-containing buffer. Soluble TH was assayed under saturating conditions (tyrosine concentration 110 μM, BH4 1.27 mM). Noradrenaline was assayed in the hippocampus. This experiment was similar in design to Experiment G .

EXPERIMENT J.

This experiment was run by Sorrel Brookes. It was similar to experiment I in all respects except the amount of previous handling of all rats before the start of behavioural training. The rats were handled daily for 4 weeks out of 6 before training was commenced, whereas in all other experiments handling was started 2-3 weeks before training. Hippocampal and hypothalamic homogenates were assayed for soluble TH at 110 μM tyrosine and 1.27 mM BH4, as in experiment I.

RESULTS:

1. Behavioural measures:

Start, run and goal times were collected as in the partial reinforcement experiments, and transformed as before to sppeds in some experiments. FIGS 8c-8f show plots of the data from Experiments G and H; and FIGS 7d and 7e (in APPENDIX II) show the results for Experiments D and E. All data from Experiments G,H and I , including drugged groups where these were present, were subjected to analysis of variance. The following summary is taken from unpublished work by N. Davis and S.Brookes.

Experiment G. (FIG 8c) Acquisition data showed a significant DAYS X PUNISHMENT interaction in the run section speeds ($F= 6.05$, df 1/572, $p < 0.025$) ; subsequent t tests showed that CR animals increased their running speed faster than PP animals ($t= 3.0$,df 572, $p < 0.01$). This was also true in the goal section of the runway. Data from the conflict phase with shock on each trial showed a PPE in all sections of the runway (see FIG 8d). The DAYS X PUNISHMENT interaction was significant in both run and goal sections; ($F= 7.59$, df 5/69, $p < 0.001$; and $F= 5.94$, df 5/69, $p < 0.001$). Subsequent t tests showed that PP animals ran faster in the run section from day 3 onwards ($t=2.05$, df 69, $p < 0.05$) .

Experiment H. (FIG 8e, but only placebo groups were assayed) There was no significant main effect of PUNISHMENT in the run section in the acquisition phase of this experiment, although in the goal data there was a significant DAYS X PUNISHMENT interaction ($F= 4.54$, df 9/477, $p < 0.001$) as the effects of punishment emerged later in the training period. Data from the goal section in the conflict phase are shown in FIG 8f. There is a significant main effect of PUNISHMENT ($F= 10.79$, df 1/53, $p < 0.01$), and a significant DAYS X PUNISHMENT interaction ($F= 16.69$,df 3/159, $p < 0.001$); subsequent t tests confirmed that PP animals were running faster than CR animals from day 2 of the conflict phase ($t= 2.57$, df 157, $p < 0.02$).

FIG. 8c Experiment G: Running speeds in runway and goal sections during acquisition at one trial per day. (Davis, 1979)

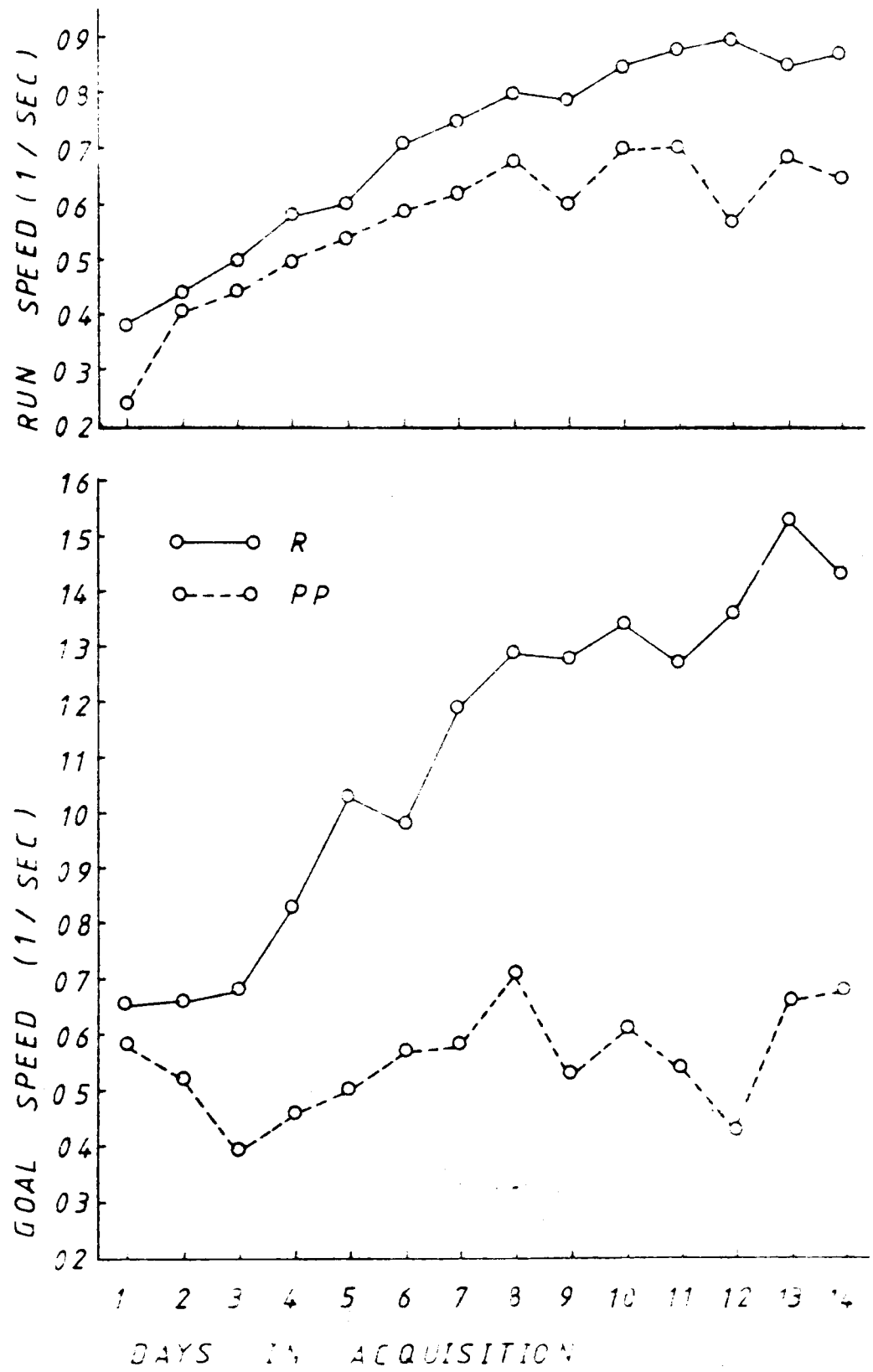


FIG. 8d Experiment G: Running speeds in start, runway and goal sections during conflict phase, showing partial punishment effect. (Davis, 1979)

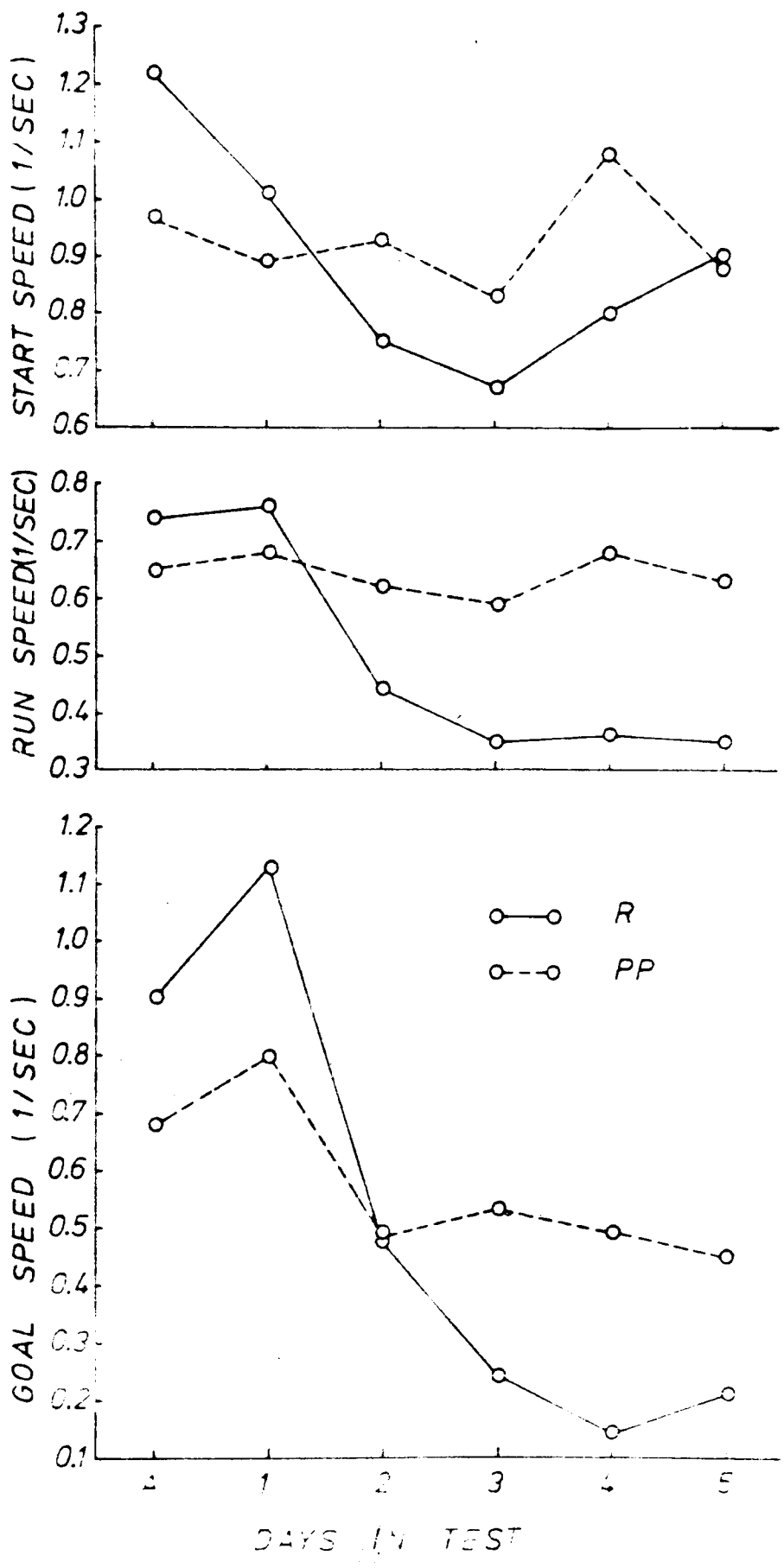


FIG. 8e Experiment H: Runway times in runway and goal sections at one trial per day during acquisition
Effect of chlordiazepoxide (CDP).
(Davis, 1979)

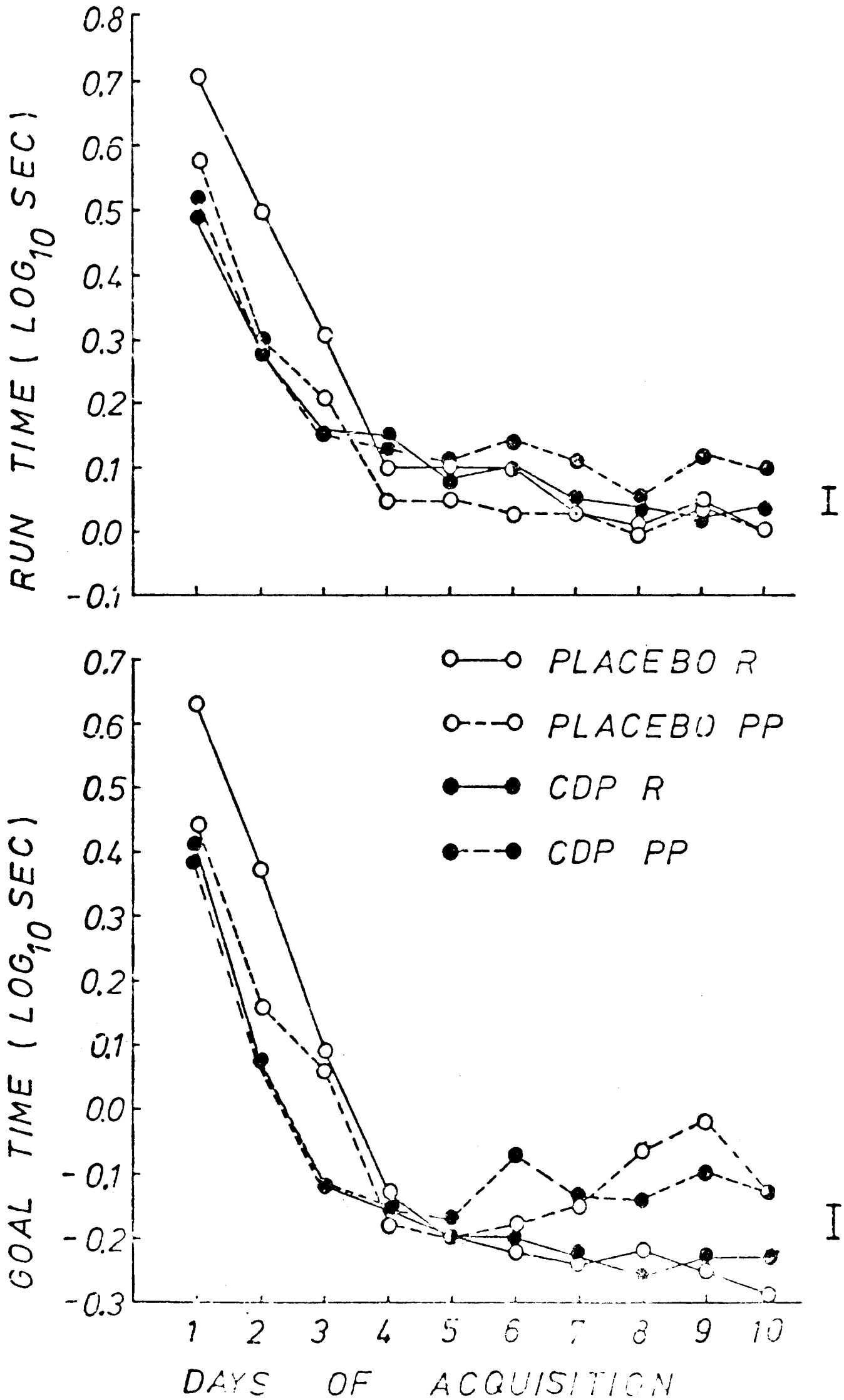
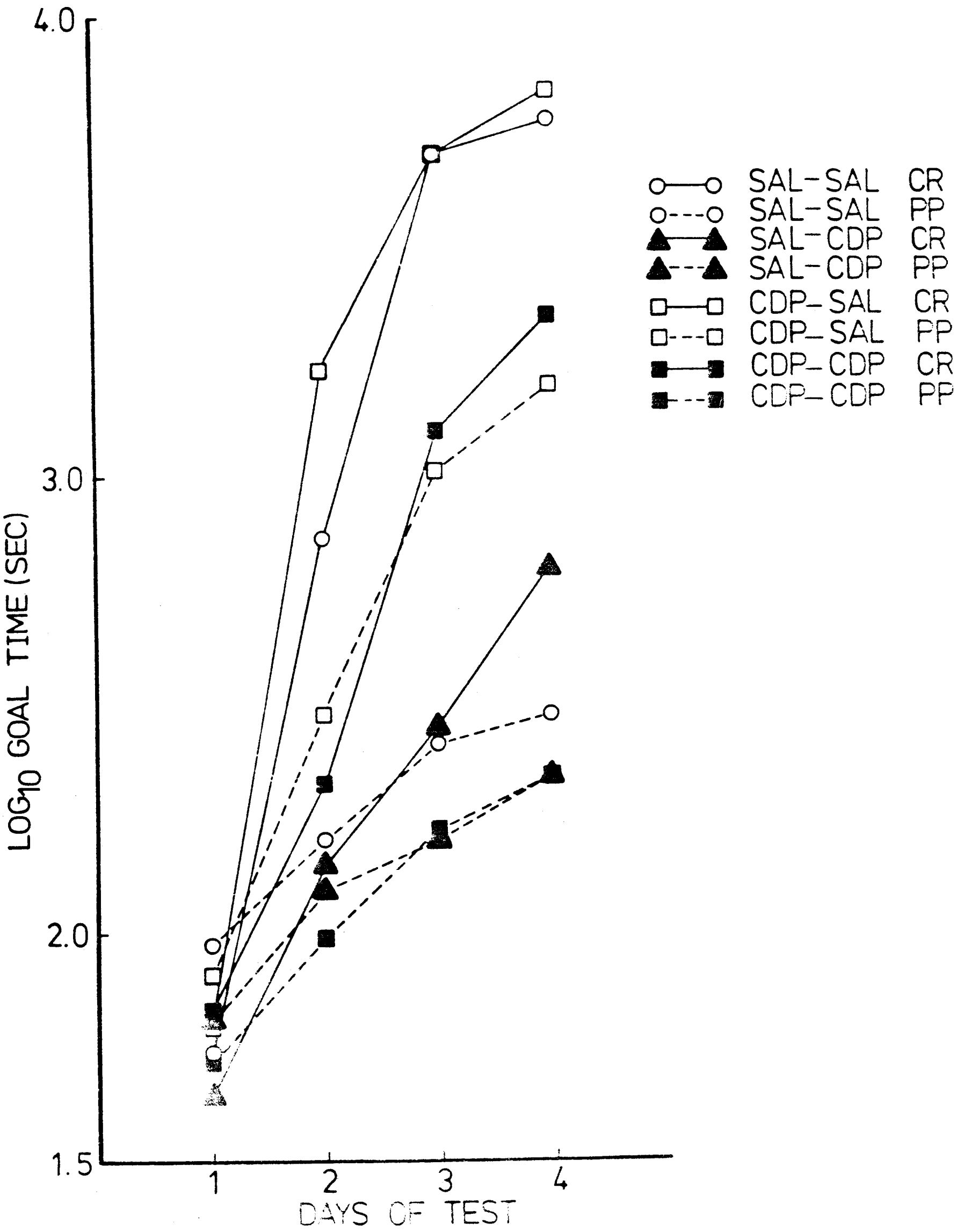


FIG. 8f Experiment H: Runway time in goal section during conflict phase at one trial per day. Effect of chlordiazepoxide during acquisition and conflict phases. (Davis, 1979)



Experiment I. Data (not displayed) from the conflict phase of this experiment showed a significant PPE in saline-treated animals, manifested by a DRUG X PUNISHMENT interaction ($F= 4.49$, df 1/56, $p < 0.05$). Subsequent t tests on the saline-treated groups showed that PP animals were faster than CR animals from the 2nd day onwards ($t= 3.01$, df 56, $p < 0.01$).

APPENDIX IV

(see CHAPTER 5, SECTION 9)

LONG TERM EFFECTS OF ELECTRICAL STIMULATION OF SEPTAL AREA ON TH ACTIVITY IN THE HIPPOCAMPUS.

DETAILS OF SURGERY, BRAIN STIMULATION,
AND BEHAVIOURAL TRAINING AND RESULTS (Holt, unpublished work).

EXPERIMENTAL DESIGN AND METHOD :

Surgery :

The rats weighed 280-550 g at the time of surgery. The anaesthetic used was a mixture of sodium pentobarbital, chloral hydrate, magnesium sulphate , ethanol and propylene glycol. Using a stereotaxic instrument, a bipolar stimulating electrode was implanted bilaterally in the lateral septal area, and a bipolar recording electrode was implanted unilaterally straddling the left dorso-medial subiculum. Stereotaxic coordinates for the septal electrode were: 0.5 mm anterior to bregma, 4.5 mm deep to surface of dura, and 0.7 mm lateral to midline. For the subicular electrode, coordinates were : 6mm posterior to bregma, 5.0 mm deep, and 2.0 mm lateral to midline. Electrodes were fastened to an Amphenol socket and cemented with dental cement and skull screws. At least two weeks recovery were allowed before commencement of testing.

Stimulation and recording :

A Vero I.C. socket was cemented to the head assembly before stimulation and recording. Bipolar stimulation was provided by an optically isolated, battery powered constant current square wave generator, and controlled by Farnell pulse frequency and pulse width modules, with a pulse

width of 0.5 msec and frequency of 7.7 Hz (interpulse interval 130 msec). For recording, a Grass model 79D polygraph with preamplifiers and a Tektronix 502A dual-beam oscilloscope were used. Artefact was controlled by a source-follower cemented into the cable system of the apparatus ; the electrode cap was connected to an overhead cable which did not impede movement of the rat within an perspex box 30 x 30 x 34 cm. Approximately 90% of the operated animals showed successful theta driving during septal stimulation with pulse duration 0.5 msec and interpulse interval of 130 msec. Minimum thresholds for driving of the theta rhythm were established for both polarities; this was done by gradually increasing the current of the stimulation pulses until recorded waves (duration > 70 msec) remained steadily in phase with the stimulation pulses. Some animals did not show theta driving at current levels up to 250 μA; these were included, along with other animals which did show the capacity for theta driving, in the unstimulated control group.

Once the thresholds had been established, stimulated animals were given bipolar stimulation at a current level 20 % above the minimum threshold for that animal; i.e. stimulation intensities ranged from 50-150 μA. Stimulation sessions were given on 10 consecutive days in a standard pattern for all experiments reported here. Each session consisted of 6-second trains of stimulating pulses, repeated 15 times during a session lasting approximately 15 minutes and programmed on an RI-30" schedule (i.e. occurring at random intervals , averaging 30 seconds). CONTROL animals were connected to the stimulating apparatus in the same way as the stimulated animals, but received no stimulation during the sessions.

In some experiments (A and B; see below) food delivery was set up during the stimulation sessions; 'UNCORRELATED' groups obtained food delivery on a random RI-30" schedule independent of the RI-30" septal stimulation; 'CORRELATED' groups obtained food at the 4-second point in

each 6-second burst of septal stimulation. 'THETA-ONLY' groups received septal stimulation at 7.7 Hz with no food delivery during the stimulation period. An extra stimulation group ('IRREGULAR') was included in experiments D and F to control for pulse frequency; these rats were stimulated via similar septal electrodes so as to give the same number of pulses as to the stimulated group, but at irregular interpulse intervals , so that theta rhythm would not occur in the hippocampus during stimulation.

Experiment D was an investigation of the long-term effects of these two kinds of brain stimulation on saturated TH activity in the hippocampus , in comparison with a third group of unstimulated controls, regardless of behavioural consequences. Two weeks after the end of the stimulation period, all three groups of rats were killed and the hippocampus was assayed.

Behaviour shaping in a modified Skinner box , equipped with a retractable lever, was started either before stimulation, as in Experiment A, or after the stimulation sessions were completed (all other experiments). Training involved learning to press a bar to obtain food reward; an FR-5 (fixed ratio) barpress schedule was introduced gradually over 3-5 days, beginning with FR-1 (reward for every bar-press) for the first day . All events were fed into a Data General NOVA 800 computer on-line.

EXTINCTION AFTER FR-5 ACQUISITION TRAINING.

In Experiments A,B and C, rewards were given during acquisition on a fixed ratio (FR-5) reinforcement schedule in which every 5th bar-press was rewarded with a food pellet (45 mg) . Subjects were given 4 trials per day with an inter trial interval of 60 seconds. Trials were initiated by presentation of

the lever, and terminated with delivery of a food pellet. For Experiments A and B, acquisition sessions were run on 15 successive days , after which the animals were put into extinction. In extinction (12 days) trials were run in the same way but no reward for bar pressing was delivered , and response rates tended to fall. In Experiment C, acquisition lasted for 21 days and extinction for 15 days.

24 hours after the last trial of extinction, rats were removed one at a time from home cages , in the order in which they had been run the previous day, and were killed by a blow to the head and immediate decapitation. The electrode cap was removed with bone forceps, and the hippocampus was dissected on ice. Brains were usually removed within 3 minutes of death ; this was longer than the usual time in un-operated rats.

EXPERIMENT A : groups were as follows:

CONTROLS (6 rats); implanted; no brain stimulation and no food in stimulation box. Acquisition on FR-5 , followed by extinction.

UNCORRELATED (6): theta driving by septal stimulation with independent delivery of food pellets. Acquisition on FR-5, followed by extinction .

CORRELATED (6): theta driving followed by delivery of food pellets. Acquisition on FR-5, followed by extinction.

Interval between end of stimulation and death; approx 30 days.

EXPERIMENT B :

CONTROLS (6): implanted; no stimulation, no food during stimulation phase. Acquisition on FR-5, followed by extinction.

FOOD CONTROLS (6): implanted; no stimulation, but food delivery in stimulation box.Acquisition on FR-5, followed by extinction.

UNCORRELATED (6) : as above.

THETA ONLY (6): theta driving stimulation without food; acquisition on FR-5, followed by extinction.

Interval between end of stimulation and death: 30 days.

EXPERIMENT C :

FOOD CONTROLS (10) : as above .

THETA ONLY (9) : as above.

Interval between end of stimulation and death : 44 days.

CONDITIONED SUPPRESSION EXPERIMENTS.

In Experiments E and F the behavioural design employed was a conditioned suppression paradigm . Rats first learned to press a bar for a reward consisting of 10 % sucrose solution, over 12 training days, gradually achieving stable performance on an RI-64" schedule. Sessions lasted 60 minutes. On day 13, a 1000 Hz tone (10 db), frequency-modulated at 15 Hz (Advance Instruments LF oscillator J2E), was sounded for 20 seconds on two occasions during the session . CER (conditioned emotional response) acquisition, in which a tone-and-shock (tone followed by shock) combination is repeatedly presented, started on day 14. Following a 20 second warning period during which the tone was sounded, footshock was delivered via a Grason-Stadler shocker and scrambler across the grid floor of the Skinner box. Six shocks were delivered in each of 12 daily sessions on a RI-8 minute schedule, each shock being preceded by a 20-second tone burst. The shock intensity was increased from 0.16 mA on the first day of CER acquisition to 0.3 on the third and subsequent days. Shock duration was 0.5 second.

As mentioned above, bar pressing comes to be suppressed in the presence of the CS alone as well as during footshock ; the tone thus seems to take on aversive stimulus properties through association with the shock (CS-UCS pairing). The ratio of the rate of bar-pressing in the CS period to the sum of the sum of the preceding baseline and the CS rates (duration of CS and pre-CS periods is 20 sec) is designated the suppression ratio and is used as an index of the CER (see above). 24 hours after the last session, rats were killed by a blow to the head and immediate decapitation.

EXPERIMENT E : groups were as follows
(all animals were subjected to footshock):

SHOCK-CONTROL (5 rats): no septal stimulation .
CER training with shock current intensity 0.16 mA on day 1 of acquisition, 0.2 mA on day 2, 0.3 mA on all subsequent days.

SHOCK + THETA (7): theta driving at 7.7 Hz for 10 days,as above. Then CER training as above.

EXPERIMENT F :
(including non-shocked controls for each of the stimulation conditions)

CS-CONTROL (4): no stimulation.
Pseudo-CER training, i.e. presentation of CS without shock.

CS-THETA (4) : theta driving at 7.7 Hz.
Pseudo-CER training as above.

CS-IRREGULAR (4): irregular septal stimulation, not inducing theta. Pseudo-CER training as above.

CER-CONTROL (4): no stimulation.
CER training with CS and shock presentation.

CER-THETA (4); theta driving at 7.7 Hz.
CER training as above.

CER-IRREGULAR (4): irregular stimulation.
CER training as above.

RESULTS:

1. Behavioural results:

EXPERIMENT A: BEHAVIOUR IN ACQUISITION AND EXTINCTION.

Response latencies were measured and logarithmically transformed for statistical analysis. Latencies to the first response, time from first to third bar press, and from third to fifth bar press were analysed separately.

FIG. 9a shows the group mean plot for the time from third to fifth bar press, plotted over days of acquisition. Analysis of variance showed a linear decrease in response latency for all animals ($F=9.69$, df 1/210, $p < 0.005$), but there was no difference between groups in acquisition.

In extinction, all animals showed a linear increase in response latency over days ($F= 101.01$, df 1/180, $p < 0.001$).

FIG. 9b indicates the group means for the time from third to fifth bar press. A difference in this measure of response latency between groups gradually emerged during extinction such that there was a linear increase in the between-group differences over days (Days x Group interaction, $F= 8.39$. df 2/180, $p< 0.001$). Subsequent t tests indicated that both the correlated and uncorrelated groups extinguished at a significantly slower rate than the implanted controls ($t= 3.06$, df 10, $p < 0.02$ and $t= 5.78$, df 10, $p < 0.001$). Also, the uncorrelated group extinguished more slowly than the correlated group ($t=2.71$, df 10, $p < 0.02$).

EXPERIMENT B: the results were similar to those of experiment A in showing, in extinction, a significant Days x Group interaction ($F=4.028$, df 3/220, $p < 0.01$) showing a between-group divergence in the linear component of the increase in time between third and fifth response . Subsequent t tests showed a significantly greater slope of the regression line in the THETA-ONLY group, compared to the very similar slopes of the CONTROLS and FOOD CONTROLS ($t=3.87$, df 10, $p < 0.01$).

FIG. 9a Experiment A : Latency from 3rd to 5th bar press: group means of logarithmically transformed latencies, over days of acquisition training.
(Holt, unpublished data)

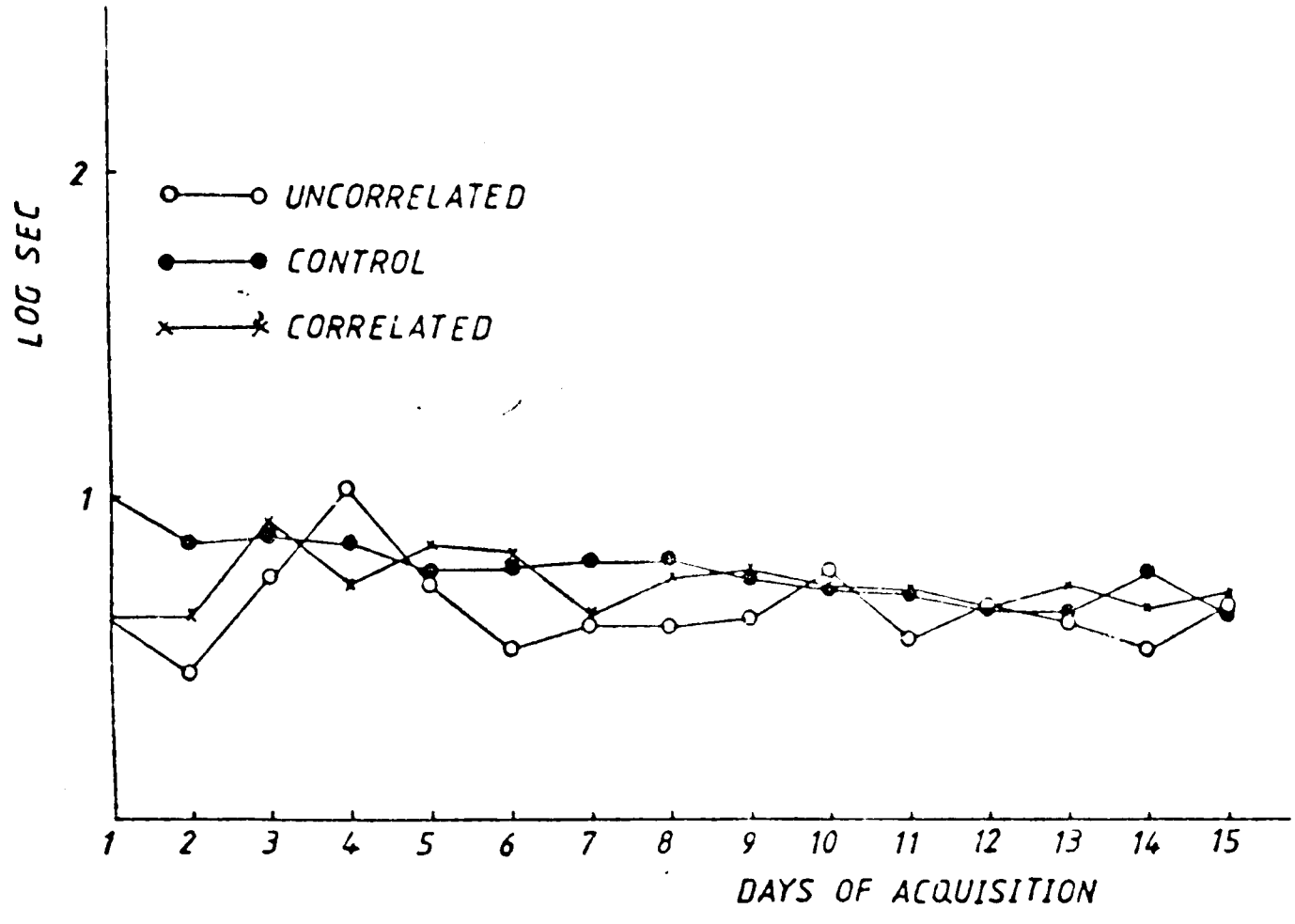
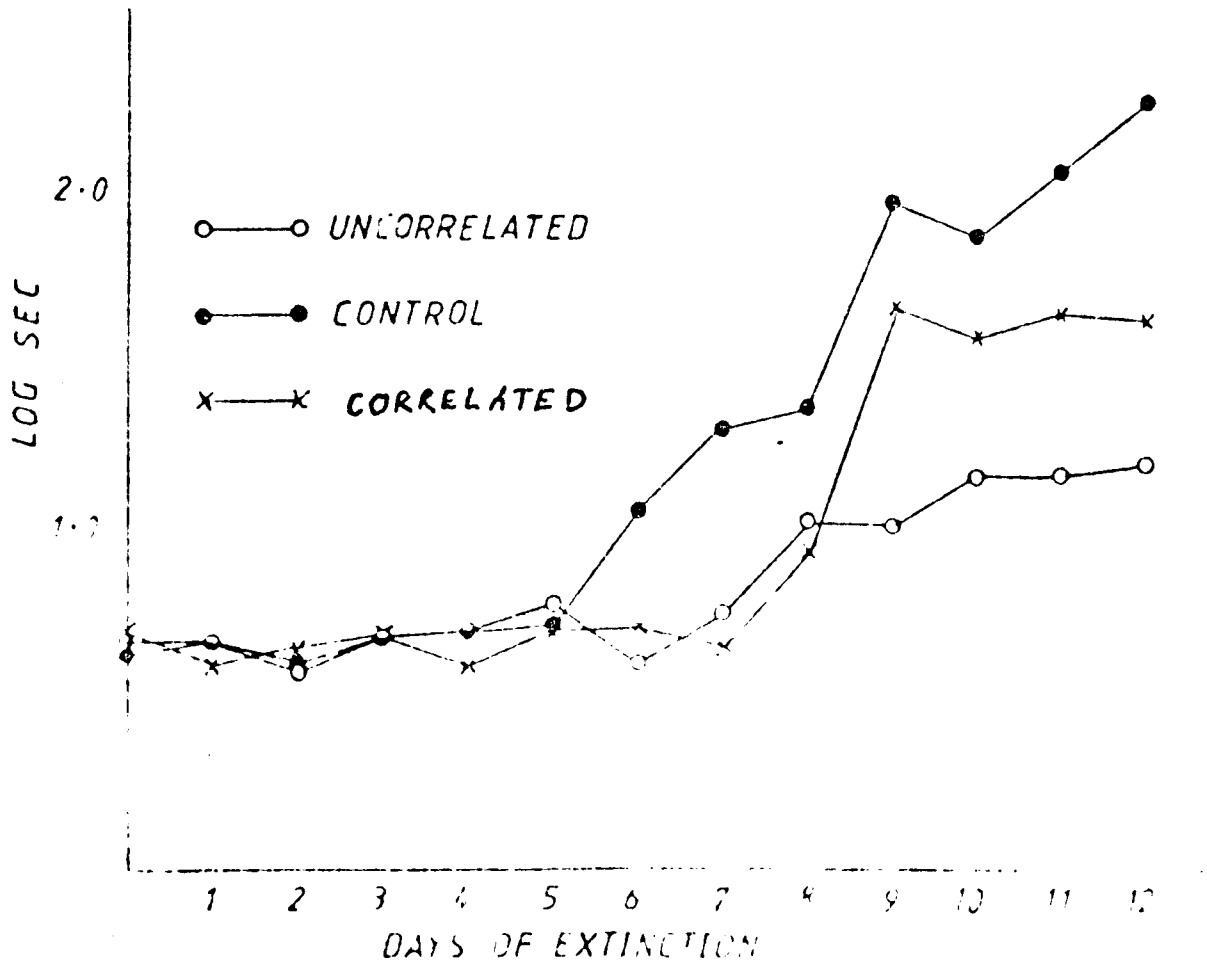


FIG. 9b Experiment A : Latency from 3rd to 5th bar press; group means over days of extinction.
(Holt, unpublished data)

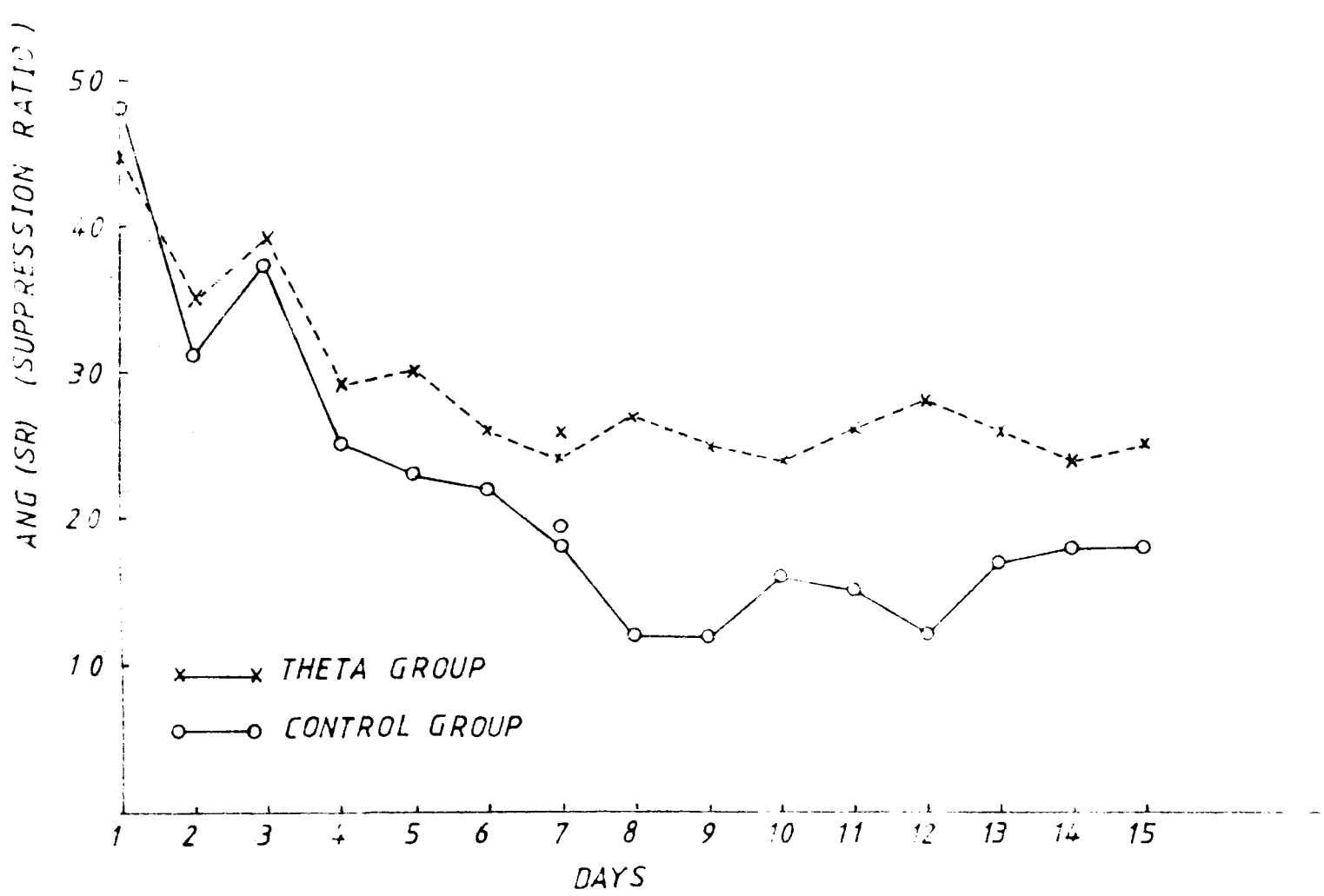


Thus, increased resistance to extinction emerged in the stimulated groups of Experiments A and B , after 8-9 days of extinction. In Experiment C, however, no increase in resistance to extinction was found in the stimulated group; the analysis of variance gave a Days x Group interaction which did not reach significance ($F=2.41$, df 2,338, $p < 0.1$). It was felt that the increased lengths of acquisition and extinction periods (and, therefore, the interval between stimulation and death) was the main point of difference between this experiment and Experiments A and B, and that the increased time lag might in some way interfere with the relationship between theta driving and resistance to extinction.

EXPERIMENT E: BEHAVIOUR DURING CONDITIONED SUPPRESSION.

The number of bar presses during a 20 second period before eacg tone burst, and during the 20 second tone burst, were measured automatically, and the suppression ratio was calculated . The data were then processed by an arcsine-square root transform with a resulting minimum value of zero, denoting total suppression, and a maximum of 45, denoting no suppression. Analysis of variance of the transformed data showed that suppression ratios were reduced over days ($F= 64.65$, df 1/138, $p < 0.001$ for the linear regression of responding on days) and this trend was shown to be statistically significant ($t= 8.01$, df 10, $p < 0.001$). There was a linear tendency for divergence of the means of the different treatment groups; ($F= 4.2$, df 1/138, $p< 0.05$). Theta-driven rats showed slower development of the suppression of responding than controls ($t=2.69$, df 10 , $p < 0.05$) (see FIG. 9c). Thus in the conditioned suppression paradigm, less suppression of responding was seen in the stimulated group than in unstimulated controls in this experiment.

FIG. 9c Experiment E : Mean suppression ratio in CER training after previous theta-driving in the hippocampus by electrical stimulation of the septal area.
(Holt, unpublished data)



EXPERIMENT F: EFFECTS OF SHOCK AND OF THETA DRIVING.

Analysis of the baseline response rates before CER training started showed a significant increase associated with previous theta driving ($F=4.23$, df 2/18, $p < 0.05$ for the main effect of stimulation).

When the suppression ratio data were analysed after covarying the baseline response rates, a significant 3-way interaction (Days x Shock x Group) was found ($F=5.58$, df 2/215, $p < 0.005$). Thus the theta driving was shown to reduce the CER if the stimulation-related effects on baseline response rate are controlled by covariance analysis.

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