INVESTIGATION OF NON-CHOLINERGIC ACETYLCHOLINESTERASE AND RELATED PEPTIDES IN AN *IN VITRO* PREPARATION OF THE SUBSTANTIA NIGRA

A thesis submitted for the degree of Doctor of Philosophy

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Trinity 2001

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ABSTRACT

The primary role of acetylcholinesterase (AChE) is hydrolysis of acetylcholine (ACh). However, observations by numerous groups have suggested that AChE may have non-cholinergic functions. Furthermore, developmental roles for AChE and its related enzyme, butyrylcholinesterase (BuChE), which is also capable of ACh hydrolysis, have been postulated.

One line of evidence to support a non-cholinergic role for AChE is the apparent disparity in several brain areas between the distribution of AChE and the cholinergic marker choline acetyltransferase. The substantia nigra (SN), an area of the ventral mesencephalon (VM), which contains the dopaminergic cells that degenerate in Parkinson's disease (PD), is an area that displays such a disparity.

One approach to treating PD involves implantation of embryonic dopaminergic VM cells into the parkinsonian brain. This procedure, known as foetal transplantation, has met with limited success, in part due to degeneration of dopaminergic cells within the donor preparation. It is known that incorporation of trophic factors into the preparation for grafting improves dopaminergic cell survival. It has previously been shown that AChE enhances survival and neurite outgrowth of postnatal dopaminergic cells in organotypic cultures of the VM. The aim of the studies in this thesis was to establish the effects of BuChE and AChE on embryonic dopaminergic neurons in a preparation analogous to that used in the animal model of foetal transplantation as a treatment for PD.

Addition of BuChE and monomeric (G_1-) and tetrameric (G_4-) forms of AChE enhanced dopaminergic neurite outgrowth. Inhibition of the active site of BuChE and AChE by echothiophate had no effect upon neurite outgrowth or cell survival, demonstrating that the trophic effects of BuChE and AChE on neurite outgrowth were not dependent upon ACh hydrolysis. In contrast, inhibition of the peripheral anionic site (PAS) of AChE by BW284c51 markedly decreased cell survival and neurite outgrowth. The mechanism of action of BW284c51 toxicity was subsequently investigated using a mixture of nicotinic ACh receptor antagonists in order to demonstrate that the chronic toxic effects of BW284c51 were not a consequence of elevated ACh resulting from inhibition of AChE.

Finally, the technique of whole-cell patch-clamp electrophysiology revealed a novel inhibitory effect of BuChE and G_1- and G_4-AChE on voltage-dependent calcium currents. It was postulated that these actions underlie the trophic effects of BuChE and AChE on embryonic dopaminergic neurons, a suggestion that was supported by the findings that established inhibitors of voltage-dependent calcium currents enhanced dopaminergic neurite outgrowth.

The findings of this thesis are discussed in the context of other studies and are related to both physiological and pathological functions of the central nervous system.

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ABBREVIATIONS

µl	Microlitre
µg	Microgram
µM	Micromolar
ΔA/min	Change in absorbance per minute
$A_{(4/8/12)}$	Asymmetric acetylcholinesterase
ABC	Avidin / biotin complex
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
Asn	Asparagine
Asp	Aspartic acid
Ba^{2+}	Barium
$BaCl_2$	Barium chloride
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
BuChE	Butyrylcholinesterase
BW84c51	1,5-bis(4-allydimethylammoniumphenyl)-pentan-3-one dibromide
$[Ca]_i$	Intracellular calcium concentration
cAMP	Cyclic adenosine monophosphate
Cd^{2+}	Cadmium
CaM	Calcium-calmodulin
ChAT	Choline acetyltransferase
CO_2	Carbon dioxide
COMT	Catechol-O-methyltransferase
CNS	Central nervous system
CsCl	Caesium chloride
CsOH	Caesium hydroxide
Cy5	Indodicarbocyanine
Cys	Cysteine
D1	Day 1
DAB	Diaminobenzidine
ddH_20	Double distilled water
DHP	Dihydropyridine
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl-sulphoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
E	Embryonic
E14	Embryonic (gestational) day 14
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(aminoethylether) N,N,N',N'-tetraacetic acid
EP	Entopeduncular nucleus
FCS	Foetal calf serum
FITC	Fluorescein

G_1-AChE	Monomeric globular AChE
G_2-AChE	Dimeric globular AChE
G_4-AChE	Tetrameric globular AChE
GABA	γ-amino butyric acid
GAD	Glutamic acid decarboxylase
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibrilliary acidic protein
Glu	Glutamate
Gly	Glycine
G^a	Amphiphilic globular AChE
G^{na}	Non-amphiphilic globular AChE
GP	Globus pallidus
GPi	Globus pallidus internal segment
Gpe	Globus pallidus external segment
H^+	Hydrogen ion
HBSS	Hank's balanced salt solution
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HEK	Human embryonic kidney
His	Histidine
HNK-1	Human natural killer-1 carbohydrate epitope
6-OHDA	6-hydroxy dopamine
IC_{50}	Half maximal inhibition
I_{max}	Maximal peak voltage-dependent calcium current
Iso-OMPA	Tetraisopropylpyro phosphoramide
K^+	Potassium
L-dopa	Levodopa
$MgCl_2$	Magnesium Chloride
MAO	Monoamine oxidase
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
MPTP	N-methyl, 4-phenyl-1,2,5,6-tetrahydropyridine
MPP^+	N-methyl, 4-phenyl-pyridinium
mRNA	Messenger ribonucleic acid
msec	Millisecond
mU	milliunits of cholinesterase activity
mV	Millivolts
n	Number
nAChR	Nicotinic acetylcholine receptor
NGF	Nerve growth factor
NMDA	N-methly-D-aspartate
$^{\bullet}O_2^-$	Superoxide anion radical (superoxide)
$^{\bullet}OH$	Hydroxyl radical
pA	picoamp
PAS	Peripheral anionic site
PBS	Phosphate buffered saline

PD	Parkinson's disease
PDL	Poly-D-Lysine
Pen/Strep	Penicillin/streptomycin
PET	Positron emission tomography
PFA	Paraformaldehyde
Phe	Phenylalanine
PN	Postnatal age
RT	Room temperature
s.e.m	Standard error of the mean
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
SBTI	Soyabean trypsin inhibitor
STN	Subthalamic nucleus
TEA	Tetra-ethyl ammonium
TEACl	Tetraethylammonium chloride
TEAOH	Tetraethylammonium hydroxide
TBS	Tris buffered saline
TH	Tyrosine hydroxylase
TH+ve	Tyrosine hydroxylase positive
TR	Texas red
Trp	Tryptophan
UV	Ultraviolet
V	Volts
VA	Ventral anterior
VL	Ventral lateral
VDCC	Voltage-dependent calcium channel
VM	Ventral mesencephalon
VP	Ventral pallidum
VTA	Ventral tegmental area

CHAPTER 1

Introduction:
The basal ganglia, Parkinson's disease and acetylcholinesterase

OVERVIEW

Parkinson's disease (PD) is a severely debilitating movement disorder in humans, and its causes, treatment and prevention are the subject of intensive research efforts. The primary pathological event underlying this disease is the degeneration of a discrete population of neuromelanin pigmented neurons which lie in the substantia nigra pars compacta (SNpc), an area within the ventral mesencephalon (VM). Neurons of the SNpc, which utilise the neurotransmitter dopamine (DA), are part of the basal ganglia circuitry within the brain. The basal ganglia are a group of functionally related subcortical nuclei, which form complex interconnections and help facilitate and co-ordinate movement. One method of treating PD involves implantation of embryonic VM cells into the parkinsonian brain in order to replace the DA lost by degeneration of the SNpc neurons (Dunnett and Björklund, 1999). This procedure, known as foetal transplantation, has met with limited success, in part due to degeneration of dopaminergic cells within the donor preparation. It is known that incorporation of trophic factors into the *in vitro* prepared graft improves dopaminergic cell survival and neurite outgrowth. Various well-established growth factors for several cell types have been utilised for this purpose, however a potent, selective trophic factor for dopaminergic cells in this role has not been identified. One such, rather unlikely candidate is the enzyme acetylcholinesterase (AChE) which has been shown to enhance the survival and neurite outgrowth of postnatal dopaminergic cells in organotypic cultures of the VM. Whilst AChE is recognised for its ability to hydrolyse acetylcholine (ACh), the possibility that it is capable of performing other functions was suggested by Silver in 1974. Furthermore, a developmental role for cholinesterase was postulated by Drews in 1975. Subsequently, the involvement of AChE, and the closely related enzyme, butyrylcholinesterase (BuChE), which is also capable of hydrolysing ACh, in development has been documented (Layer,

1983; Layer *et al.*, 1987; Layer and Sporns, 1987; Layer, 1990; Willbold and Layer, 1992; Layer *et al.*, 1993; Layer and Willbold, 1995). Identification of a trophic ability and a developmental form of AChE make it an ideal candidate as a trophic factor of dopaminergic cells in an embryonic VM preparation.

The purpose of this thesis is to investigate the putative non-cholinergic actions of AChE, and its related enzyme BuChE, on developing dopaminergic neurons in a preparation analogous to that used in the animal model of foetal transplantation as a treatment for PD. Chapters 3, 4 and 5 are concerned with the effects of AChE, BuChE, and AChE inhibitors on cell morphology; investigations in these chapters examine the effects of exogenously applied AChE, BuChE and AChE inhibitors upon cell survival and neurite outgrowth of embryonic dopaminergic cells in dissociated VM cultures. Chapters 7, 8 and 9 are concerned with the effects of AChE and BuChE on cell physiology; these chapters investigate a possible mechanism of action for AChE and BuChE on voltage-dependent calcium channels (VDCC) of embryonic cells in dissociated cultures of the VM.

This chapter (Chapter 1) aims to introduce the topics that are relevant to all of the following experimental chapters (Chapters 3, 4, 5, 7, 8 and 9). Thus, this chapter will provide an overview of firstly the basic functional architecture of the basal ganglia and the function of the SNpc within it. Secondly of PD; its clinical manifestations, aetiology and current treatments, including foetal transplantation, and finally, the structure and functions of AChE, and comparison with BuChE. An introduction to Chapters 7, 8 and 9 outlining the structure and function of VDCC, and evidence for a potential role of AChE in VDCC modulation is provided in Chapter 6.

1.1 THE BASAL GANGLIA

The basal ganglia consist of several interconnected subcortical nuclei, which are intimately involved in a variety of processes including motor, associative, cognitive and mnemonic functions. The principle nuclei of the "dorsal" basal ganglia are the neostriatum (or caudate-putamen, hereafter referred to as the striatum), the globus pallidus (GP) or its equivalent in primates, the external segment of the globus pallidus (GPe), the entopeduncular nucleus (EP) or its homologous structure in primates the internal segment of the globus pallidus (GPi), the subthalamic nucleus (STN) and the substantia nigra (SN), which comprises the substantia nigra pars reticulata (SNpr) and the substantia nigra pars compacta (SNpc). These structures are associated with motor and associative functions, whilst the "ventral" division of the basal ganglia, comprising the ventral striatum or nucleus accumbens, olfactory tubercle, ventral pallidum (VP) and ventral tegmental area (VTA) are associated with limbic functions. The studies detailed in this thesis are concerned with the dopaminergic cells of the SNpc and their role in the dorsal (associative and motor-function related) aspects of the basal ganglia during neurodegeneration in Parkinson's disease. Thus the ventral region of the basal ganglia will not be referred to further here. Since rodents were used exclusively in the studies detailed here, terminology appropriate to the rodent basal ganglia will be used. Thus the term 'globus pallidus' (GP) will not only refer to this structure in rodents, but includes the primate equivalent, the GPe. The term 'basal ganglia output nuclei' will refer to the EP or the primate equivalent the GPi, and the SNpr.

1.1.1 The basal ganglia circuitry

The anatomy of the basal ganglia connections suggest that the structures comprising it operate as part of recurrent circuits with the cerebral cortex. In very simplistic terms, the

basal ganglia receives input via the striatum from the cerebral cortex, processes this input and sends information back to the cortex via connections in the midbrain and thalamus. Subdivisions within these cortico-basal ganglia-thalamocortical circuits suggest that different circuits relate to different cortical functions (Alexander and Crutcher, 1990; Parent and Hazrati, 1995a and b; Joel and Weiner, 2000). The importance of the "motor circuit" of the basal ganglia, just one of the cortico-basal ganglia-thalamocortical circuits, is particularly apparent since neurodegenerative diseases of the basal ganglia such as Parkinson's disease and Huntington's disease result in profound movement disorders (DeLong 1990, Albin *et al.*, 1995).

A model was proposed for the basal ganglia circuitry that accounts for both normal and abnormal motor function (Albin *et al.*, 1989). It has been expanded and updated by many authors since its conceptualisation (Alexander and Crutcher, 1990; DeLong, 1990; Chesselet and Delfs, 1996; Gerfen and Wilson, 1996; Levy *et al.*, 1997; Smith *et al.*, 1998; Obeso *et al.*, 2000). The model is based upon there being two distinct pathways of cortical information flow through the basal ganglia; the 'direct' and 'indirect' pathways as illustrated by Figure 1.1A.

Cortical afferents arrive at the principal integrating neurons of the striatum which are called the medium-size densely spiny neurons (MSN), so called because of the extensive spiny processes which exist on the dendrites. The MSN constitute 80-90 % of the total population of the striatum, and utilise the neurotransmitter γ-amino-butyric acid (GABA). The massive input which the MSNs receive from the cortex is primarily glutamatergic and asymmetric in nature (Carlsson and Carlsson, 1990; Graybiel 1990; Gerfen, 1992). According to the

direct/indirect pathway model, cortical information reaching the MSN during movement is then conveyed to the output nuclei of the basal ganglia via two pathways.

1.1.1.1 The direct pathway

This pathway involves a direct projection from the striatum to the EP/SNpr complex, which is predominantly GABA-ergic in nature (Graybiel, 1990; Smith and Bolam, 1990), although GABA is co-localised with Substance P and dynorphin (Haber and Elde, 1981; Graybiel, 1990). The EP and SNpr, which are separated by the internal capsule, project to the thalamic relay nuclei, also known as the ventral anterior (VA) and ventral lateral (VL) nuclei (Parent and Hazrati, 1995b). The pathway from the EP/SNpr to the thalamus represents the major output of the basal ganglia and again utilises GABA (Beckstead *et al.*, 1979; Haber *et al.*, 1985; Haber *et al.*, 1993). The glutamatergic projection of the thalamic nuclei back to the cortex completes the direct pathway (Alexander and Crutcher, 1990; Smith and Bolam, 1990).

1.1.1.2 The indirect pathway

The indirect pathway projects from the striatum to the GP. It is also GABA-ergic, but in contrast to the direct pathway, GABA is co-localised with enkephalin (Alexander and Crutcher, 1990; Graybiel 1990; Smith and Bolam, 1990; Gerfen, 1992). The GP in turn projects to the STN via GABA-ergic efferents (Parent and Hazrati, 1995b). The indirect pathway then joins the direct pathway by a glutamatergic projection from the STN to the EP/SNpr, and then follows the direct pathway to the thalamus (GABA-ergic) and back to the cortex (glutamatergic).

According to the direct/indirect pathway model, the direct pathway provides positive feedback between the basal ganglia and the thalamus and the indirect pathway provides negative feedback between the basal ganglia and the thalamus. These efferent pathways have functionally opposing effects on the basal ganglia output nuclei and thus on thalamic targets of these nuclei. Activation of the direct pathway leads to a disinhibition of the thalamus, thereby increasing thalamo-cortical activity, whereas activation of the indirect pathway leads to an inhibition of thalamocortical neurons. As a result, activation of the direct pathway facilitates movement, whereas activation of the indirect pathway inhibits movement.

This scheme is a gross simplification of the systems involved in the basal ganglia, and ignores, due to simplification of the circuitry models, the SNpc, which is a fundamental component of the basal ganglia, as it is extensively involved in control of the other basal ganglia nuclei.

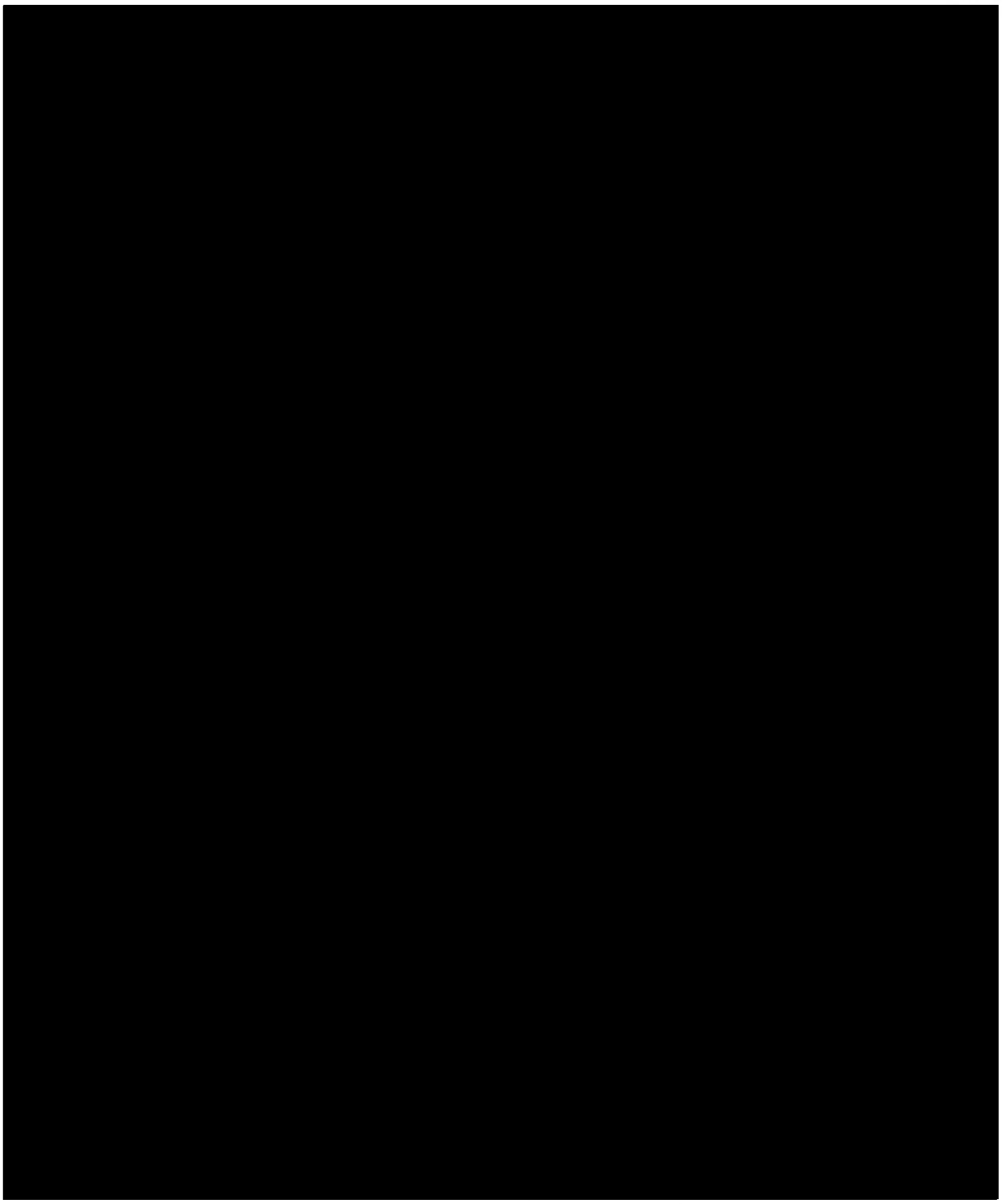

Figure 1.1 Diagrammatic representation of the motor loop in the basal ganglia, grossly simplified.

*Excitatory connections are in red, and inhibitory in black. (**A**) represents the normal condition and (**B**) that of Parkinson's disease, showing the massively diminished SNpc, the thickness of the connecting lines representing the changes in the level of input. The loss of the SNpc results in increased striatal output via the indirect pathway, whilst there is a decrease in the striatal output via the direct pathway. The net effect is to cause a significant decrease in the reinforcing thalamo-cortical projection, resulting in decreased motor co-ordinating output to the brainstem and spinal cord from the cortex. Structure abbreviations are given in section 1.1.1. Other abbreviations; enk = enkephalin, Subs P = Substance P. Adapted from Alexander and Crutcher, (1990).*

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1.1.2 The SNpc

1.1.2.1 SNpc organisation

The SN consists of three anatomical subdivions; the pars compacta, the pars lateralis, and the pars reticulata, which lies ventral to the pars compacta. The pars reticulata is predominantly GABA-ergic in nature and, as discussed above, is part of the basal ganglia output nuclei. The pars compacta consists of ~ 7200 densely packed neurons (Oorschot, 1996). The majority (> 95 %) of these are dopaminergic which project principally to the striatum. A non-dopaminergic nigro-striatal pathway has also been suggested (van der Kooy *et al.*, 1981; Gerfen *et al.*, 1987b; Lacey *et al.*, 1989) and a study Rodríguez and González-Hernández (1999) using retrograde labelling following striatal injection of a tracer revealed that 5-8 % of nigrostriatal cells were immunoreactive for GABA. The pars lateralis is dopaminergic in nature.

The dopaminergic cells of the SNpc (which can be referred to as TH+ve cells because they can be immunocytochemically identified using an antibody for tyrosine hydroxylase (TH), the rate limiting enzyme in catecholamine synthesis; Appendix 2) can be subdivided into dorsal and ventral tiers based on their melanin content (in primates; Gibb and Lees, 1991) and calcium binding protein levels (Gerfen *et al.*, 1987a; Yamada *et al.*, 1990; Damier *et al.*, 1999a and b). The publication by Gibb and Lees (1991) showed that TH+ve neurons in the dorsal tier of the primate SNpc are heavily neuromelanin pigmented, whilst those in the ventral tier are only lightly pigmented. The significance of this is discussed in the context of Parkinson's disease in Section 1.2.3.3.i of this chapter. Nigral TH+ve neurons within the dorsal tier extend dendrites in the medio-lateral plane within the pars compacta, whilst ventral neurons have been shown to possess large dendritic processes which extend ventrally

into the SNpr (Gerfen *et al.*, 1987a) and exhibit the non-classical feature of dendritic DA release (Geffen *et al.*, 1976; Nieoullon, *et al.*, 1977, Cheramy *et al.*, 1981, Cragg *et al.*, 1997). Ventral dopaminergic neurons of the pars compacta may therefore act locally in an automodulatory fashion within the substantia nigra to modulate the activity of pars reticulata output neurons (Groves *et al.*, 1975; Lacey *et al.*, 1987). The dorsal/ventral tier division of the SNpc extends to differing striatal targets (Section 1.1.2.2 below), and also to calcium binding protein immunoreactivity, with the dorsal tier showing calbindin D28K binding whilst the ventral tier does not (Gerfen *et al.*, 1987a; Celio, 1990; Damier *et al.*, 1999a). This again has implications for Parkinson's disease, as studies in post-mortem Parkinson's disease human brains reveal that calbindin-positive dopamine neurons are spared relative to the calbindin-negative ones (Yamada *et al.*, 1990; Hirsch *et al.*, 1992; Damier *et al.*, 1999a; Section 1.2.3.3.ii of this chapter)

1.1.2.2 SNpc efferents

The primary axonal target of the dopamine neurons in the SNpc is the striatum. The nigrostriatal pathway represents one of the major inputs to the striatum. The striatum is anatomically and functionally highly heterogenous. It consists of two compartments; the matrix and the striosomes (also referred to as patch), which are histologically distinct, representing the heterogenous distribution of neuroanatomical markers. For example, matrix regions are enriched with acetylcholinesterase-immunoreactive and calbindin-immunoreactive neurons (Gerfen *et al.*, 1985; Graybiel, 1990; Gerfen, 1992), whilst striosome compartments are enriched with μ opiate receptors (Gerfen 1987b). The dorsal tier of the SNpc projects largely to the matrix of the striatum and the ventral tier projects largely

to the striosomes (Gerfen *et al.*, 1987a and b; Voorn *et al.*, 1988; Joel and Weiner, 2000; Prensa and Parent, 2001).

Whilst the nigro-striatal projection constitutes the majority of SNpc efferents, dopamine neurons of the SNpc also extend axons to other target structures. For example, the dorsal raphé nucleus (Beckstead *et al.*, 1979), cingulate cortex (Takada and Hattori, 1986), globus pallidus (Lindvall and Björklund, 1979; Jan *et al.*, 2000), subthalamic nucleus (Hassani *et al.*, 1997) and pedunculopontine nucleus (Beckstead *et al.*, 1979; for review, see Smith and Kieval, 2000).

1.1.2.3 SNpc afferents

The SNpc receives substantial innervation from various brain regions. The most significant input is that of the striato-nigral pathway which comes via the SNpr, where the projecting neurons from the striatum arborize and then intertwine with the ventrally extending dendrites of the ventral tier of the SNpc (Parent and Hazrati, 1995a; Section 1.1.2.2 above). The striatal input to the SNpc projects solely from the striosomes; the matrix do not project to the pars compacta neurons (Gerfen *et al.*, 1985; Gerfen *et al.*, 1987a; Graybiel, 1990). Other afferents to the SNpc include the STN (Kita and Kitai, 1987), a GABA-ergic input from the SNpr (Hajós and Greenfield, 1994; Tepper *et al.*, 1995), a serotonergic input from the dorsal raphé nucleus (Gervais and Rouillard, 2000), and mild GABA-ergic inputs from the EP and GP (Gerfen, 1992). There is also a cholinergic/glutamatergic input to the SNpc from the pedunculopontine nucleus (Clarke *et al.*, 1987; Bolam *et al.*, 1991; Di Loreto *et al.*, 1992) and a substantial serotonergic afferentation of the SNpc from the dorsal and median raphé nuclei (Lavoie and Parent, 1990; Corvaja *et al.*, 1993).

1.1.2.4 Role of the SNpc in the basal ganglia

The dopaminergic input to the striatum plays a central role in modulating the motor, cognitive and emotional functions of the basal ganglia nuclei. The MSN that comprise the two striatal output pathways (the direct and the indirect) preferentially express different subtypes of dopamine receptor and so are affected differently by the dopaminergic projection from the SNpc to the striatum. The dopaminergic projection from the SNpc exerts a net excitatory effect on the MSN that give rise to the direct pathway, by activation of D_1 receptors, and a net inhibitory effect on the MSN that give rise to the indirect pathway, by activation of D_2 receptors. Despite disparate synaptic actions, the dopaminergic inputs to the striatum result in the same effect; a reduction in the inhibition of thalamocortical neurons, thus facilitating movements initiated in the cortex (Alexander and Crutcher, 1990; Figure 1.1A). The functional significance of this innervation is exemplified by the marked disturbances in motor control that occur due to loss of TH+ve nigral neurons in Parkinson's disease. Degeneration of the nigrostriatal pathway results in overactivity of the striatopallidal projection and reduced activity of the striato-SNpr projection. The former situation leads to excessive inhibition of GP neurons and hence, abnormal and inappropriate disinhibition of STN neurons, ultimately resulting in excessive glutamatergic drive to the output nuclei (Figure 1.1B). Reduced activity of the direct pathway serves to further disinhibit the output nuclei so an imbalance in activity of the direct and indirect pathways occurs in favour of the indirect pathway (Figure 1.1B). Since the output nuclei are GABA-ergic, hyperactivity in these structures leads to excessive inhibition and effectively to shutdown of the targets of the basal ganglia with the ultimate result of marked motor disturbances. The excessive thalamic inhibition leads to suppression of the cortical motor system resulting in a loss of motor activity. The overactive inhibitory projection to the

brainstem locomotor area may further contribute to the movement abnormalities seen in Parkinson's disease.

1.2 PARKINSON'S DISEASE

1.2.1 Clinical symptoms

James Parkinson, in 1817, described, in his 'Essay on the Shaking Palsy' the classical symptoms of a disease now known as Parkinson's disease (PD). The symptoms were expressed as a slowness or absence of spontaneous movement (bradykinesia or akinesia), rigidity, and a resting tremor of the limbs. PD is a progressive neurodegenerative disorder with an average onset of 60 years, and affects 2 % of the population over 65. Other typical symptoms include postural instability, gait disturbances and several non-motor features including depression, excessive sleepiness and dementia.

1.2.2 Pathology

The primary degenerative event in PD is the loss of the dopaminergic neurons of the mesencephalon. The loss varies from region to region with the initial, and ultimately the greatest loss from the SNpc (~ 77 %; Hirsch *et al.*, 1989; Figure 1.2). Clinical manifestations of the disease are apparent from when ~ 50 % of the TH+ve cells in the SNpc have degenerated (for reviews see Greenfield, 1992; Lang and Lozano, 1998a). TH+ve cells are also lost from the substantia nigra pars lateralis (~ 34 %) and the ventral tegmental area (~ 55 %) as the disease progresses. Within the SNpc there is a gradient of degeneration, with the heavily neuromelanin pigmented neurons of the dorsal tier preferentially spared over the ventral cells which have a lower-melanin content. As will be discussed in Section 1.2.3.3.i, below, it is possible that production of melanin, which is a by-product of dopamine

metabolism, is linked to degeneration. Whether SNpc degeneration is the principal event in PD, or whether the disease initiates elsewhere before causing degeneration of this nucleus is unknown. In the later stages of PD cell loss extends to the entire striatum (Hornykiewicz, 1998; Lang and Lozano, 1998a and b), the locus coeruleus (Paulus and Jellinger, 1991), and the STN (Hornykiewicz, 1998). Neurons in other brain regions, including the cerebral cortex, brainstem and spinal cord may also be progressively lost, leading to non-motor dysfunctions such as genitourinary dysautonomia and cognitive decline (Lang and Lozano, 1998b).

Idiopathic PD is characterised by hyaline filamentous inclusions in the cytoplasm of nigral dopamine neurons. These Lewy bodies, named after Lewy, who first described them in 1912, are spherical inclusions, 5-25 μm in diameter, seen as a dense eosinophilic core with a pale surrounding halo in the cytoplasm of affected neurons (Figure 1.3A). Although found in other neurodegenerative conditions, the presence of Lewy bodies in the SN, in conjunction with nigral cell loss, is generally considered to be the defining neuropathological feature in idiopathic PD, to distinguish it from other PD-like motor disorders. Lewy bodies are immunoreactive for ubiquitin, a small protein that is known to 'tag' cells for degradation by the proteasome (Figure 1.3B). One of the primary constituents of Lewy bodies is α-synuclein, a soluble 140 amino acid protein. In PD, α-synuclein folds, and aggregates into insoluble fibrils to form Lewy bodies, which become ubiquitinated (see Mezey *et al.*, 1998; Kaytor and Warren, 1999). The presence of ubiquitin-immunoreactive inclusions upon post-mortem examination suggests that the degradation system has failed within the parkinsonian brain.



Figure 1.2 Post-mortem parkinsonian and non-parkinsonian human midbrain sections.

*Sections of human midbrain showing the substantia nigra of a parkinsonian (**A**) in comparison with a non-parkinsonian (**B**) patient. In the non-parkinsonian brain, the substantia nigra pars compacta is clearly visible as a thin band of heavily pigmented cells on either side of the lower part of the brain. In the parkinsonian brain this cell group has degenerated, therefore the pigmented band is no longer visible. Courtesy of Dr. S. Cragg.*

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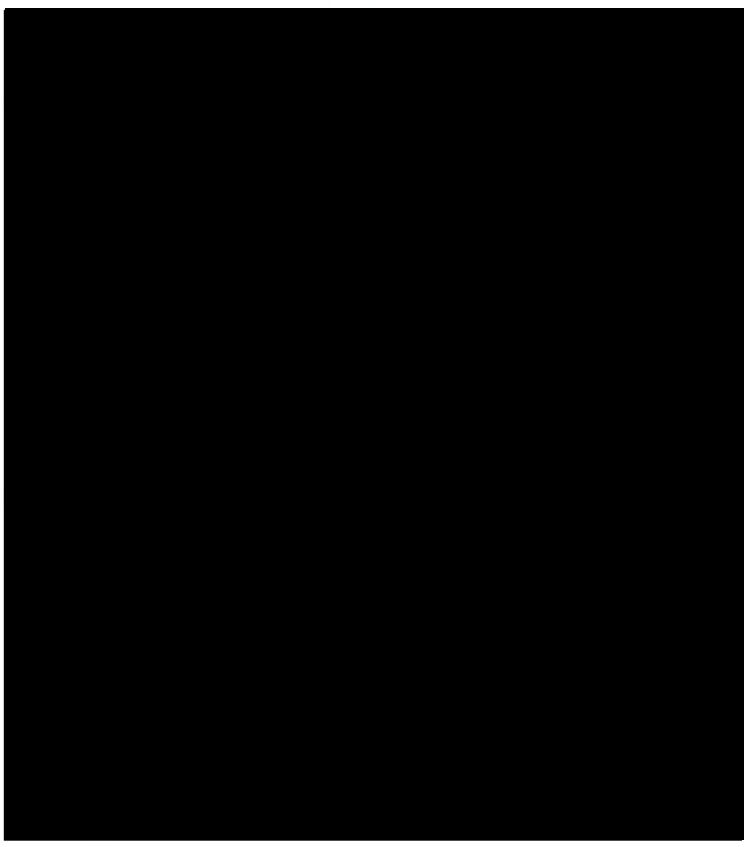

Figure 1.3 Identification of Lewy bodies in post-mortem brain.

*(**A**) Round and lobated, eosinophillic Lewy bodies surrounded by pale halos in pigmented neurons of the locus coeruleus.* x *600. (**B**) Immunohistochemistry for ubiquitin revealed around Lewy body with densely positive central core in sympathetic ganglion.* x *600. From Hague et al. (1997).*

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1.2.3 Aetiology

The precise reasons and the pathological mechanisms responsible for the preferential degeneration of the TH+ve neurons of the SNpc in PD remain unknown. Both environmental and genetic factors have been implicated in the disease's initiation.

1.2.3.1 Environmental factors

Evidence suggesting an environmental cause of PD arose following an incident involving a group of Californian drug addicts who developed parkinsonian symptoms after taking a synthetic form of heroin. The drug was subsequently found to contain 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which is metabolised to the *N*-methyl-4-phenylpyridinium ion (MPP^+) (Salach *et al.*, 1984). Accumulation of MPP^+ in dopaminergic neurons occurs via the dopamine re-uptake pathway (Javitch *et al.*, 1985). MPP+ is then taken up by mitochondria where it inhibits oxidative phosphorylation resulting in ATP depletion and cell death (Vyas *et al.*, 1986). An environmental toxin was also responsible for the increased incidence of parkinsonian symptoms on the Island of Guam, where it was revealed that the flour used by the islanders was derived from the cycad seed. This contains β-*N*-methylamino-L-alanine (Spencer *et al.*, 1987) which becomes excitotoxic (Section 1.2.3.3.ii, below) at physiological pH and causes selective degeneration of nigral dopaminergic neurons. Rural residency (Schoenberg *et al.*, 1988) and other environmental factors (Melcon *et al.*, 1997; McCann *et al.*, 1998) may be involved in the initiation or development of PD. Epidemiological studies have implicated that drinking of water from wells, which is common in certain rural locations, may contribute to regional distribution of PD. Well water may be a source of toxins (e.g. metal ions such as iron, manganese and aluminium) which are thought to initiate parkinsonian degeneration. Indeed, miners of

manganese in Chile and Cuba have an increased incidence of PD (review by Barbeau *et al.*, 1996). Intake of these metal ions could lead to cell death via free radical formation (see Section 1.2.3.3.i). Exposure to certain pesticides is also thought to be a causative factor in PD. Bocchetta and Corsini (1986) reported the first cases of PD in relation with the use of pesticides, and subsequently a correlation between PD and exposure to pesticides has been demonstrated by the study of Menegon *et al.* (1998). Furthermore, a study by Betarbet *et al.* (2000) showed that chronic exposure of rats to rotenone, a lipophillic pesticide commonly used in vegetable gardens and lakes, caused highly selective nigrostriatal degeneration and behavioural motor deficits that were concordat with animal models of PD.

1.2.3.2 Genetic factors

Approximately 5-10 % of PD patients have a familial form of parkinsonism. Mutations in several genes have been shown to segregate with the PD phenotype (Table 1.1; for reviews see Dunnett and Björklund, 1999; Olanow and Tatton, 1999; de Silva *et al.*, 2000; Haass and Kahle, 2001). Firstly, the findings of several studies provide evidence that a single mutation in the human α-synuclein gene is sufficient to account for the PD phenotype. Following linkage of autosomal dominant PD in an Italian-American kindred, and three Greek kindreds to chromosome 4q21-q23, a missense mutation in exon 4 of the α-synuclein gene (known as aSYN, SYCA or PARK1) was found to segregate with the disease (Polymeropoulos *et al.*, 1997). Further to this, a second missense mutation in the α-synuclein protein was described in a German parkinsonian family (Krüger *et al.*, 1998). As discussed in Section 1.2.2, above, α-synuclein is one of the primary components of Lewy bodies, which are considered to be the pathological hallmark of PD.

Secondly, autosomal-recessive juvenile Parkinsonism (AR-JP) is characterised by early-onset (< 40 years) of clinical features, neuronal loss that is restricted to the substantia nigra and locus coeruleus, and an absence of Lewy bodies. The genetic locus for AR-JP was identified in consanguineous Japanese families, which led to the identification of homozygous deletions in the *parkin* gene (PARK2) on chromosome 6q25.2-127 (Kitada *et al.*, 1998). The *parkin* gene encodes for a protein of 465 amino acids with moderate similarity to ubiquitin at the amino terminus. Whilst the function of parkin is unknown, its similarity to ubiquitin suggests a comparable role to ubiquitin in the mediation of proteasomal degradation of proteins. It is currently believed that mutations in the α-synuclein and *parkin* genes that give rise to parkinsonism are not connected. However, a recent paper by Shimura *et al.* (2001) may change this belief, as it provides evidence that parkin regulates the degradation of an unusual form of α-synuclein through the attachment of ubiquitin. It is postulated by Shimura *et al.* (2001) that mutations in parkin may result in accumulation of non-ubiquitinated α-synuclein in the brain, as seen in AR-JP.

Impaired proteasomal degradation of abnormal proteins seems to underlie the pathogenesis of familial PD. This assertion is supported by the detection of a missense mutation in the ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) gene located on chromosome 4p14, in a small German pedigree (Leroy *et al.*, 1998). UCH-L1 belongs to a family of de-ubiquitinating enzymes that cleave polymeric ubiquitin to monomers.

Two further loci have been implicated in autosomal-dominant idiopathic PD. A chromosome 4p haplotype, distinct from UCH-L1, segregates with both PD and essential tremor in a North

American Iowan kindred (Farrer *et al.*, 1999), and chromosome 2p13 linkage has been detected in a Danish/German family with autosomal-dominant PD (Gasser *et al.*, 1998). The genetic defects at both loci remain undetected.

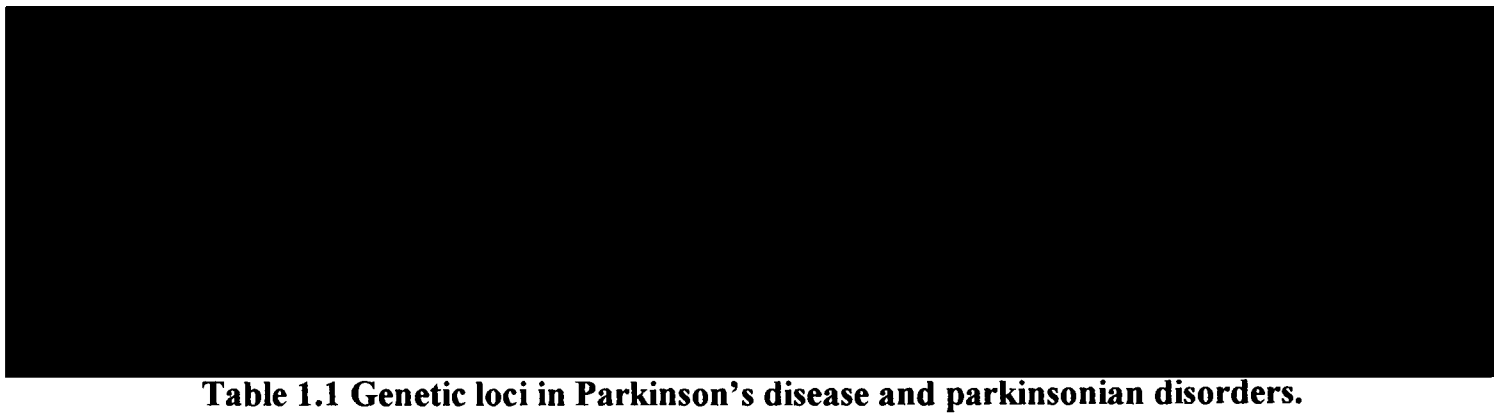

Table 1.1 Genetic loci in Parkinson's disease and parkinsonian disorders.

From de Silva et al. (2000).

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The association of PD with both environmental toxins and gene mutations suggests that either an environmental or a genetic factor can cause PD. However, in the majority of cases it is unlikely that PD will be explained by a single cause, but rather will have a multifactorial aetiology. This raises the possibility that individuals that develop PD have a genetic susceptibility to exposure to environmental toxins. Evidence to support this theory is provided by the work of Menegon *et al.* (1998), who demonstrated a correlation between PD in patients who report exposure to pesticides, and variants in the gene encoding for a glutathione transferase enzyme (GST). It was postulated in this study that the polymorphisms of the GST gene influenced the ability of GST to detoxify pesticides that may then have had a neurotoxic effect, resulting in PD. Several other functionally relevant associations in genes that could have a potential role in PD pathogenesis have been proposed (Table 1.2), but these continue to be inconclusive due to the frequent failure to reproduce the findings using different study groups (for review see de Silva *et al.*, 2000). The question of why the SNpc dopaminergic neurons are targeted in PD remains unanswered, and from this

follows the postulation that midbrain TH+ve neurons are either innately vulnerable to metabolic stress and/or exist within an environment that predisposes them to degeneration. As such, many experimental observations suggest the involvement of oxidative stress or excitotoxicity in mechanisms of parkinsonian cell death.



Table 1.2 Genes with reported allelic association in sporadic Parkinson's disease.

From de Silva et al. (2000)

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1.2.3.3 Mechanisms of cell death

(i) Oxidative stress

A great deal of work has been directed towards investigating the links between oxidative stress, mitochondrial dysfunction and cell death in PD. This is largely due to the potential of oxidative metabolism of dopamine, catalysed by monoamine-oxidase (MAO) to yield oxygen based free radicals (Halliwell and Gutteridge, 1985; Hirsch, *et al.*, 1989; Olanow 1993; Schapira, 1995; Figure 1.4 a) which may cause cell death through processes such as lipid peroxidation. Furthermore, according to the Fenton (Figure 1.4 b) and Haber-Wiess (Figure 1.4 c) reactions, the products of oxidative dopamine metabolism may, in the presence of iron or other transition metal ions, be converted to unstable, highly reactive and damaging hydroxyl radicals (Olanow 1993, Schapira, 1995). Support for the involvement of iron in PD pathology arises from findings that iron levels are elevated in the SN of PD patients (Jellinger *et al.*, 1990; Hirsch *et al.*, 1991).

(a) $\text{Dopamine} + O_2 \xrightarrow{\text{MAO}} \text{semiquinone} + \bullet O_2^- + H^+$

$\text{Dopamine} + O_2^- + 2H^+ \longrightarrow \text{semiquinone} + H_2O_2$

(b) $H_2O_2 + Fe^{2+} \longrightarrow \bullet OH + OH^- + Fe^{3+}$

(c) $\bullet O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$

Figure 1.4 *Oxidative metabolism of dopamine results in the formation of hydrogen peroxide (H_2O_2) (**a**). An increase in the steady-state concentration of H_2O_2 can lead to a reaction with ferrous iron that generates the highly reactive and potentially cytotoxic hydroxyl radical (OH•) according to the Fenton (**b**) Haber-Wiess (**c**) reactions.*

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Neuromelanin, which is also synthesised by auto-oxidation of dopamine, is capable of binding iron, and has thus been thought to increase free radical production through the Fenton and Haber-Weiss reactions. However, Gibb and Lees (1991) showed that the more heavily melanin pigmented dorsal tier of the SNpc is actually preserved compared to the weaker pigmented ventral tier. The recent work by Sulzer *et al.* (2000) may resolve some of the issues concerning the role of neuromelanin. This paper suggests that neuromelanin may provide a novel anti-oxidant mechanism in dopamine cells by trapping cytosolic quinones and semi-quinones in lysosome-associated organelles so that they are no longer reactive with cytosolic components. This would account for the apparent neuroprotective properties of neuromelanin.

Other biochemical abnormalities have also been detected within the SN, including increased superoxide dismutase levels (Saggu *et al.*, 1989; Jenner and Olanow, 1996), and decreased levels of glutathione (Perry *et al.*, 1982, Olanow 1993, Olanow and Tatton, 1999) and glutathione peroxidase (Damier *et al.*, 1993). Superoxide dismutase converts superoxide radicals to H_2O_2, and glutathione peroxidase protects against recurrent free radical formation

by using H_2O_2 to convert glutathione from a reduced state to an oxidised state (Figure 1.5). Alterations in the levels of these markers in parkinsonian SN suggest that oxidative stress is occurring in dopaminergic neurons.

$$2 \text{ reduced glutathione} + H_2O_2 \xrightarrow{\text{Glutathione peroxidase}} \text{glutathione disulphide} + 2H_2O$$

Figure 1.5 *Oxidation of reduced glutathione with glutathione peroxidase*

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Mitochondrial dysfunction has also been implicated in PD. The work of Mizuno *et al.* (1989), Schapira *et al.* (1990) and Schapira (1994) reported a 25-38 % deficiency in the function of mitochondrial enzyme complex I in post-mortem examination of parkinsonian SN. Furthermore, the pesticide rotenone, which reproduces the anatomical, neurochemical, behavioural and neuropathological features of PD in rats is a high-affinity specific inhibitor of mitochondrial complex I (Betarbet *et al.*, 2000). As mitochondrial function will be affected by free radical damage (Zhang *et al.*, 1990), oxidative stress and mitochondrial dysfunction may act in synergy to cause dopamine cell death.

(ii) Excitotoxicity

Excitotoxicity refers to neuronal cell death caused by overactivation of excitatory amino acid receptors. Cell death occurs due to an excessive influx of calcium as a result of receptor stimulation. The increase in intracellular calcium levels activates a self-destructive cellular cascade involving calcium-dependent enzymes such as phosphatases (e.g. calcineurin), proteases (e.g. calpains) and lipases (see Nicotera and Orrenius, 1998). These cause autodestructive processes leading to plasma membrane 'blebbing' due to disruption of cytoskeletal organisation, mitochondrial swelling and subsequent cell death (Olney, 1969;

Boobis *et al.*, 1989). Lipid peroxidation can also cause production of free radicals, which damage vital cellular proteins and cause cell death (Boobis *et al.*, 1989).

The fact that the SNpc receives glutamatergic input from the pedunculopontine nucleus and STN, and serotonergic afferentation from the raphé nuclei is anatomically consistent with a possible excitotoxic mechanism. Under normal circumstances glial cells and axon terminals remove excesses of excitatory neurotransmitters (Meldrum and Garthwaite, 1990). However, if these processes are disrupted under, for example, a state of metabolic stress, glutamate present in the extracellular space could trigger further release of glutamate (Meldrum and Garthwaite, 1990), initiating an excitotoxic cycle that results in cell death (see Greenfield, 1992).

Evidence implicating excitotoxicity in PD is manifold. *In vitro* animal studies have revealed neurotoxic effects of glutamate upon dopaminergic neurons in mesencephalic cultures. These effects appear to be mediated by the NMDA receptor (Kikuchi and Kim, 1993; Schierle and Brundin, 1999). Furthermore NMDA antagonists protect against dopamine cell loss resulting from MPP+ infusion into the SNpc of rats (Turski *et al.*, 1991) and MPTP treatment in primates (Greenmayre *et al.*, 1994). In humans, elevated levels of calpains have been found in the SN of PD brains (Hirsch *et al.*, 1997). Additionally, many anti-parkinsonian drugs (amantadine, memantine, trihexyphenidyl, ethopropazine, procyclidine) are non-competitive NMDA antagonists (Kornhuber *et al.*, 1991), a property that is thought to contribute to their anti-parkinsonian effects (Klockgether and Turski, 1989; Ossaka, 1994). The correlation of dopamine cell survival with calbindin in PD also supports the role of excitotoxicity in PD pathology. Calbindin is a calcium-binding protein thought to provide a

buffering capacity against excitotoxicity (Yamada *et al.*, 1990; Philips *et al.*, 1999). Finally, excitotoxic mechanisms provide an explanation for the increased incidence of Parkinson's disease on the island of Guam.

The factors cited in this section as possible mechanisms for cell death in Parkinson's disease are by no means mutually exclusive. Rather, it is more likely that when many (or all) of these factors combine, a threshold is reached that tips the balance from physiology to pathology. Such a multifaceted explanation for parkinsonian cell death serves to complicate attempts at providing both treatment and cure for the disease.

1.2.4 Treatment

Treatment of PD is confounded by several factors. Firstly, the cause of the disease remains unknown. Secondly, due to the remarkable compensatory capacity of the basal ganglia, clinical symptoms are not apparent until 50 % or more of SNpc dopamine neurons have degenerated (Lang and Lozano, 1998a; Bezard *et al.*, 2001). Finally, as with all disorders of the CNS, the failure of drugs to cross the blood-brain barrier (BBB) presents a problem.

1.2.4.1 Drug therapy

Pharmacological treatment of PD was largely ineffective until the 1960s and the discovery that dopamine was severely depleted in the basal ganglia of parkinsonian brains. A dopamine replacement therapy was therefore instigated with the dopamine precursor molecule dihydroxyphenylalanine (DOPA; Cotzias *et al.*, 1967). The L-isomer of DOPA became the practical choice for pharmacological development of an oral agent since, unlike dopamine, it is able to cross the blood brain barrier and be utilised by the remaining SNpc neurons

(Marsden, 1990; Weiner, 1999). L-DOPA is also, however, converted to dopamine in the periphery via DOPA decarboxylase, and this gives rise to side effects such as nausea, hypotension and cardiac arrhythmias. In order to prevent such side effects, DOPA-decarboxylase inhibitors are used in conjunction with L-DOPA therapy. Inhibitors such as carbidopa and benzerazide increase the amount of L-DOPA crossing the blood-brain barrier while reducing the side effects. This combined therapy is marketed as Sinemet^{TM} and is in use today. L-DOPA is also metabolised in the peripherary by Catechol-O-methyltransferase (COMT) to produce 3-O-methyldopa (3-OMD). COMT-inhibitors such as Entacapone and Tolcapone have recently been introduced as a treatment for PD, again, to prevent metabolism of L-DOPA in the periphery (Vieira-Coelho and Soares-da-Silva, 1999; Piccini *et al.*, 2000; Gerlach *et al.*, 2001). Whilst these therapies relieve the symptoms of PD, they have no impact on the underlying cause of the disease. Furthermore, successively larger doses of L-DOPA are required as the number of dopamine neurons available to utilise it continues to decline and the remaining dopamine neurons become desensitised to the drug. This leads to severe and rapid oscillations in mobility (known as on-off effects) and choreic and dystonic involuntary movements (dyskinesias). Therefore, a second generation of dopaminergic antiparkinson drugs, based on direct dopamine agonists has been investigated. Agonists are theoretically a superior treatment for PD than L-DOPA because they are pharmacologically active and not dependent on enzymatic conversion. However, agonists such as apomorphine or bromocriptine have been shown to lack the therapeutic potency of L-DOPA (Olanow *et al.*, 1987). The impact of these agonists has been limited and only patients with advanced PD benefit from using L-DOPA and agonists in synergistic combination to modulate motor fluctuations or to temporarily stabilise disability.

An alternative approach to dopamine replacement therapy involves protecting neurons from the degenerative processes. This strategy involves administration of monoamine oxidase (MAO) inhibitors. While somewhat controversial, MAO inhibitors (such as deprenyl and selegiline) are used frequently in early PD, and presumably enhance the effect of dopamine by slowing dopamine's catabolism (Golbe *et al.*, 1988). It is also thought that MAO inhibitors may slow the progression of PD by reducing oxidative metabolism of dopamine, and thus the potential for generation of reactive and potentially toxic oxidative products (Ahlskog, 1999). Other protective treatments include the use of antioxidants, such as vitamins C and E with a view to possible removal of free radicals (Fahn, 1992) and iron-chelating agents (Grünblatt *et al.*, 2001).

1.2.4.2 Surgical therapy

Surgery as a means of treating PD has its origins in the observation by James Parkinson in 1817 that a stroke abolished contralateral tremor. According to the direct/indirect pathway model of the parkinsonian basal ganglia (Figure 1.1B), degeneration of the nigrostriatal pathway results in overactivity of the striatopallidal projection and reduced activity of the striato-SNpr projection. It was thus postulated that lesioning of other nuclei within the basal ganglia circuitry may compensate for the dopaminergic deficit and restore the balance of neurotransmitters required for motor control. Surgical targets have included the GP, thalamus and STN and have produced varying improvements in rigidity, tremor, bradykinesia and dyskinesia (for review see Koller *et al.*, 1999). The disadvantages of such radical surgery include the incomplete improvement of parkinsonian symptoms, the permanency of the procedure and the potential of irreversible side effects. In recognition of these problems, the most recent advances in surgical intervention in PD have involved deep

brain stimulation (DBS). This procedure also attempts to compensate for the imbalance in the basal ganglia circuitry caused by nigral dopamine loss by altering the activity of other basal ganglia nuclei. High frequency (135-300 Hz) stimulators are placed within the target basal ganglia nucleus and stimulated via a subcutaneously implanted pulse generator that the patient can turn on or off using a hand-held magnet. Target nuclei are as for lesioning studies and whilst improvements in tremor are noticeable, akinesia, bradykinesia and dystonia generally persist (Barcia-Salorio *et al.*, 1999; Hariz *et al.*, 1999; Jahanshahi *et al.*, 2000). The precise mechanism of action of DBS is unknown; persistent depolarisation and neuronal jamming have been proposed as possible mechanisms. The advantages of DBS over ablative surgery include reversibility (no destructive lesion) and adaptability (changing of stimulus parameters to increase efficacy or decrease adverse reactions). Disadvantages include the cost of the equipment and surgery, and the risk of infection.

1.2.4.3 Foetal transplant therapy

Neural transplantation in PD is based upon the idea that dopamine supplied from cells implanted into the striatum can substitute for the lost nigrostriatal neurons. Animal models of foetal transplantation involve unilateral destruction of the nigrostriatal pathway using 6-hydroxy dopamine (6-OHDA) in the rodent model (Sauer and Oertel, 1994) and MPTP in the primate model (Javitch *et al.*, 1985) to create a parkinsonian state. This is followed by transplantation of donor ventral mesencephalic cells either as a cell suspension, or as a solid tissue into the host striatum (Figure 1.6). To survive, the transplanted cells must be obtained at an early stage of development (in rat, embryonic day 14-15 (E14-E15), and in primate/human, 5.5-5.9 weeks post conception). Animal models of foetal transplantation have been shown to survive and extend neurites into the host striatum (Björklund *et al.*, 1980

and 1983; Clarke *et al.*, 1988; Walters *et al.*, 1992; Nikkah *et al.*, 1994). Synaptic contacts between grafted TH+ve cells and host striatal neurons (Freund *et al.*, 1985), and afferent connections on to grafted TH+ve neurons are also apparent (Mahalik *et al.*, 1985; Bolam *et al.*, 1987; Clarke *et al.*, 1988). Grafts have been shown to restore dopaminergic neurotransmission in 6-OHDA lesioned animals (Schmidt *et al.*, 1982 and 1983; Brundin *et al.*, 1988), contain spontaneously active neurons similar to those of the SNpc *in situ* (Wuerthele *et al.*, 1981), and provide functional recovery of several behavioural deficits caused by 6-OHDA lesions (Björklund *et al.*, 1980 and 1981; Clarke *et al.*, 1988; Stern *et al.*, 1992; Walters *et al.*, 1992). However, nigral grafts in animals are not capable of restoring all behavioural deficits, particularly following bilateral destruction of the nigrostriatal pathway. As the study by Dunnett *et al.* (1983) illustrated, transplantation of donor neurons into several host striatal sites following lesioning alleviated akinesia and sensorimotor neglect, but had no effect upon aphagia or adipsia (lack of desire to eat and drink). Studies of post-mortem animal brains that have undergone transplantation illustrate that only 5-20 % of grafted TH+ve cells survive (Nikkah *et al.*, 1994; Barker *et al.*, 1996), with the vast majority lost during the first 7-days following transplantation (Barker *et al.*, 1996; Emgård *et al.*, 1999).

Clinical trials in human PD patients that have undergone foetal transplant therapy have shown that transplanted mesencephalic dopamine neurons can survive and are functional (Freed *et al.*, 1992). Positron emission tomography (PET) scans have illustrated a significant increase in the [^{18}F]fluorodopa uptake in the area around the graft that has been maintained for at least six years in some patients (Wenning *et al.*, 1997; Dunnett and Bjorklund 1999; Figure 1.7). Long-lasting symptomatic improvement has been reported in many patients that

have undergone transplantation, and in the most successful cases it has been possible to withdraw L-DOPA treatment (Lindvall *et al.*, 1990; Freed *et al.*, 1992; Jacques *et al.*, 1999). However, functional recovery is incomplete and highly variable, with about 60 % of patients reporting a partial recovery of motor function and a few showing improvements of gait, language and balance.



Figure 1.6 Two alternative procedures used for grafting human foetal tissue into the striatum of patients with Parkinson's disease.

This schematic diagram shows both the cell suspension (left) and the solid graft (right) techniques for foetal transplant grafts. For the cell suspension method, the foetal ventral mesencephalon is dissociated prior to implantation. For the solid graft method, the foetal VM is divided into pieces and then implanted. From Lindvall, (1991).

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Figure 1.7 Image of foetal dopaminergic cells surgically implanted in the brain of a patient with PD.

In this PET scan the precursor ^{18}F(F-DOPA) was used to identify dopaminergic foetal mesencephalic cells that had been implanted in the putamen of a patient with PD. The PET image has been superimposed on an anatomical MRI image. The site of the craniotomy and the three needle tracks left from the surgical implantation of the cells can also be seen. From Kandel et al. (2000).

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The low survival rate of grafted dopamine cells is a critical drawback of the technique of foetal transplantation, particularly as dopaminergic neurons constitute only 5-10 % of the original mesencephalic cell population of the graft (Mayer *et al.*, 1993a). This poor cell survival rate leads to a greater requirement of tissue per transplant. As the primary source of human foetal cells is aborted human foetuses, the large amounts of embryonic mesencephalic tissue that are needed to obtain therapeutic effects in patients result in ethical and practical limitations.

Several reasons have been suggested for the poor survival of grafted cells both in animals and humans. Firstly, cells are grafted into the striatum in an attempt to replace the loss of dopamine caused by degeneration of the nigrostriatal pathway. This means that appropriate afferent inputs to the grafted dopaminergic neurons will not be established. Secondly, the donor cell preparation technique is highly traumatic. Preparation of either a cell suspension

or solid pieces of tissue for grafting requires removal of the cells from their environment, and in the former case enzymatic and mechanical dissociation of the resultant tissue. The trauma caused by dissociation results in death of ~ 30 % of TH+ve cells in the preparation (Fawcett *et al.*, 1995). Finally, the death of cells that survive the preparation process has been suggested to be due to oxidative stress. In support of this, addition of free-radical scavengers and caspase inhibitors to the foetal cell preparation have been shown to increase dopamine neuron survival 2 - 3 fold (Schierle *et al.*, 1999).

Further to this, attempts to improve the survival and outgrowth of dopaminergic cells within grafted tissue by addition of trophic factors to cell preparations has provided the focus for much of the recent work on foetal transplantation. Several growth factors have been shown to improve the survival and outgrowth of TH+ve cells both within cultures and *in vivo* grafts. Glial cell-derived neurotrophic factor (GDNF) which is a 33 - 45 kDa basic glycoprotein was isolated by Lin *et al.* (1993) from the rat B49 glial cell line. GDNF was shown by Lin *et al.* (1993) to increase survival, differentiation and transmitter uptake of midbrain dopaminergic neurons. GDNF has been shown to protect dopamine cells against 6-OHDA toxicity *in vitro* (Eggert *et al.*, 1999). Administration of GDNF near the SN also protects SNpc dopaminergic neurons against infusions of 6-OHDA (Sauer *et al.*, 1995; Choi-Lundberg *et al.*, 1997; Mandel *et al.*, 1997) and MPTP (Tomac *et al.*, 1995) in rodents. Incorporation of GDNF into transplanted foetal grafts has also been shown to enhance the survival and outgrowth of TH+ve cells within the grafts. (Törnqvist *et al.*, 2000).

A second example of a factor that has been demonstrated as having a stimulatory effect on dopamine cell survival is nerve growth factor (NGF), a member of the neurotrophin family.

NGF was first discovered following experiments over 40 years ago involving implantation of mouse sarcoma tissue into mouse brain. This resulted in a dramatic increase in size of sympathetic ganglia in the vicinity of the sarcoma implant. A diffusable factor, NGF, was isolated from the sarcoma and purified. It was subsequently discovered that snake venom gland and mouse submaxillary gland are rich in NGF (see Montalcini and Angeletti, 1968). Striatal implantation of segments of peripheral nerves that secrete NGF has been shown to increase the number of surviving TH+ve neurons following MPTP administration in mice (Date *et al.*, 1990; Date *et al.*, 1994). Injection of NGF into the cerebral ventricle of mice following MPTP treatment increased levels of striatal dopamine in comparison to MPTP-treated, non-NGF treated controls in a study by Garcia *et al.* (1992). As NGF injection into non-MPTP treated mice in this study had no effect upon striatal dopamine levels, it was concluded that NGF was having a stimulatory effect upon damaged dopamine cells.

A third well-characterised growth factor known to enhance dopamine cell survival is basic fibroblast growth factor (bFGF). This is a 16.4 kDa protein that was identified and isolated from bovine brain using heparin affinity chromatography (Lobb and Fett, 1984). bFGF is present in high concentrations in the brain, including in the SNpc (Caspar *et al.*, 1994; Lolova, 1995). bFGF has been shown to promote survival and outgrowth of VM cells *in vitro* (Knusel *et al.*, 1990; Engele and Bohn, 1991; Mayer *et al.*, 1993a; Silani *et al.*, 1994). Furthermore, a neuroprotective role for bFGF has also been described *in vivo* since bFGF enhanced the recovery of the dopaminergic nigrostriatal system following MPTP lesions in mice (Otto and Unsicker, 1990; Date *et al.*, 1993). The action of bFGF on mesencephalic grafts has also been investigated. Pre-treatment of foetal rat graft tissue with bFGF moderately increased survival of dopaminergic neurons within the graft following

transplantation into 6-OHDA–lesioned rats (Mayer *et al.*, 1993b; Zawada *et al.*, 1998) and accelerated recovery of transplanted animals in tests of drug-induced rotational symmetry (Mayer *et al.*, 1993b; Zeng *et al.*, 1996; Törnqvist *et al.*, 2000). It has been postulated that the neurotrophic effects of bFGF are dependent upon the presence of glia, due to its actions being indirectly mediated through glial cells (Knusel *et al.*, 1990; Engele and Bohn, 1991; Silani *et al.*, 1994; Fawcett *et al.*, 1995; Zeng *et al.*, 1996). However, the study by Unsicker *et al.* (1992) demonstrated that death of embryonic chick ciliary ganglia in single neuron cultures in the absence of non-neuronal cells could be prevented by addition of bFGF. The growth factor bFGF is utilised in Chapter 3 of this thesis, to compare the neurotrophic actions of bFGF with the effects of test substances on dopamine cells within a dissociated culture of foetal ventral mesencephalic neurons.

Foetal transplantation is obviously not a cure for PD, and many inherent problems exist with the procedure, the foremost of which being that the donor cells are transplanted into the hostile environment of the ongoing disease process. However, the noticeable improvements in many of the patients having undergone the procedure warrant its continued use, and research is currently directed towards overcoming many of the problems that limit the procedure. Alternatives to aborted foetal tissue are being investigated, such as xeno-transplants or the expansion of neuronal progenitors of dopamine producing cells *in vitro* before transplantation into the parkinsonian striatum. Whilst death of dopamine cells within the graft reduces its efficacy, it is apparent that addition of trophic factors to transplanted cell preparations improves and prolongs the survival of TH+ve cells within the grafts, contributing significantly to the effect of the graft upon functional recovery of the patient. Factors that have been incorporated in cell preparations for grafting such as bFGF and GDNF

are well established trophic factors for many cell types. A further and perhaps surprising candidate as a neurotrophic substance for midbrain dopaminergic neurons is acetylcholinesterase (AChE). Therefore the following section will describe the characteristics of AChE and reasons for suggesting alternative functions for this familiar enzyme.

1.3 ACETYLCHOLINESTERASE

AChE is a serine hydrolase, whose primary role is hydrolysis of acetylcholine (ACh), thus rapidly terminating cholinergic transmission (see Massoulie *et al.*, 1993).

The existence of a substance that was capable of breaking down ACh was first hypothesised by Dale in 1914 and confirmed by Loewi and Navratil in 1926. In 1937, Marney and Nachmansohn demonstrated that high concentrations of AChE were present in the neuromuscular junction of *Torpedo* and *Electrophorus*. Butyrylcholinesterase (BuChE), an enzyme homologous to AChE, is also able to hydrolyse ACh. Hydrolysis of ACh by AChE is so efficient that it is close to the maximal theoretical limit set by molecular diffusion of the substrate (see Quinn, 1987). However, by comparison, substrate hydrolysis by BuChE is much slower. BuChE can also hydrolyse ACh-analogues, whilst AChE is specific for ACh (Chatonnet and Lockridge, 1989). The existence of a highly efficient enzyme for the hydrolysis of ACh, AChE, has meant that the physiological role of BuChE is ambiguous.

A study by Layer *et al.* (1993) demonstrated a non-cholinergic action of both BuChE and AChE in embryonic avian tissue, suggestive of a role for cholinesterases in development. However, a non-cholinergic role for AChE in electrophysiological and behavioural studies

has been proposed due to the consistent ineffectiveness of BuChE in these studies. As such, many studies have examined non-cholinergic aspects of AChE (see Massoulié *et al.*, 1993; Appleyard, 1994; Greenfield, 1995 and 1998). Therefore, whilst the evidence for roles of both BuChE and AChE in development will be discussed in due course (Section 1.3.4.1.3), the following sections provide an overview of the structure of AChE, with reference to BuChE, and the evidence for a non-cholinergic role of AChE.

1.3.1 Structure of AChE

AChE belongs to a class of proteins that have a large central β sheet surrounded by 14 α helices (Sussman *et al.*, 1991; Figure 1.8). BuChE has 53 % similarity with AChE, with the first 534 residues aligned (Sussman *et al.*, 1991; Massoulié *et al.*, 1993).



Figure 1.8 The ribbon structure of AChE.

Showing the β sheet structure (blue) surrounded by 14 α helices (red). Courtesy of Dr. K. Giles.

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1.3.2 Multiple forms of AChE

Both BuChE and AChE are structurally polymorphic and may exist as soluble, membrane bound and basal lamina-associated forms (Massoulié *et al.*, 1993). Endogenous, vertebrate AChE and BuChE are composed of glycoproteic subunits (70-80 kDa) which associate into

dimers through inter-subunit disulphide bonding (Heider and Brodbeck, 1992) and tetrameric forms by hydrophobic interaction (Giles, 1997). The C-terminal domains of AChE define the post-translational processing and ultimately dictate where the enzyme will be located (Massoulié *et al.*, 1993).

The majority of the AChE and BuChE sequence in individual species is highly conserved, forming a globular protein without any transmembrane or other cellular anchoring features. However, in addition, some forms of AChE may be disulphide bonded to collagenic tail 'Q' subunits or a hydrophobic membrane anchor; 'P' subunits (Massoulié *et al.*, 1993).

In vertebrates there are two cholinesterase genes, one for BuChE and one for AChE. The BuChE gene is located on chromosome 3 (3q26.1; Gnatt *et al.*, 1990). The AChE gene is located on chromosome 7 (7q22; Getman *et al.*, 1992), and generates multiple types of transcripts through a combination of different processes: the choice of several promoters; alternatively splicing in the 5' untranslated region; alternatively splicing at the 3' extremity of the coding region and the choice of several polyadenylation sites, including variable lengths of 3' untranslated sequence (Massoulié *et al.*, 1993). Different types of coding sequences may generate distinct C-terminal domains, called H, (hydrophobic), T (tailed), R (read-through: not spliced) and in snakes, S (which is soluble).

The three spliced variants of AChE, H, T and S are homomeric and may form monomeric, (G_1), dimeric (G_2) or tetrameric (G_4) globular subunit associations (Massoulié *et al.*, 1993; Figure 1.9). The C-terminal region of BuChE is not subject to alternative splicing and is closely homologous to the vertebrate T-spliced AChE. Historically, variations within the

globular categories of AChE have been classified on the basis of amphiphilicity; either amphiphillic (G^a) or non-amphiphillic (G^{na}). Whilst this nomenclature is useful and still used today, it does not allow researchers to establish the molecular forms of AChE, only the likelihood that they are derived from the membrane.

When co-expressed with Q subunits the T-subunits of mammalian AChE are assembled into collagenic-tailed forms, which are described as asymmetric forms (Duval *et al.*, 1992; Figure 1.9), which may contain one (A_4), two (A_8) or three (A_{12}) catalytic tetramers. Asymmetric AChE is the predominant form involved in the termination of neurotransmission at the neuromuscular junction in vertebrates (Vigny *et al.*, 1976; Busker *et al.*, 1994).



Figure 1.9 The major forms of cholinesterases

From Giles, (1997), after Massoulié et al. (1993).

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The globular form of AChE is assembled in rough endoplasmic reticulum with the assembly of asymmetric forms occurring in the distal elements of the Golgi apparatus (Rotundo, 1984).

Once processed, AChE is packaged in coated vesicles and transported to the plasma membrane, where the vesicles fuse, resulting in either secretion of AChE or insertion of AChE to the membrane (Rotundo, 1984; Rotundo and Carbonetto, 1987).

1.3.3 Functional sites of AChE

The functional sites of AChE have been elucidated using a combination of various techniques including x-ray crystallography (Sussman *et al.*, 1991; Bourne *et al.*, 1999), mutation studies (Shafferman *et al.*, 1992a; Barak *et al.*, 1994; Radić *et al.*, 1997; Morel *et al.*, 1999), and ligand binding studies (Radić *et al.*, 1991; Eichler *et al.*, 1994; Barak *et al.*, 1995; Hosea *et al.*, 1995; Radić *et al.*, 1997; Mallender *et al.*, 1999).

Since hydrolytic activity is one of the simplest characteristics of AChE to examine, work has focused almost exclusively on sites of AChE related to this function. In brief, there are three main sites of the AChE molecule, which are involved in the hydrolysis of ACh: the active site (esteratic), it's associated anionic sub-site and the more distantly located peripheral anionic site (PAS). In contrast, BuChE possesses an active site but lacks a PAS. It should be noted that the sequence numbering in the following sections is for *Torpedo* cholinesterase.

1.3.3.1 The active site: esteratic sub-site

Hydrolysis of ACh occurs via an acyl-deacyl-enzyme mechanism that involves nucleophilic (Serine) and general acid-base (Histidine) elements (Quinn, 1987; Figure 1.10). Crystallographic studies have demonstrated that the esteratic site is located 4 Å from the bottom of a 20 Å deep gorge which penetrates half way into the protein and is lined with rings of 14 aromatic residues (Sussman *et al.*, 1991; Figure 1.11). Sequence alignment has

revealed that 6 of the 14 aromatic residues are absent in BuChE (Doctor *et al.*, 1991). Several functional sites have been identified within the active centre gorge, including the catalytic triad, Serine-200-Histidine-440-Glutamate-327 (Ser-200-His-440-Glu-327), which is responsible for substrate hydrolysis (Sussman *et al.*, 1991; Shafferman *et al.*, 1992a and 1992b), the acyl pocket, Phenylalanine-288 (Phe-288) and Phe-290 (Ordentlich *et al.*, 1993; Taylor and Radić, 1994), and a hydrophobic sub-site. Site directed mutagenesis studies have revealed that replacement of the catalytic triad with Cysteine-Histidine-Glutamate (Cys-His-Glu) or Serine-Histidine-Aspartic acid (Ser-His-Asp) practically abolishes the hydrolytic activity of AChE (Shafferman *et al.*, 1992a).



Figure 1.10 Acylenzyme mechanism for hydrolysis of ACh.

The serine hydroxyl group (O-H) causes nucleophilic attack of ACh to form a transient ACh-tetrahedral intermediate with the subsequent loss of choline and formation of the acylenzyme. The acylenzyme is then stabilised by a deacylation step involving attack by H_2O and the liberation of acetate.

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Figure 1.11 3-dimensional structure of *Torpedo californica* AChE looking down the 'gorge' lined with aromatic residues (purple).
Courtesy of Dr. K. Giles.

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1.3.3.2 The active site: anionic subsite

The anionic sub-site of the active site is more than 4.7 Å from the esteratic site and binds the quaternary ammonium residue of ACh at Tryptophan-84 (Trp-84) (Quinn, 1987; Sussman *et al.*, 1991). Kinetic ligand-binding protocols have demonstrated hydrophobic and electrostatic properties of the anionic site, which are involved with ACh binding (Shafferman *et al.*, 1992a; Velan *et al.*, 1993; Radić *et al.*, 1997).

1.3.3.3 The peripheral anionic site

The existence of an additional anionic site was first indicated through observations that the bis-quaternary ammonium cholinesterase inhibitor, propidium, exhibited increased fluorescence when incubated with AChE (Taylor and Lappi, 1975). This observation revealed a number of quaternary ammonium ligands, which could displace propidium from an apparent anionic binding site. Studies by Berman and co-workers in 1980 using fluorescence-transfer energy measurements revealed that the PAS lies at the mouth of the catalytic gorge, 20 Å from the active site; this was subsequently confirmed by x-ray analysis

of AChE (Sussman *et al.*, 1993; Figure 1.12). The exact role of the PAS, to date, is unclear. However, substrate inhibition of AChE at high ACh concentrations is well documented and has been suggested to occur by binding of substrate to the PAS (Radić *et al.*, 1991; Shafferman *et al.*, 1992b). Several proposals have been made as to the mechanism underlying substrate inhibition including conformational change (Barak *et al.*, 1995) and steric hindrance (Szegletes *et al.*, 1998; Rosenberry *et al.*, 1999; Szegletes *et al.*, 1999).



Figure 1.12 Molecular representation of AChE based on the x-ray crystallographic structure by Sussman *et al.* (1991)

This is a monomeric unit, which has been cut in half so it is possible to observe the active site. Courtesy of Dr. S. Emmett.

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1.3.4 Non-classical actions of AChE

The possibility that AChE may possess functions other than the hydrolysis of ACh was first proposed by Silver in 1974. Evidence for this postulation has since grown, initially from observations that AChE is not confined to tissues that participate in cholinergic transmission. This is illustrated by the disparity in distribution of AChE and the synthesising enzyme for ACh, choline acetyltransferase (ChAT) in certain rat brain structures, including the hypothalamus (Satoh *et al.*, 1983), raphé nucleus (Satoh *et al.*, 1983), cingulate cortex (Lysakowski *et al.*, 1989) and SN (Henderson and Greenfield 1987).

The second piece of evidence suggesting a non-hydrolytic function of AChE was the discovery of a soluble, secreted form of the enzyme in cisterna magna cerebrospinal fluid (CSF) following stimulation of the ox adrenal gland (Chubb and Smith, 1975a). Later studies by Greenfield and Smith (1979) showed that following electrical stimulation of certain neuronal structures, including the caudate nucleus, hypothalamus, and SN, there was an increase in CSF AChE levels. Subsequently, the release of AChE has been examined from a number of brain regions, including the SNpc. AChE release can be evoked from the SNpc by potassium (K^+) (Greenfield *et al.*, 1983) and may be independent of cholinergic receptor stimulation (Weston and Greenfield, 1986). AChE secretion is a calcium-dependent mechanism (Llinás and Greenfield, 1987), suggesting synaptic terminals as the source of release. It is thought that secreted AChE may underlie a novel form of neuronal signalling, involving an action independent of ACh hydrolysis (Taylor and Greenfield, 1989, Greenfield, 1995), and evidence for this postulation arises from behavioural and electrophysiological studies.

1.3.4.1 Non-cholinergic actions of AChE

1.3.4.1.1 Behavioural studies

A number of behavioural experiments have examined the direct effects of AChE on the SNpc. Bilateral infusions of AChE, but not BuChE into the SNpc induced stereotypical behaviours such as chewing and sniffing (Weston and Greenfield, 1985) which are characteristic of dopamine release in the striatum (Parker *et al.*, 1993). Furthermore, studies using the rotational model of dopaminergic functioning (Ungerstedt and Arbuthnott, 1970; Ungerstedt, 1971) have revealed long term excitatory effects of AChE on the nigrostriatal pathway (Hawkins and Greenfield, 1992a and 1992b; Hawkins and Greenfield, 1995). Based

upon current knowledge of the neurochemical architecture of the SNpc with respect to the other basal ganglia structures, the excitatory actions of AChE are likely to be independent of cholinergic activity. However, Hawkins and Greenfield (1996) reported that AChE infusion into the posterior SNpc evoked contralateral rotation, while anterior infusions resulted in ipsilateral circling. This is suggestive of different populations of cells having different sensitivities to AChE. It is important to consider, however, that these studies utilised a number of different molecular forms of the enzyme, from unpurified eel AChE to mouse recombinant AChE and this disparity may account for some of the variations seen.

1.3.4.1.2 Electrophysiological studies

Various electrophysiological approaches have been utilised to examine the direct effects of AChE on neuronal cell populations. In cerebellar cortex slices, application of AChE enhanced the synaptic response of Purkinje cells to excitatory amino acids and to climbing fibre stimulation. This effect was independent of catalytic activity since pre-treatment of AChE with the irreversible AChE inhibitor 1,2,2-trimethyl-propyl-methylphophonofluoridate (soman) did not abolish the enhancement, and BuChE had no effect (Appleyard and Jahnsen, 1992). In the SN, AChE has been shown to cause hyperpolarisation of neurons located in the rostral SNpc (Webb *et al.*, 1996). However, these AChE-sensitive neurons are unlikely to have been dopaminergic, as they possessed the same physiological characteristics as medial STN neurons (Overton *et al.*, 1995).

1.3.4.1.3 A developmental role for cholinesterases

A role in neuronal development has also been ascribed to non-cholinergic AChE and BuChE. 'Developmental cholinesterase' was a phrase first coined by Drews (1975) to describe the

high expression of cholinesterase in migratory cells of vertebrates. Several lines of evidence now support a non-cholinergic role for BuChE, and AChE in neuro-developmental processes (see Layer and Willbold, 1994 and 1995). These include the transient expression of cholinesterases in the developing brain before the onset of synaptogenesis, and the variation in expression of different molecular forms of cholinesterases during development.

(i) Transient expression of cholinesterases

Alterations in, or even disappearance of, the expression of AChE and BuChE during the course of development is well documented. Transient expression of AChE has been described in ferret retina (Hutchins *et al.*, 1995), rat, monkey and human cortex (Robertson *et al.*, 1985; Robertson *et al.*, 1988; Hanes *et al.*, 1992; Barone *et al.*, 1994) and the thalamus (Robertson *et al.*, 1989; Brimijoin and Hammond 1996). BuChE expression in the developing avian nervous system has been shown to be transient and to spatially foreshadow AChE expression (Layer 1983; Layer *et al.*, 1987; Layer *et al.*, 1988; Layer 1990; Willbold and Layer, 1992).

It has been proposed that 'embryonic' AChE has non-cholinergic actions, since ChAT expression patterns are dissimilar, and lesions that inhibit AChE in projection fields do not change ChAT levels (Robertson and Yu, 1993). AChE is also present in several areas before the ingrowth of extrinsic afferents (Drews, 1975; Layer 1983). A developmental pattern of AChE reactivity has also been reported in the SNpc and striatum using histochemical visualisation techniques (Butcher and Hodge, 1976). The maximum intensity of cell body and dendrite staining of cells of the SNpc was found between postnatal day six and fifteen in the rat. Thereafter the staining intensity decreases to produce the more diffuse adult pattern

of AChE reactivity. The same study illustrated that AChE immunoreactivity in the striatum during the same developmental period was located within 'islands'. This observation was later confirmed when Graybiel established that AChE labelling corresponded to patch-matrix- innervation from the SNpc (Graybiel, 1984).

(ii) Developmental forms of cholinesterases

Developmental studies have illustrated that a temporal shift occurs in the expression of soluble, globular AChE from G_1 and G_2 forms to the higher molecular weight tetrameric form G_4. This has been shown to occur in many species including the chick (Layer *et al.*, 1987), mouse (Inestrosa *et al.*, 1994), rat (Rieger and Vigny, 1976; Muller *et al.*, 1985) and human (Arendt *et al.*, 1992). A study by Layer *et al.* (1987) also demonstrated a similar shift in the expression of BuChE molecular forms in embryonic chick retina.

(iii) Developmental functions of cholinesterases

The coincidence of BuChE expression with the end of cell proliferation and the start of cell differentiation in the embryonic brain has been proposed as evidence for a role of BuChE in development (Layer, 1983; Layer *et al.*, 1987; Layer and Sporns, 1987; Layer, 1990; Willbold and Layer, 1992). Furthermore, a study by Layer *et al.* (1993) demonstrated that BuChE promotes neurite outgrowth of embryonic chick tectal cells independently of substrate hydrolysis, suggestive of a non-cholinergic role of BuChE in development.

A morphogenic role for AChE in development is supported by evidence from numerous *in vitro* studies, which have examined the correlation between AChE and neurite outgrowth. Layer (1990) demonstrated that neurons in the developing chick spinal cord express AChE

during extension. Moreover, Gupta and Bigbee (1992) observed different levels of AChE expression by rat dorsal root ganglia grown on substrates that promoted or hindered neurite outgrowth. A study by Brimijoin and Hammond (1996) correlated increased AChE activity with axonal outgrowth in postnatal rat thalamic neurons. Transfection of murine neuroblastoma cells with murine AChE cDNA increased neurite length and the percentage of neurite-bearing neurons (Koenigsberger *et al.*, 1997). Addition of recombinant forms of G_4- and G_1-AChE increased outgrowth of postnatal dopaminergic cells in mesencephalon organotypic cultures (Holmes *et al.*, 1997). Furthermore, antisense suppression of AChE in PC12 cells resulted in impaired neurite outgrowth upon NGF-mediated differentiation (Grifman *et al.*, 1998), and transfection of murine neuroblastoma cells with antisense AChE cDNA resulted in a reduction in neurite outgrowth (Koenigsberger *et al.*, 1997). Addition of an inhibitor of the active site and the PAS of AChE (1,5-bis-(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51) resulted in retardation of neuritic outgrowth and neuronal migration of dorsal root ganglion neurons (Dupree and Bigbee, 1994), retardation of neurite outgrowth in embryonic chick tectal cells (Layer *et al.*, 1993), reduction in the stimulatory effect of AChE upon neurite outgrowth of *Aplysia* neurons (Srivatsan and Peretz, 1997) and decreased neurite outgrowth and survival of TH+ve cells in postnatal organotypic cultures of the VM (Jones *et al.*, 1995). In all of the studies that utilised BW284c51, cultures were also incubated with phospholine iodide (echothiophate), an inhibitor of the active site of AChE. Addition of echothiophate had no effect upon cell survival or neurite outgrowth in any of the studies described, suggesting that the involvement of AChE in neurite outgrowth is not dependent upon its hydrolytic activity (for review see Grisaru *et al.*, 1999).

The precise mechanisms underlying the effects of non-cholinergic BuChE and AChE upon neurite outgrowth and cell migration are not fully understood. However, the fact that cholinesterases share a high degree of sequence similarity with several cell-adhesion molecules (CAM) has led to proposals that the effects of AChE on neurite outgrowth may be mediated through an adhesion property (Layer and Kaulich 1991, Layer *et al.*, 1993; Sharma and Bigbee, 1998). For example, BuChE and AChE have been found to express the HNK-1 epitope (Bon *et al.*, 1987). This carbohydrate moiety was first described by Abo and Balch (1981) who reported binding of a monoclonal antibody to human natural killer cells, and consequently called the antibody HNK-1. Since this initial discovery, the HNK-1 epitope has been shown to be a common motif of many CAMs (McGarry *et al.*, 1983; Kruse *et al.*, 1984 and 1985; Schachner and Martini, 1995; Voshol *et al.*, 1996). The HNK-1 epitope has subsequently been found on many different forms of AChE (see Massoulié *et al.*, 1993). Weikert and Layer (1994) showed that the HNK-1 epitope is present on inactive BuChE and on active brain BuChE from chick. A histological study by Layer and Kaulich (1991) also illustrated that the HNK-1 epitope is strongly expressed by migrating neural crest cells as they approach their target.

More recently, work has focused on the homology of AChE with known cell-cell adhesion proteins, the neuroligins (Andres *et al.*, 1997; Grifman *et al.*, 1998; Tsigelny *et al.*, 2000). Neuroligins constitute a multigenic family of brain-specific proteins that have been suggested to exert overlapping functions in mediating recognition processes between neurons (Ichtchenko *et al.*, 1996; Song *et al.*, 1999; Scheiffele *et al.*, 2000; for review see Rao *et al.*, 2000). Neuroligins bind to a specific subset of neurexins, which are polymorphic neuronal cell surface proteins believed to play a role in neuronal differentiation and axogenesis

(Ichtchenko *et al.*, 1995; Püschel and Betz, 1995; Nguyen and Südhof, 1997). Both neuroligins and neurexins are transmembrane proteins and possess cytoplasmic PDZ domains (named after the three proteins in which they were first identified: PSD-95, the DLG tumour suppressor protein in *Drosophila*, and a protein termed ZO-1), which mediate protein-protein interactions. As such, the PDZ motifs provide a signalling capacity of neuroligins and neurexins that mediates transduction of growth signals directly into the cell (for review see Cantallops and Cline, 2000). Studies on neuroligin 1, 2 and 3 have revealed an extracellular domain homologous to esterases such as AChE (Ichtchenko *et al.*, 1995 and 1996). Furthermore, a study by Tsigelny *et al.* (2000) uncovered a common EF-hand motif (a motif formed by E and F helices of the protein which are positioned like the forefinger and thumb of the right hand with a connecting loop; Strynadka and James, 1989) in BuChE, AChE and neuroligins. It has been proposed that AChE may replace, or compete with certain types of neuroligins for binding to neurexins, and in doing so, affect intracellular events, with corresponding repercussions on developmental processes such as neurite outgrowth (see Grisaru *et al.*, 1999). Evidence for this theory has been provided by the work of Grifman *et al.* (1998), who demonstrated that overexpression of neuroligin-1 restored both neurite extension and neurexin expression following antisense suppression of AChE in PC12 cells. Furthermore, neurite outgrowth was partially rescued by transfection of cells with catalytically inactive AChE variants, suggesting that this function of AChE does not require the ability to hydrolyse ACh.

A role for cholinesterases in development is emphasised by the study of Greenspan *et al.* (1980), which demonstrated that deletion of the gene encoding AChE in *Drosophila,* which lack BuChE, resulted in embryonic lethality. In contrast to this, a recent study demonstrated

that the targeted deletion of the AChE gene in mice totally eliminated AChE activity, whilst BuChE activity remained normal (Xie *et al.*, 2000). Although nullizygous mice were born alive and lived for up to 21 days, their physical development was impaired; they were nutritionally deficient and developed calorie deficient tremulous movement (Xie *et al.*, 2000; Figure 1.13). The survival of mice that are totally deficient in AChE for any length of time is however, surprising, and suggests that BuChE may be playing a compensatory role. In accordance with this, a study by Li *et al.* (2000) demonstrated that BuChE activity was not altered in tissues of AChE knockout mice, confirming that BuChE can compensate for a deficit in AChE and suggesting a physiological function for BuChE, particularly during development.



Figure 1.13 AChE knock-out mouse.

At 12 days old AChE nullizygous (-/-) mice (A) are caloritically deficient with respect to their wild-type litter mate (B), and die prematurely at 21 days old. From Xie et al. (2000).

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1.4 SUMMARY

This chapter has presented various lines of evidence supporting a non-cholinergic role of AChE and the involvement of non-cholinergic AChE, and BuChE in development. Whilst the actions of BuChE and AChE in adult and postnatal SN have been explored, effects of application of BuChE and AChE to developing embryonic nigral dopaminergic cells have not

been studied. Given the importance of embryonic dopaminergic cells in PD transplantation therapy, and the understanding that AChE is present in the extracellular space of the SN and has a trophic effect on postnatal dopaminergic cells *in vitro*, the question of the effects of AChE upon embryonic dopaminergic cells is a salient one to ask. Therefore, this thesis utilises a preparation of embryonic rat VM cells analogous to the preparation used in the animal model of foetal transplantation in PD to investigate the putative non-cholinergic effects of AChE upon TH+ve cells *in vitro*. The effects of exogenously applied AChE and BuChE, (Chapter 3) and various AChE inhibitors (Chapters 4 and 5) upon dopaminergic cells in culture are initially examined using immunocytochemistry. Chapters 7, 8 and 9 then address the question of the involvement of calcium in the effects of AChE and BuChE upon VM cells using electrophysiology and immunocytochemistry.

CHAPTER 2

Methods

2.1 INTRODUCTION

This chapter is concerned with the principal techniques used in the studies presented in this thesis. It will therefore detail the protocols used for tissue preparation, immunocytochemistry, neuronal survival and outgrowth quantification, and neuronal recording. Variations upon the immunocytochemical techniques required for post-recording neuronal identification are also given. Details of other procedures used are provided in relevant experimental chapters.

All of the experiments described in this thesis used dissociated embryonic cultures of the VM. This preparation is used in the animal model of foetal transplantation as a treatment for PD (Chapter 1, Section 1.2.4.3). Foetal transplantation in humans as a treatment for PD has met with limited success, in part due to degeneration of dopamine cells within the donor preparation. Identification of factors that affect survival, and furthering knowledge of the properties of dopaminergic cells within the *in vitro* embryonic preparation described in this chapter may therefore improve the survival of grafted cells in the animal model of PD, which has implications for foetal transplant surgery in Parkinson's disease patients. The advantages and disadvantages of the dissociated embryonic culture preparation of the VM as a method for studying dopamine cell response to test substances will therefore be discussed.

2.1.1 *In vitro* versus *in vivo* preparations

The anatomical and functional complexity of the central nervous system presents a significant drawback for interpreting data from *in vivo* studies. Furthermore, variables are introduced to *in vivo* studies from experimental procedures performed, such as the interactions of anaesthesia, the accessibility of the test drug to its site of action and drug

metabolism. *In vitro* studies markedly reduce the complexity of the system being studied and by rigorously controlling and manipulating the environment of the cultured tissue, allow the objective and systematic measurement of the effect of factors on cell function and viability. Such advantages of reductionist *in vitro* culture systems also, of course, confer disadvantages. The environment provided for the growing cells does not closely parallel the *in vivo* situation. This is an important consideration when using embryonic or neonatal tissue, which will develop *in vitro*, but not necessarily at the same rate or in the same manner as *in vivo* due to alterations caused by culturing, such as loss of afferent connectivity and changes in release of neurotransmitters. Thus, a degree of caution must be exercised when interpreting findings, particularly when endeavouring to apply a physiological meaning to data obtained *in vitro*. Extrapolation of such data to the *in vivo* situation must be done with care since fundamental characteristics may have changed under *in vitro* conditions.

2.1.2 *In vitro* cell culture systems for the study of CNS development

Historically, culture systems were first prepared to determine whether tissue would survive outside of its natural environment and if so, to what extent the tissue resembled its living counterpart. In the early 1900's Harrison was among the first scientists to succeed in maintaining cells outside of the living organism, and in doing so, determined that tissue samples could survive removal from their natural microenvironment provided they were maintained under appropriate conditions (Harrison, 1907 and 1910). As a result, culture techniques enjoyed a refinement of conditions. Gradually the poorly enriched saline solutions used to maintain cells were reformed, thus lengthening the lifetime of the cultures and bringing about a better understanding of the concept that a living system requires a complex mixture of nutrients to survive and grow. *In vitro* culture techniques have now

become widespread and invaluable research tools, which have allowed exact and highly specific measurement of many fundamental biological processes including cell adhesion and migration, process outgrowth and synapse formation (Gähwiler, 1988; Layer *et al.*, 1993; Schacher *et al.*, 1999). Cultivation of nervous tissue gives access to individual neurons that continue to differentiate and develop *in vitro*. One hundred years after Harrison's first rudimentary experiments with embryonic frog tissue, many cell culture preparations exist and their use depends on the parameter of interest.

2.1.3 Dissociated cultures

Dissociated cultures involve creating a suspension of individual cells following dissociation of an isolated brain region. Cells are then plated, at a known density, onto a suitable surface to which they can adhere and grow, for example, astrocyte or poly-D-lysine-coated coverslips. Many neuronal populations have been studied using this technique including purkinje cells (Brorson *et al.*, 1991; Gillard *et al.*, 1997) and granule cells (Courtney *et al.*, 1990) of the cerebellum, noradrenergic cells of the locus coeruleus (Masuko *et al.*, 1986) and dopaminergic cells of the VM (Sanchez-Ramos *et al.*, 1988; O' Malley *et al.*, 1992; Shimoda *et al.*, 1992; Fawcett *et al.*, 1995; Barker *et al.*, 1996).

The dissociated cell culture system offers the advantage of a rigorously controlled environment, which allows easy addition of test substances and quantification of the effects they may have using biochemical approaches and microscopy. The number of cells present before treatment (the plating density) is known and individual cells are easily visualised; an important consideration for electrophysiology. There are several drawbacks to the technique however. Firstly, preparation of the suspension involves complete destruction of the tissue

matrix. This means that constituent cells will be devoid of their original connectivity, and cell-cell interactions will depend upon the plating density, which has been found to be a crucial factor in cell survival. Engele *et al.* (1991) found that increasing cell density enhanced cell survival in dissociated cultures of embryonic rat mesencephalon cells, and Fawcett *et al.* (1995) showed that a much higher percentage of TH+ve cells died in monolayer VM cultures in comparison to three-dimensional VM cultures where there was a closer association of cells. Another problem with dissociated cultures is the immense trauma placed on the cells during dissection and dissociation. Comparisons of cell counts in intact brain, and in tissue which has been enzymatically dissociated then immediately fixed and stained, shows approximately 30 % of TH+ve cells are lost during the dissociation process (Fawcett *et al.*, 1995). Furthermore this does not account for the loss due to mechanical dissociation, although this has been reported to be modest (Fawcett *et al.*, 1995). The other inherent disadvantage of dissociated cultures is the fact that they are often composed of a heterogenous population of cells. This can complicate studies of one particular cell type, since it becomes necessary to identify the constituent cell populations.

2.1.3.1 Dissociated VM culture

The VM cell suspension used in this thesis was prepared according to the method of Branton *et al.* (1998) using embryonic day 14 (ED14) rat pups. Age was verified by a crown-rump length (CRL) of 13-14 mm (Figure 2.1). This is documented as the optimum time for removal of the VM to maximise the number and survival of TH+ve cells in culture (Shimoda *et al.*, 1992; Dunnett and Björklund, 1997). The neurons of the substantia nigra arise from the ventricular zone and appear to migrate along radial glial tracts to the ventral surface of the developing mesencephalon (Shults *et al.*, 1990) where they then extend axons towards

their forebrain targets (Nakamura *et al.*, 2000). The cells are generated from day 12 to day 15 of gestation with ED13 and 14 corresponding to the peak of neurogenesis (Marchand and Poirier, 1983; Dunnett and Björklund, 1992).



Figure 2.1 Illustration of crown-rump length (CRL) of a rat embryo.

Crown-rump length (CRL) defined as 'the greatest length of the embryo as measured in a straight line (i.e. calliper length) without any attempt to straighten the natural curvature of the specimen'. For some purposes the head length (HL) provides additional precision. From Dunnett and Björklund, (1992).

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Dopamine cells grown in primary dissociated cell cultures have been shown to mature morphologically, biochemically and electrophysiologically; they become capable of dopamine synthesis and release (Mount *et al.*, 1989; Shimoda *et al.*, 1992), respond to a variety of neurotrophic factors (Morrison *et al.*, 1988; Collier *et al.*, 1990; Casper *et al.*, 1991; Lin *et al.*, 1993) and become electrophysiologically competent (Ort *et al.*, 1988).

The cultures in this thesis were maintained for a maximum of 7 days. This culture period was chosen partly due to the documented poor rate of survival of dopamine cells in dissociated cultures beyond this period (Lin *et al.*, 1993; Takeshima *et al.*, 1994), which has been attributed to a lack of trophic factors as well as the problems associated with cell preparation described in Section 2.1.3, above.

As it has been shown by O' Malley *et al.* (1992) that plating of embryonic mesencephalon cells onto a type I astrocyte matrix enhances cell survival, this procedure was adopted in the studies used here. An astrocyte matrix confers the advantage over surfaces such as poly-D-lysine of providing the closest parallel to the *in vivo* situation. As such, it will supply some of the trophic factors that would be secreted *in situ* such as glial cell-line derived neurotrophic factor (GDNF), as well as providing a substratum similar to that *in situ* for adherence of dissociated cells.

The primary purpose of using the dissociated VM culture technique in this thesis was to investigate the effect of various test substances upon developing dopaminergic cell survival and neurite outgrowth. However the area described as the ventral mesencephalon is composed of several brain structures; the STN, pedunculopontine nucleus, SNpr and SNpc, and the ventral tegmental area which, to varying extents, will have been incorporated into the preparations used for these studies. Incorporation of several brain regions thus gives rise to a heterogenous cell population, composed, for the large majority of cells that stain positively for glutamic acid decarboxylase (GAD; the enzyme involved in the synthesis of γ–amino butyric acid; GABA. An antibody against GAD will therefore detect both glutamatergic and GABA-ergic cells; Appendix 2). These cells are derived primarily from the STN (glutamatergic) and SNpr (GABA-ergic). A far smaller percentage of the population will be cholinergic cells (from the pedunculopontine nucleus and tegmental nucleus) and dopaminergic cells (from the SNpc; constituting ~2-6 % of the total cell population; Mayer *et al.*, 1993a; Silani *et al.*, 1994; Castilho *et al.*, 2000). Whilst the presence of the other cell types in this preparation is unavoidable, and indeed is likely to contribute positively to TH+ve cell health, a study by Masuko *et al.* (1992) documented a higher percentage of

TH+ve cells in VM cultures due to a more specific area being dissected. However, the preparation used in the study of Masuko *et al.* (1992) was derived from postnatal substantia nigra. As the experiments described in this thesis are concerned with the preparation used for the animal model of foetal transplantation as a treatment for Parkinson's disease, and factors that may contribute to the survival of developing TH+ve cells within such a preparation, the use of a postnatal TH+ve culture procedure would be of little relevance.

2.1.4 Patch-clamp electrophysiology

The principal technique utilised in the latter chapters of this thesis (Chapters 7 and 8) is that of whole-cell patch-clamp electrophysiology. The patch-clamp technique is a refinement of early work by Neher and colleagues (Neher and Sakmann, 1976; Neher *et al.*, 1978) who pressed a small glass micropipette filled with a salt solution resembling that normally found in extracellular fluid against the membrane of a muscle fiber. A metal electrode in contact with the electrolyte in the pipette connected the pipette to an electrical circuit that measured the current flowing through channels in the membrane under the pipette tip. Such electrically 'loose' measurements of cell-attached patches of frog muscle were used to show acetylcholine-activated channel currents. In 1980 it was discovered that suction applied through the pipette allowed formation of a giga-Ohm seal or 'gigaseal'; an extremely high resistance seal between the pipette glass and the cell membrane (Sigworth and Neher, 1980). The gigaseal ensured that an electrical current flowing through the pipette was identical to the current flowing through the membrane covered by the pipette. Figure 2.2 illustrates the relation of the pipette to the cell and the equivalent electrical circuit during patch-clamp recording.



Figure 2.2 Schematic illustration of the patch-clamp technique.

A indicates the relation of the pipette to the cell. B illustrates the equivalent electrical circuit during patch-clamp recording. The opening of an ion channel is represented as the closing of the switch. The pipette resistance and seal resistance are in series between the amplifier and earth (the extracellular solution) and the patch and cell resistances are in parallel with the seal resistance in this path. Adapted from Magistreti et al. (1996).

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Various types of patch-clamp configurations are possible including excised patches, where the pipette is withdrawn sharply to rupture the membrane within it, resulting in a small patch of membrane attached to the pipette through which the activity of channels within this excised patch can be monitored. Alternatively, the cell-attached method can be used, with whole-cell access gained by rupturing the membrane patch, thus producing a low resistance pathway between the pipette and the interior of the cell, with the cell contents equilibrating over time with the solution within the pipette. The activity of the channels in the entire

plasma membrane can be monitored using this technique, which was the chosen method for the studies presented in this thesis. A schematic diagram of the whole-cell procedure is given in Figure 2.3



Figure 2.3 Procedure for whole-cell patch clamp.

The pipette is lowered onto the cell (a), a high-resistance seal is formed by between the pipette and a small patch of membrane by suction (b), and the membrane patch is broken by increased suction (c).

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In the experiments presented in Chapters 7 and 8 of this thesis, following whole-cell access, the cell was voltage clamped. Hodgkin and Huxley used this procedure in the early 1950s in a series of experiments that revealed the ionic mechanisms underlying the action potential. The basic function of the voltage clamp is to interrupt the interaction between the membrane potential and the opening and closing of voltage-dependent ion channels, which would otherwise make it impossible to achieve a stable membrane potential. This is done by injecting a current into the cell that is equal and opposite to the current flowing through the voltage-dependent membrane channels. The voltage-dependent ion channels continue to open or close in response to changes in membrane potential but the voltage clamp prevents the resultant changes in membrane current from influencing the membrane potential. Thus voltage-clamping permits measurement of the effect of changes in membrane potential on the conductance of the membrane to individual ion species. Under physiological conditions,

depolarisation of the clamped cell's membrane would result in opening of sodium, potassium and calcium voltage-dependent channels. However, the purpose of the experiments presented in the Chapters 7 and 8 was to examine the effects of test substances upon only voltage-dependent calcium currents, so solutions used deliberately excluded potassium and sodium.

2.2 EXPERIMENTAL PROCEDURES

A few essential conditions were necessary at all stages of the procedures described in Sections 2.2.1 and 2.2.2 below, including a strict sterile environment. All dissecting equipment was sterilised in an oven for 4 hours at 200 °C and all solutions were passed through a sterile filter (pore size 0.2 μm).

2.2.1 Astrocyte preparation

Type I astrocytes were prepared according to the method of Dr. M. Rumsby (personal communication). Postnatal day 1 rat pups were anaesthetised with halothane in a gassing chamber before being decapitated. Brains were removed and placed onto sterile filter paper. Cerebella were removed and the cerebra were rolled on the filter paper to remove meninges. Cerebra were then chopped on a McIlwain tissue chopper (The Mickle Lab. Engineering Company Ltd., Surrey, UK) to produce slices of 0.5 mm thickness. Tissue was then rotated through 90° and chopped again. The resultant tissue was then placed into 1 ml of media (see Appendix 1) warmed to 37 °C for each brain and dissociated using a sterile glass pipette before being centrifuged for 6 minutes at 1000 rpm (167 g). The supernatant was discarded and the pellet re-suspended in 1 ml/brain of media and dissociated again. 1 ml of the suspension was then added to 14 mls of media in a 75 cm^2 flask with a vented lid pre-coated with poly-D-lysine (Appendix 1) and incubated at 37 °C in a humidified atmosphere comprising 95 % air: 5 % CO_2 for 7 days.

After 7 days the astrocytes were passaged. Media was removed and the cell layer was washed twice with Hank's balanced salt solution (HBSS; Life Technologies Ltd., Paisley UK). 2 mls of 1 % trypsin were added and flasks were agitated to encourage cell removal.

The suspension was poured into 3 mls of media to prevent further trypsinisation and centrifuged for 6 minutes at 1000 rpm (167 g). The supernatant was discarded and the pellet re-suspended in 1 ml of media and dissociated using a sterile glass pipette. The suspension was then placed into a fresh PDL-coated flask containing 14 mls of media, returned to the incubator and allowed to reach confluency (6-8 days).

Astrocytes flasks were then trypsinised as described above and plated (one confluent flask was used for four 8-well chamberslides or 20 coverslips) onto PDL-coated 8-well chamber slides or PDL-coated 13 mm coverslips and incubated for 6 days to produce a confluent extracellular matrix before addition of the dissociated nigral preparation.

2.2.2 Preparation and maintenance of dissociated ventral mesencephalic (VM) cultures

Cultures were prepared according to the method of Branton *et al.* (1998). Time-mated female Wistar rats at gestational day 14 were obtained from Harlan UK Ltd. (Oxford) and kept under standard conditions (12 hr light/dark cycle with food and water *ad libitum*). Rats were anaesthetised intraperitoneally using terminal doses of pentobarbitone (200 mg/ml, dose 140 mg/kg) and decapitated once the animal was no longer responsive to noxious stimulus (a foot pinch). Uterine horns were removed after abdominal labarotomy, and collected in sterile HBSS. The region of the ventral mesencephalon containing the substantia nigra was removed and stored in 10 mls HBSS containing 0.05 % deoxyribonuclease (DNAse) (HBSS/DNAse) on ice. A schematic diagram of the dissection procedure is shown in Figure 2.4.

Pooled explants were then incubated in 10 mls trypsin/DNAse (0.1 % trypsin, 0.05 % DNAse in HBSS) for 20 minutes at 37 °C. Excess trypsin was then removed and the explants were rinsed with 10 mls 0.1 % soyabean trypsin inhibitor in HBSS/DNAse at 37 °C prior to rinsing 3 x in HBSS/DNAse. Explants were dissociated gently by trituration in 1 ml of HBSS/DNAse using 1000 µl and 100 µl pipette tips. Viability was estimated using the trypan blue exclusion method and cell densities calculated. Cells were then plated at a density of 2.5 x 10^4 cells/ml onto either 8-well chamber-slides for outgrowth and survival experiments, or 13 mm diameter coverslips placed inside sterile 28 mm diameter petri dishes (Life Technologies Ltd., Paisley, UK) for electrophysiology, on which a confluent layer of astrocytes had been grown (Section 2.2.1 above). Cultures were placed in the incubator at 37 °C, 95 % air: 5 % CO_2 humidified atmosphere for two hours. After 2 hours the surplus media was gently removed from the cultures, leaving cells adhered to the astrocyte matrix, and fresh media, containing treatments where appropriate, was added to the cultures (300 µl/well for chamber-slides and 2 mls/coverslip) and they were returned to the incubator. Every 48 hours, 150 µl of media was removed from each culture and replaced with the same amount of fresh media containing, where appropriate, the test substance. Cultures used to assess the effects of test substances on cell health were maintained in culture for 1 day (24 hours), 3 days (72 hours) or 7 days (168 hours). Cultures were used for electrophysiological purposes throughout the 7 day period.

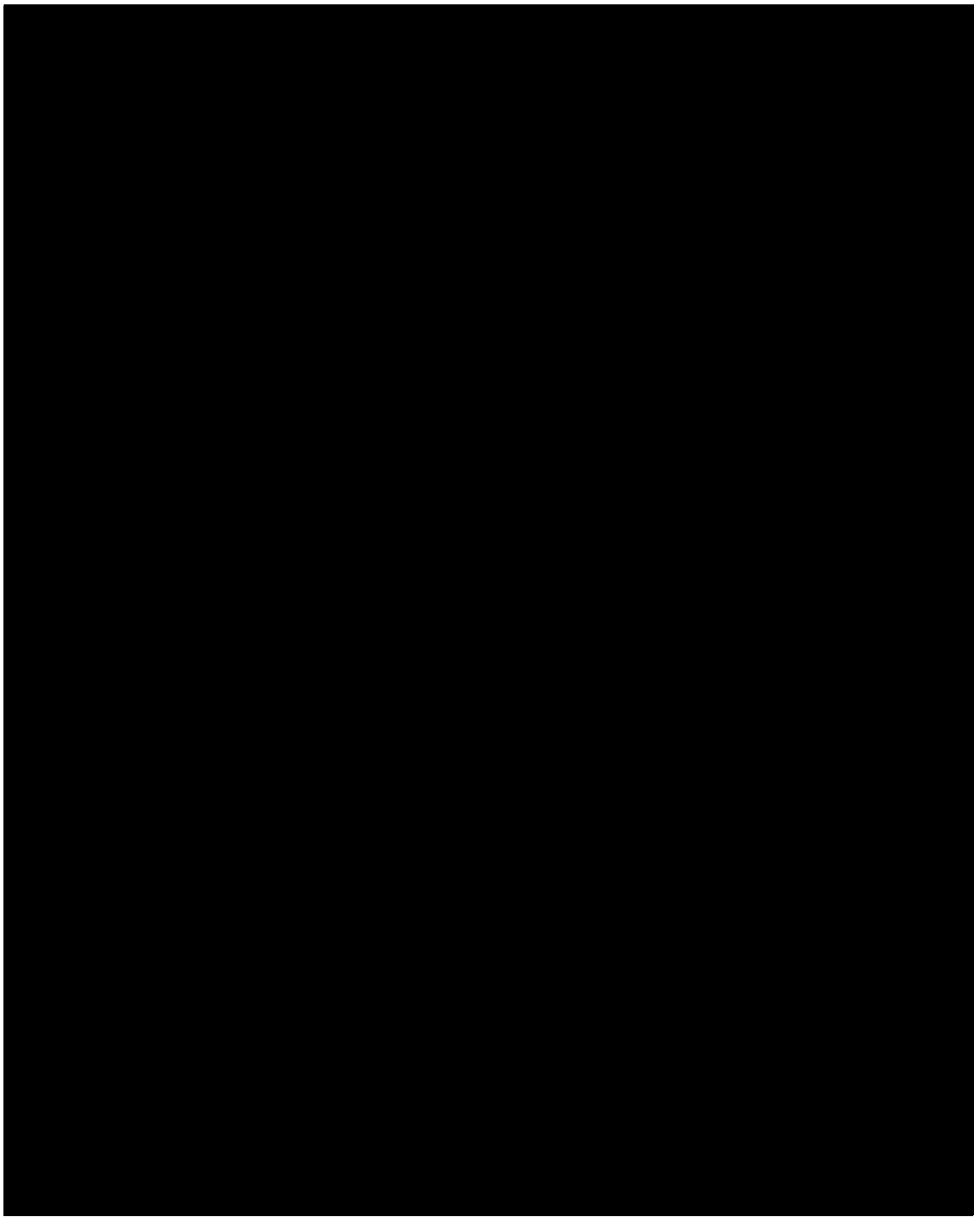

Figure 2.4 Dissection procedure for removal of the ventral mesencephalon.

A, *Removal of the brain from an E14 rat embryo* ***B,*** *Location of developing substantia nigra (sn) with respect to noradrenaline cells of the locus coeruleus (lc), serotonin cells of the pontine (pr), and medullary (mr) raphé nuclei in an E14 embryo (based on Olson and Seiger).* ***C****, Dissection of substantia nigra from the ventral mesencephalon (**a**) as viewed from the ventrolateral perspective and (**b**) the lateral perspective, showing the angle of the first two cuts of the dissection, with imaginary lines (arrows) continued to intersect the dorsal surface of the tectum and the thalamus. Abbreviations; m mesencephalic flexure; sn, ventral mesencephalon including the substantia nigra; tc, tectum; th, thalamus. From Dunnett and Björklund, (1992).*

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2.2.3 Immunocytochemistry

On completion of the incubation period cultures used to assess the effects of test substances on neurite outgrowth and cell survival were fixed and processed for TH immunoreactivity by the biotin-avidin peroxidase method. TH is the rate-limiting enzyme in catecholamine synthesis (Appendix 2) and will therefore be present in adrenergic, noradrenergic, and dopaminergic neurons (see Hornykiewicz, 1966). However, no adrenergic neurons have been detected in the mesencephalon using an antibody to the noradrenaline/adrenaline synthesising enzyme dopamine-β-hydroxylase (Hartman *et al.*, 1972), thus it is widely accepted that TH+ve is a reliable marker of dopaminergic neurons within the VM.

Cultures were rinsed with warmed phosphate buffered saline (PBS), and fixed with 4 % paraformaldehyde (PFA) for 60 mins at room temperature. Cells were then washed twice in PBS. Non-specific binding sites were blocked by incubation with 10 % goat serum in PBS for 60 mins at room temperature. Cultures were incubated with monoclonal anti-TH antibody raised in mouse (Chemicon International Ltd., UK) (1:1000 dilution in PBS), overnight at 4 °C. Cultures were then washed for 3 x 10 mins in PBS and biotinylated goat-anti-mouse secondary antibody (Novocastra laboratories Ltd., UK) (1:300 dilution in PBS) was applied for two hours at room temperature. Following 3 x 10 min washes with PBS, a solution of 10 % methanol and 0.3 % hydrogen peroxide in PBS was used to quench any endogenous peroxidase activity (10 min at room temperature). Following further washes cultures were incubated for 60 mins at room temperature with avidin-biotin complex (ABC elite kit, Novocastra laboratories Ltd., UK). Cultures were then washed for 3 x 10 mins with tris buffered saline (TBS; Appendix 1). The complex formed between the mouse monoclonal antibody for TH, the anti-mouse secondary antibody, and the avidin-biotin compound was

visualised using a diaminobenzidine (DAB) chromagen with 0.015 % hydrogen peroxide in TBS. Stained cultures were dehydrated by placing in successively increasing ethanol concentrations (50 %, 75 %, 90 %, 95 % and 100 %) for 2 mins followed by immersion in xylene for 8 mins. Cultures were then mounted in Depex (BDH/Merck Chemcials Ltd., Warwickshire, UK). A schematic representation of this method is given in Figure 2.5.

Fix (4 % PFA) 1 hour at room temperature

↓

Block non-specific binding (10 % goat serum in PBS)

↓

Incubate in primary antibody 24 hours at 4 °C

↓

Incubate in secondary antibody 2 hours at room temperature

↓

Quench endogenous peroxidases (0.3 % hydrogen peroxide, 10 % methanol in PBS)

↓

Incubate in ABC reagent, 60 minute at room temperature

↓*

Develop (0.05 % DAB, 0.015 % hydrogen peroxide in TBS)

↓*

Dehydrate by washing cultures in ethanol from 50 % to 100 %, 2 minutes each. Followed by 8 minutes in xylene and mount using Depex

Figure 2.5 Schematic representation outlining the immunocytochemical staining procedure for the identification of TH+ve cells in dissociated embryonic ventral mesencephalon cultures.

Fixed cultures were processed using the avidin-biotin method using a monoclonal antibody directed towards tyrosine hydroxylase (TH). Each arrow (↓) represents three ten-minute washes in PBS, where PBS = Phosphate buffered saline. (↓) represents three ten-minute washes in TBS, where TBS = Tris buffered saline*

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2.2.4 Assessment of survival and growth of TH+ve neurons

For experimental Chapters 3, 4, 5 and 9 assessments of cell survival and outgrowth of TH+ve neurons within dissociated cultures were required. Quantification of cell survival and

outgrowth are commonly used for assessing the effect of a substance upon the development of cells in culture (Holmes *et al.*, 1995, 1997).

2.2.4.1 Number of cells

The number of TH+ve cells surviving in each culture was determined by counting the total number of cells per culture well of the chamber-slide for each treatment at each time point using a light microscope. For all experiments involving treatment of cultures with test substances, data was normalised to the untreated control.

2.2.4.2 Neurite length

Camera Lucida drawings were made of every TH+ve cell in the chamber-slide well for each treatment group at each time point. Length of each cell's longest neurite was assessed (Figure 2.6) using a sports route planner and results for each treatment group were normalised to the untreated control.

Experiments involving exposure of cultures to test substances were repeated a minimum of three times using foetuses from different mothers. Each treatment group contained a minimum of 4 chamber wells, giving a total of at least 12 cultures for each treatment group ($n \geq 12$). For both parameters data were collected in a blind fashion to prevent sampling bias. For both parameters, values for each data group were expressed as mean ± standard error of the mean (s.e.m.) The distribution of the control population was examined, and statistical significance was assessed using analysis of variance (ANOVA) for normally distributed data followed by a Dunnett's multiple comparison t-test, or the Kruskal-Wallis non-parametric test for non-normally distributed data.

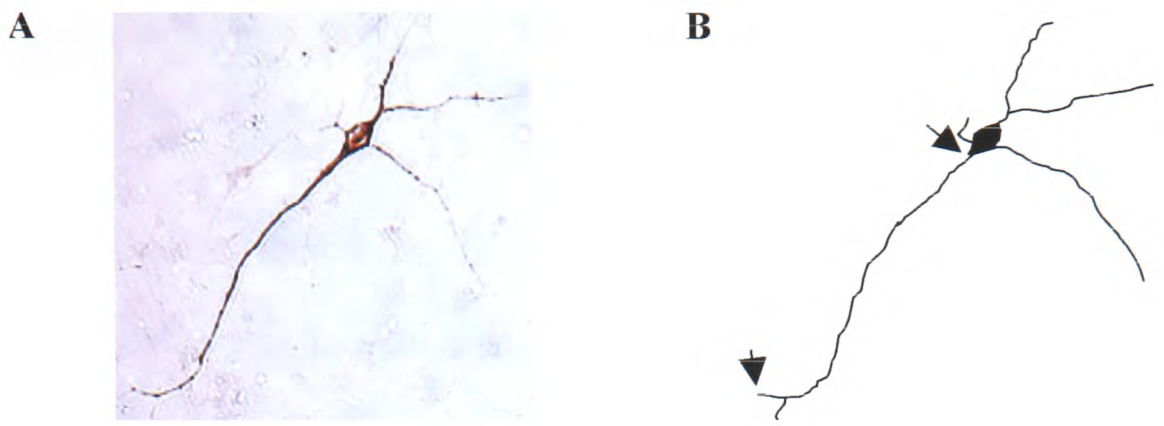

Figure 2.6 Measuring neurite outgrowth of a TH+ve cell from a dissociated embryonic culture of the VM.

(A) A single cell from a VM culture that has been immunocytochemically processed for TH. (B) Camera Lucida drawings were made of each cell. The length of the longest neurite, as indicated by the arrows, was measured

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2.2.5 ELECTROPHYSIOLOGY

Voltage-clamp studies of whole-cell calcium currents in dissociated embryonic VM cells were studied using whole-cell patch-clamp electrophysiology. Cultures were grown on 13 mm astrocyte-covered coverslips and used from day 1 *in vitro.*

2.2.5.1 Recording chamber

Coverslips were placed into the recording chamber, which consisted of a perspex well (approximate volume 1.5 ml) which had a glass coverslip base. Cultures were fully submerged and perfused with extracellular solution at a rate of 2.6 mls/min. Perfusate entered the bath at one end and was suction drained at the other.

2.2.5.2 Extracellular perfusate

The extracellular solution used was that of Gillard *et al.* (1997). The solution contained (in mM): tetraethylammonium chloride (TEACl), 143; Barium chloride ($BaCl_2$), 5; Magnesium

Chloride ($MgCl_2$), 1; N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), 10; glucose, 10, and was adjusted to pH 7.4 with tetraethylammonium hydroxide. The osmolarity was adjusted to 315 mOsm/kg with TEACl. Barium (Ba^{2+}) was used as the charge carrying ion due to the calcium-dependent inactivation of calcium currents effect often documented with the use of calcium (Ca^{2+}) as the charge carrier (Zhang *et al.*, 1993; Catterall, 2000).

2.2.5.3 Pipette fabrication

Pipettes were made from glass microelectrodes; – borosilicate glass 1.0 mm outer diameter, 0.58 mm internal diameter with an internal capillary (Harvard Apparatus Ltd., Kent, UK). Pipettes were pulled on a Sutter Instruments P-87, Flaming/Brown horizontal puller fitted with a trough filament (Sutter Instruments Inc., USA) using a four step pull. This consistently gave resistances of 2-4 MΩ. Pipette tips were suction filled and then backfilled with intracellular solution using 'Microfil' needles (World Precision Instruments, USA).

2.2.5.4 Intracellular solution

The intracellular solution used to fill the patch pipettes was that of Gillard *et al.* (1997). The solution consisted of (in mM): Caesium chloride (CsCl), 135; $MgCl_2$, 1; HEPES, 10; triphosphocreatinine, 14; MgATP, 3.6 and 50 µl/ml creatinine phosphokinase, adjusted to pH 7.1 with caesium hydroxide (CsOH), and 290 mOsm/kg with CsCl. This solution was frozen in 1 ml aliquots. A fresh aliquot was used for each day's recordings. The aliquot was filtered using 0.2 µm pore size millipore filter and 0.2 % biocytin (Sigma-Aldrich Company Ltd. Poole, UK) was added to enable labelling of the cell for identification post recording.

2.2.5.5 Electrophysiology set-up

The set-up consisted of an Axopatch 200A whole-cell patch-clamp amplifier (Axon Instruments, USA) connected to a CED 1401 (Cambridge Electronics, Cambridge, UK). These were run by a Dell XPS personal computer using Strathclyde Electrophysiology Whole-Cell Program Software (Dempster, UK). Traces were visualised both on the computer and on a digital recording oscilloscope (Gould Inc. UK). The amplifier was used to provide continuous current and voltage commands, whilst the computer was used to generate command pulses. Currents were filtered by a four-pole, low-pass Bessel filter and were stored on hard disk for subsequent analysis.

2.2.5.6 Patching a cell

Individual cells in the culture placed into the recording chamber were visualised with x 10 and x 40 magnification objectives on a Zeiss Axioscope-2 fixed-stage light microscope (Carl Zeiss Ltd., UK) aided by differential interference contrast (DIC) optics.

A glass pipette was inserted into the pipette holder, which was controlled using a manual Narishige micromanipulator (model MX-1) that allowed fine movements in the Y and Z planes. A cell was chosen for patching and the pipette was lowered into the bath and located near the cell whilst gentle positive pressure was applied to the end of the pipette to prevent the accumulation of debris. The reference potential for all measurements was the zero-current potential of the pipette before establishment of the seal. No correction was made for junctional potentials between bath and the electrode solutions, which were < 5 mV. A regular 5 mV test pulse was switched on (the 'seal-test command'). The electrode was lowered onto the cell surface with contact detectable by a distinct increase in membrane

resistance and a change in the baseline current, at which point the pipette pressure was released, and gentle suction applied. When the resistance had increased to $\geq 10^9\ \Omega$ (a giga-Ohm seal) suction was stopped and capacitance transients associated with the bath and pipette compensated for. The cell was then ruptured using small positive current pulses and suction, and was detectable by the sudden appearance of large capacitance transients. The observed sequence of changes in resistance whilst forming a seal (A), and capacity transients when rupturing the cell (B), are illustrated in Figure 2.7. Upon whole-cell access the cell was voltage-clamped at a holding potential of –60 mV. Series resistance and cell capacitance were determined by analysing instantaneous membrane responses to 5 mV, 30 msec voltage steps. A 'step-test' of nine, 100 msec depolarisation steps of increasing 10 mV increments (-60 mV to –50 mV; -60 mV to –40 mV etc. through to –60 to +30 mV) was then used to establish the current-voltage (IV) relationship for each cell. Cells that displayed poor voltage-clamp control were not used. Cells were then depolarised to a test potential of 0 mV for a duration of 100 msec every 30 sec. After stable whole-cell currents were obtained, test substances (as detailed in Chapters 7 and 8) were delivered to the recording chamber in extracellular solution via a three-way tap system. Complete exchange of the bath solution occurred in about 1.5 mins; figures have not been corrected for this delay. Reductions in current size due to test substance were measured as a percentage of the control current.

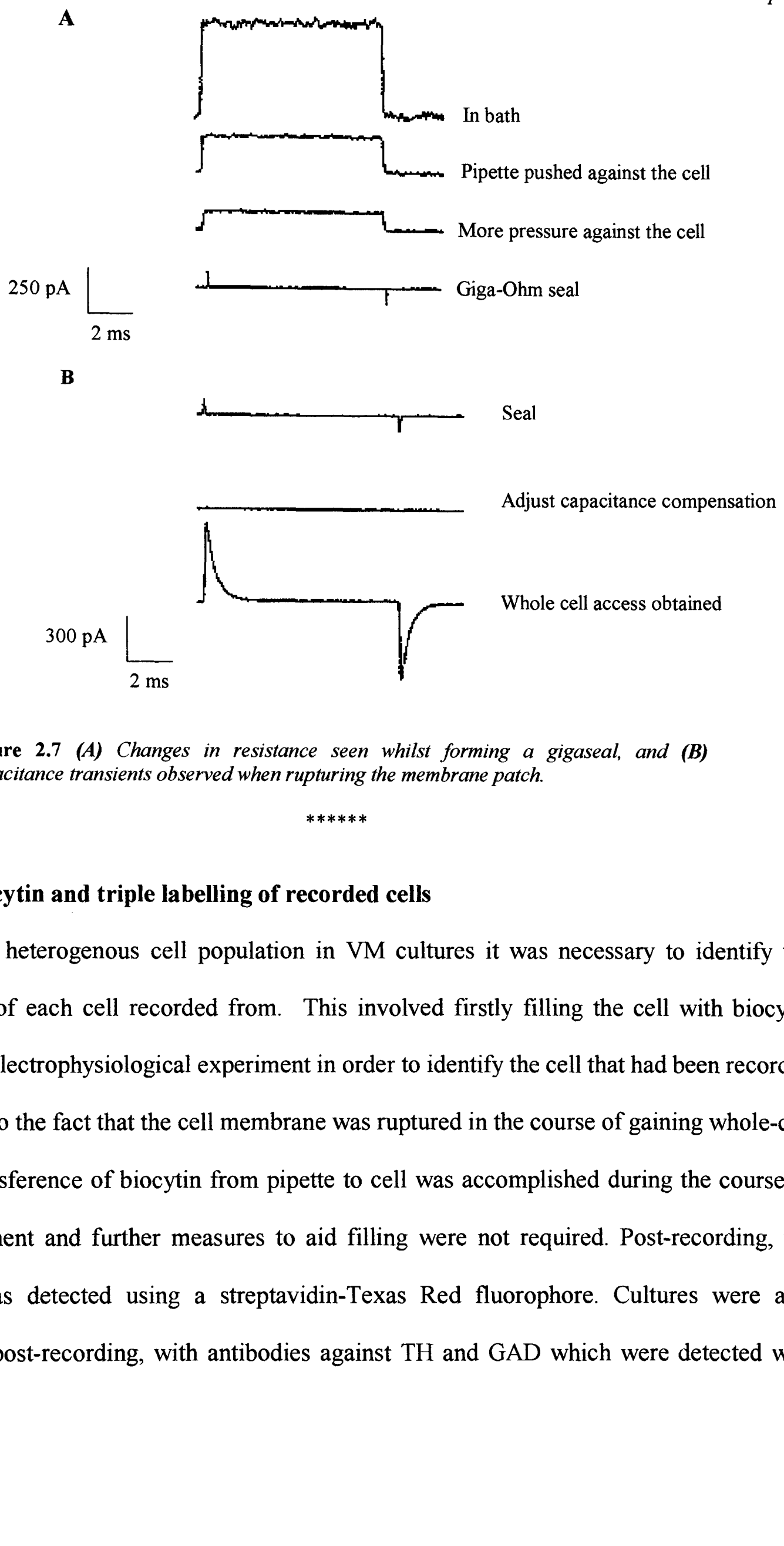

Figure 2.7 *(A) Changes in resistance seen whilst forming a gigaseal, and (B) capacitance transients observed when rupturing the membrane patch.*

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2.2.5.7 Biocytin and triple labelling of recorded cells

Due to the heterogenous cell population in VM cultures it was necessary to identify the phenotype of each cell recorded from. This involved firstly filling the cell with biocytin during the electrophysiological experiment in order to identify the cell that had been recorded from. Due to the fact that the cell membrane was ruptured in the course of gaining whole-cell access, transference of biocytin from pipette to cell was accomplished during the course of the experiment and further measures to aid filling were not required. Post-recording, the biocytin was detected using a streptavidin-Texas Red fluorophore. Cultures were also incubated, post-recording, with antibodies against TH and GAD which were detected with

fluorescein (FITC) and Indodicarbocyanine (Cy5) fluorophores respectively in order to identify the phenotype of the cell that had been recorded from. One cell only per culture was recorded from. Details of the immunocytochemical procedures required to fluorescent label the cell recorded from are provided below.

Following recording the pipette was gently withdrawn and the coverslip removed from the perfusion chamber and fixed by placing into PFA for 60 mins at room temperature as for biotinylated labelling of TH+ve cells in Section 2.2.3. Following fixation, coverslips were washed for 3 x 10 mins with PBS. Streptavidin–Texas Red (Amersham Pharmacia Biotech. Ltd., UK) was then added at a concentration of 1:100 overnight at 4 °C in order to label the biocytin-filled cell with a Texas Red fluorophore. Coverslips were then washed for 3 x 30 mins in PBS and 20 % donkey serum (Jackson Immunoresearch Laboratories Inc., USA) in PBS was applied for 120 mins to block any non-specific binding sites. Monoclonal anti-TH antibody raised in mouse, and polyclonal anti-GAD antibody raised in rabbit (both from Chemicon International Ltd., UK, both at 1:1000 dilution in 1 % donkey serum in PBS) were then co-applied overnight at 4 °C. Coverslips were then washed with PBS for 3 x 30 mins and FITC-conjugated donkey-anti-mouse and Cy5-conjugated donkey anti-rabbit secondary antibodies (both from Jackson Immunoresearch Laboratories Inc., USA; both 1:100 dilution in 1 % donkey serum in PBS) were applied. Table 2.1 illustrates the wavelength of excitation for the antibodies used. Coverslips were then washed for 3 x 30 mins, dipped once in distilled water and inverted into Vectashield (Novocastra laboratories Ltd., UK). After excess fluid had dried, coverslips were sealed with nail varnish and stored in the dark at 4 °C until visualised.

Fluorophore	Wavelength (nm)		Emitted colour
	Excitation	Emission	
Fluorescein (FITC)	492	510	Green
Texas Red (TR)	596	620	Red
Indodicarbocyanine (Cy5)	650	670	Orange

Table 2.1 Peak wavelengths of excitation and emission for fluorophore-conjugated antibodies used in this study

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2.2.5.8 Detection of triple labelling

Labelled cells from each electrophysiology experiment were examined using a Zeiss Axioplan 1 microscope (Carl Zeiss Ltd., UK), with a Zeiss 02 DAPI filter set (Texas Red, 10; Cy5, 15; FITC, 25; Carl Zeiss Ltd., UK). A Kodak Spot 2 CCD Camera and Kodak Image Capture software were used to record images.

2.3 SUMMARY

The purpose of this thesis is to investigate the putative trophic, non-cholinergic effects of AChE and BuChE, and the mechanism of these effects upon developing dopaminergic cells. The experiments described in the following chapters aimed to provide further information about the potential of AChE to act as a trophic factor for grafted cells in the animal model of foetal transplantation in Parkinson's Disease. Therefore, the use of a preparation similar to that used for grafting was necessary, hence the method of Branton *et al.* (1998) was utilised to provide an embryonic dissociated culture containing TH+ve cells. It was also decided to plate the embryonic cells onto an astrocyte matrix (Figure 2.8), as this had been documented as improving neuronal cell survival (O' Malley *et al.*, 1992) and would provide a more analogous situation to that of cells grafted into the brain than using a PDL substratum. The

main disadvantage of the dissociated VM culture was that it comprised a heterogenous population where only 2-5 % of the total cell number per culture were dopaminergic, with the vast majority of the remainder staining positively for GAD (Figure 2.9). The heterogeneity did not present a problem for studies using biotinylated antibodies to identify TH+ve cells, as the stained TH+ve cells and their neurites were very apparent from the unstained population (Figure 2.10). However, it meant that every cell recorded from in an electrophysiology experiment had to be triple fluorescent labelled post recording (as described in Section 2.2.5.7 above), as it was not possible to definitively identify the cell phenotype otherwise (Chapters 7 and 8).

Thus, the following experimental chapters utilise a preparation of embryonic rat VM cells analogous to the preparation used in the animal model of foetal transplantation in PD to investigate the putative non-cholinergic effects of AChE, and BuChE on TH+ve cells *in vitro*. The effects of exogenously applied AChE and BuChE (Chapter 3) and cholinesterase inhibitors (Chapters 4 and 5) upon dopaminergic cells in culture are initially examined using immunocytochemistry. Chapters 7, 8 and 9 then address the question of the involvement of calcium in the effects of AChE upon VM cells using electrophysiology and immunocytochemistry.

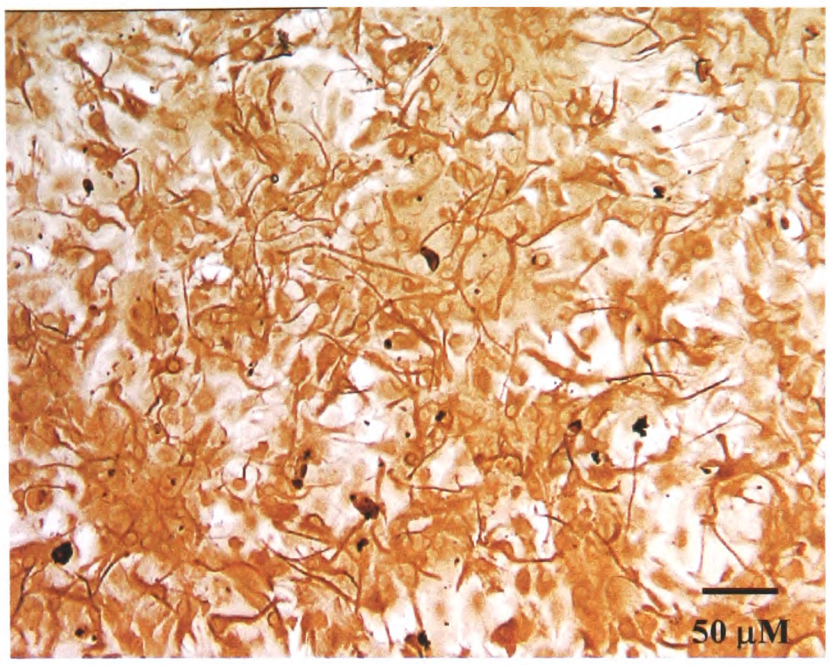

Figure 2.8 Confluent type-I astrocyte culture

Prepared according to the method of Dr. M. Rusmby (personal communication). Stained with glial-fibrillary acidic protein (GFAP).

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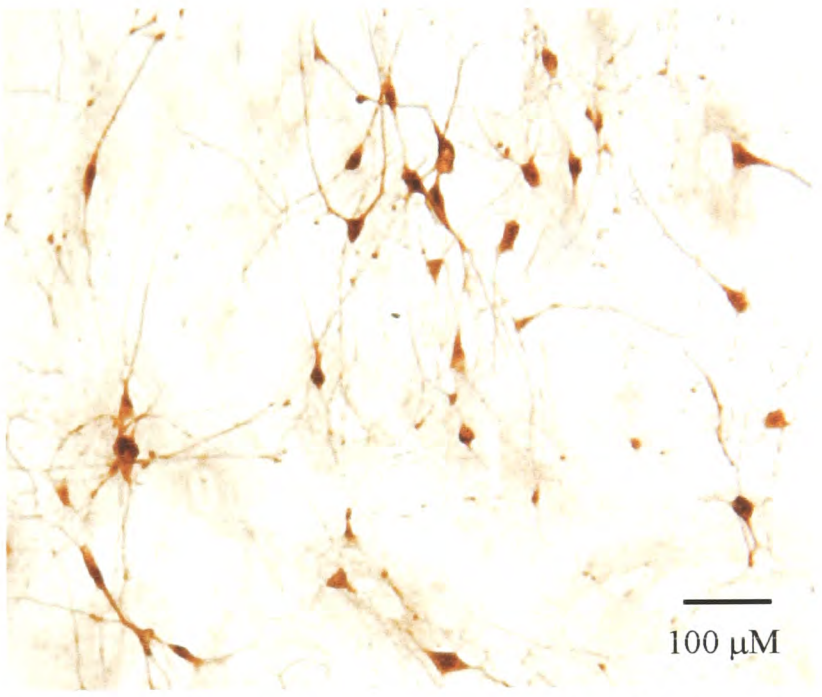

Figure 2.9 GAD+ve cells in an embryonic dissociated culture of the ventral mesencephalon at day 3 *in vitro*.

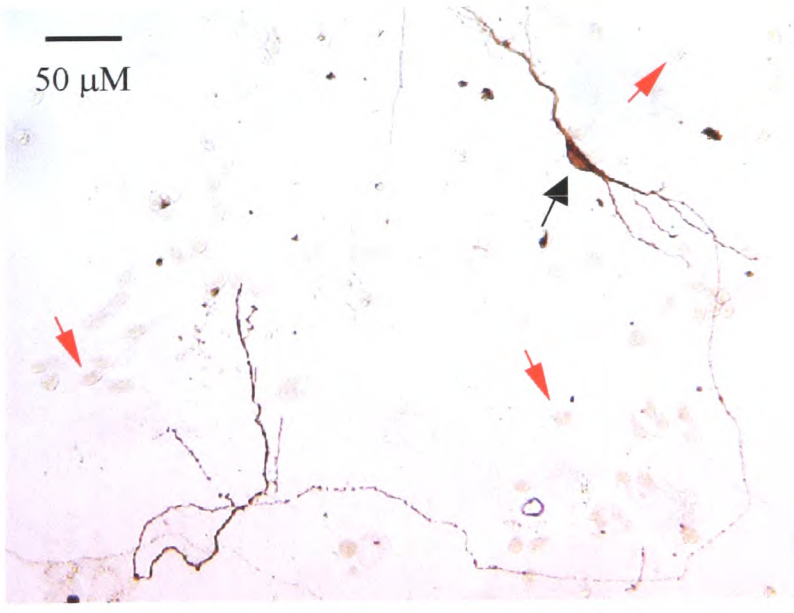

Figure 2.10 TH+ve cell labelled by immunocytochemistry, with unlabelled non-dopaminergic cells visible.

↓ *Indicates a TH+ve cell*

↓ *Indicates an unlabelled cell*

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CHAPTER 3

The effect of AChE and controls on survival and outgrowth of embryonic dopaminergic cells in dissociated cultures of the VM

3.1 INTRODUCTION

The proposal that AChE may be capable of performing functions other than hydrolysis of ACh was first made by Silver in 1974. Since then, evidence for the idea has grown. Reasons for suggesting a non-cholinergic function of AChE are numerous (Chapter 1 Section 1.3.4) and include its distribution (Satoh *et al.*, 1983; Henderson and Greenfield, 1987; Lysakowski *et al.*, 1989), polymorphism (see Massoulié *et al.*, 1993) transient expression during development (Layer, 1983; Robertson *et al.*, 1985, 1988 and 1989; Hanes *et al.*, 1992; Barone *et al.*, 1994; Hutchins *et al.*, 1995; Brimijoin and Hammond 1996), and the identification of a soluble, secreted form (Chubb and Smith, 1975b; Chubb *et al.*, 1976). Furthermore, AChE, acting though a non-cholinergic mechanism, has been shown by several studies to be involved in neurite outgrowth (for review see Grisaru *et al.*, 1999). Of particular relevance to the work presented in this thesis are the studies by Jones *et al.* (1995) and Holmes *et al.* (1997), which demonstrate that recombinant forms of G_4- and G_1-AChE, again acting through a non-cholinergic mechanism, are trophic to postnatal dopaminergic cells in mesencephalic organotypic cultures.

The effects of AChE upon embryonic dopaminergic cells have not, however, been examined. Such an investigation is particularly relevant, as implantation of embryonic VM cells is used as a treatment for Parkinson's disease (Chapter 1, Section 1.2.4.3). It is known that the survival rate of implanted cells is poor, and a factor that is trophic to embryonic dopamine cells would be of considerable importance. Thus, the aim of the experiments described in this chapter is to investigate the putative non-cholinergic trophic actions of G_4- and G_1-AChE upon the survival and neurite outgrowth of embryonic dopaminergic neurons in dissociated cultures of the VM.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Dissociated cultures of the VM: preparation and staining

Suspensions of ventral mesencephalon cells from E14 rats were prepared as described in Chapter 2 (Section 2.2.2). Cultures were chronically treated for 1, 3 or 7 days with media supplemented with molecular forms of cholinesterase or relevant controls. Every 48 hours 150 µl of media was removed and replaced with 150 µl of fresh media containing, where appropriate, the test substance. Cultures were fixed and processed for TH immunoreactivity as described in Chapter 2 (Section 2.2.3) at days 1, 3 and 7 *in vitro.*

3.2.2 Measuring cholinesterase activity in the cell culture system

3.2.2.1 Measurement of total cholinesterase activity using the method of Ellman *et al.* (1961) (The 'Ellman Assay')

The Ellman assay involves incubating test samples with acetylthiocholine and 5,5`-dithio-bis(2-nitrobenzoic acid) (DTNB, Sigma-Aldrich Company Ltd. Poole, UK). Any cholinesterase present in the sample will cleave the acetylthiocholine, yielding acetate and thiocholine. Thiocholine reacts with DTNB to produce the yellow anion of 5-thio-2-nitro-benzoic acid, which can be detected by spectrophotometry (Figure 3.1). The procedure for this assay was as follows; aliquots of 25 µl of each sample were added to individual wells of a 96 well microtitre plate. 175 µl of Ellman reagent (Appendix 1) was added to each well. Absorbance was read at 405 nm over a 10 minute time span using a Molecular Devices plate reader (Alpha Laboratories Ltd., Hampshire, UK). Multiplication of the Vmax value for each sample by 1.026 and any dilution factor gives a value for cholinesterase activity in mU/ml, as illustrated in Figure 3.2. For each assay, plate blanks containing Hanks balanced salt

solution (HBSS, Life Technologies Ltd., Paisley UK) were used to control for the colour of the phenol red within the culture medium.

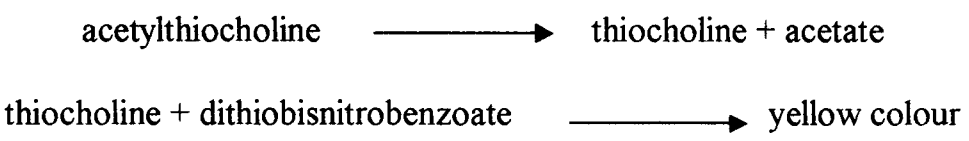

Figure 3.1 Reactions that are utilised in the Ellman assay to quantify cholinesterase activity

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$$V_{MAX} \text{ x } 1.026 \text{ x dilution factor} = \text{mU/ml}$$

Figure 3.2 Ellman Assay Equation

Where one unit of cholinesterase hydrolyse 1 μM of ACh per minute.

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3.2.2.2 Use of cholinesterase inhibitors to identify the activity of the total cholinesterase sample attributable to either BuChE or AChE

BuChE inhibitor tetra-isopropyl pyrophosphoramide (iso-OMPA) and AChE inhibitor, 1,5-bis(4-allydimethylammoniumphenyl)-pentan-3-one dibromide (BW284c51) were used as tools to identify the proportions of the total cholinesterase activity of the media tested that were attributable to AChE and BuChE.

Iso-OMPA selectively and irreversibly inhibits BuChE by binding to the active site (Masson *et al.*, 1997). BW284c51 is a bisquarternary ammonium compound that specifically inhibits AChE by spanning the catalytic and peripheral anionic sites of the enzyme, thus restricting substrate entry into the catalytic gorge and its hydrolysis at the active site. BW284c51 has been used in Chapters 4 and 5 of this thesis as a tool to investigate the mechanism of action of AChE upon developing dopamine cells, and the compound will be discussed in greater

detail in Chapter 4. To determine whether the cholinesterase present in media samples was AChE or BuChE, the activity of the samples was measured in the presence of 100 μM iso-OMPA (to inhibit BuChE) or 100 μM BW284c51 (to inhibit AChE).

Cholinesterase inhibitors and the Ellman assay were used to determine (i) the cholinesterase levels present in untreated culture medium. (ii) The change in AChE and BuChE activity in samples of culture medium incubated at 37 °C for 7 days, following addition of 3 U/ml BuChE or AChE at day 0.

3.2.3 Purification of cholinesterase

3.2.3.1 Soluble tetrameric AChE (G_4)

The purification of G_4-AChE was carried out by Dr. Jane Loughlin (Department of Pharmacology, University of Oxford), from a commercial stock of electric eel (*Electrophorus electricus*) AChE (Sigma-Aldrich Company Ltd. Poole, UK, type V-S, 1000 U/ml) using procainamide-ECH sepharose affinity chromatography (Giles, 1994; Appendix 3). The washed column was loaded with AChE in PBS at 12 ml per hour in a cold room and left to recycle overnight. The column was then washed with [A 0.0] and [A 0.4] (Appendix 3) and AChE was eluted with 400 mM tetraethylammonium (TEA) and 10 mM decamethonium in [A 0.0]. Eluted fractions were assessed for cholinesterase activity by the Ellman assay, followed by protein determination using the Lowry method (Appendix 3). The purity of fragments was assessed using gel filtration with ultraviolet (UV) detection (SMART^{TM} machine, Amersham Pharmacia Biotech. Ltd., UK; sepharose G25 PD10 column) (Figure 3.3A).

3.2.3.2 Truncated (T548) monomeric AChE (G_1-AChE)

3.2.3.2.a Preparation and maintenance of the G_1 producing HEK cell line

Mouse recombinant AChE was kindly provided by Professor Palmer Taylor (Department of Pharmacology, University of California, San Diego, USA). The enzymes were produced from stable transfections of constructs from the mouse pcDNA-3 clone (personal communication, Dr. Kanter. University of California, USA). All preparations were expressed in a HEK cell line and shipped to Oxford on dry ice. On arrival cells were slowly thawed in a water bath at 37 °C, washed in growth medium (10 % foetal calf serum; Life technologies Ltd., Paisley, UK) in DMEM (Life technologies Ltd., Paisley, UK) with 1 % penicillin/streptomycin solution (Sigma-Aldrich company Ltd, Poole, UK)) and centrifuged at room temperature (5 minutes at 1000 rpm; 167 g). Cells were initially grown in 75 cm^2 sterile tissue culture flasks (37 °C, 5 % CO_2 : 95 % O_2) until 100 % confluent. For harvesting, cells were passaged (1:3) and placed into triple layer harvesting flasks and maintained in growth medium until 100 % confluent. Cells were then washed with PBS and placed in serum-free 'ultraculture' medium (Biowhittaker, USA). Medium was harvested and centrifuged (10 minutes at 3000 rpm; 1499 g) and the resulting supernatant was analysed for cholinergic activity by the Ellman assay.

3.2.3.2.b Purification of truncated (T_{548}) G_1-AChE

To purify G_1-AChE from the harvested medium; a meta inhibitor affinity resin was synthesised (Appendix 3). The affinity resin was added to the harvested medium and stirred for 8 hours at 4 °C. To ensure binding of the AChE in the harvested medium to the resin, an Ellman assay was performed repeatedly until over 80 % binding was achieved. The resin was

loaded onto the column and rinsed with bicarbonate buffer (1 ml/hour). To elute the AChE, decamethonium bromide was loaded on top of the settled resin. Each fraction (40 drops = 1 ml) was measured for AChE content by the Ellman assay and the active fragments subjected to dialysis using a 14-16 KDa cut-off dialysis tubing. Eluted samples were assessed for cholinesterase activity by the Ellman assay, followed by protein determination using the Lowry method (Appendix 3). The purity of fragments was assessed using gel filtration with UV detection (SMART^{TM} machine, Amersham Pharmacia Biotech. Ltd., UK; superose 6 PC/3.2 30 column) (Figure 3.3B).

3.2.3.3 Purification of BuChE

Purification of equine BuChE from a commercial stock (Sigma-Aldrich Company Ltd., Poole, UK, 500 U/ml) was carried out by Dr. Cathy Holmes (Department of Pharmacology, University of Oxford) using procainamide ECH sepharose affinity chromatography, as described in Section 3.2.3.1 of this chapter. The elution buffer used, however, contained 12 mM edrophonium chloride instead of 10mM decamethonium as used in G_4-AChE purification. Eluted BuChE fractions were assessed for cholinesterase activity by the Ellman assay, followed by protein determination using the Lowry method (Appendix 3). The purity of fragments was assessed using gel filtration with UV detection (SMART^{TM} machine, Amersham Pharmacia Biotech. Ltd., UK; sepharose 4B column) (Figure 3.3C).

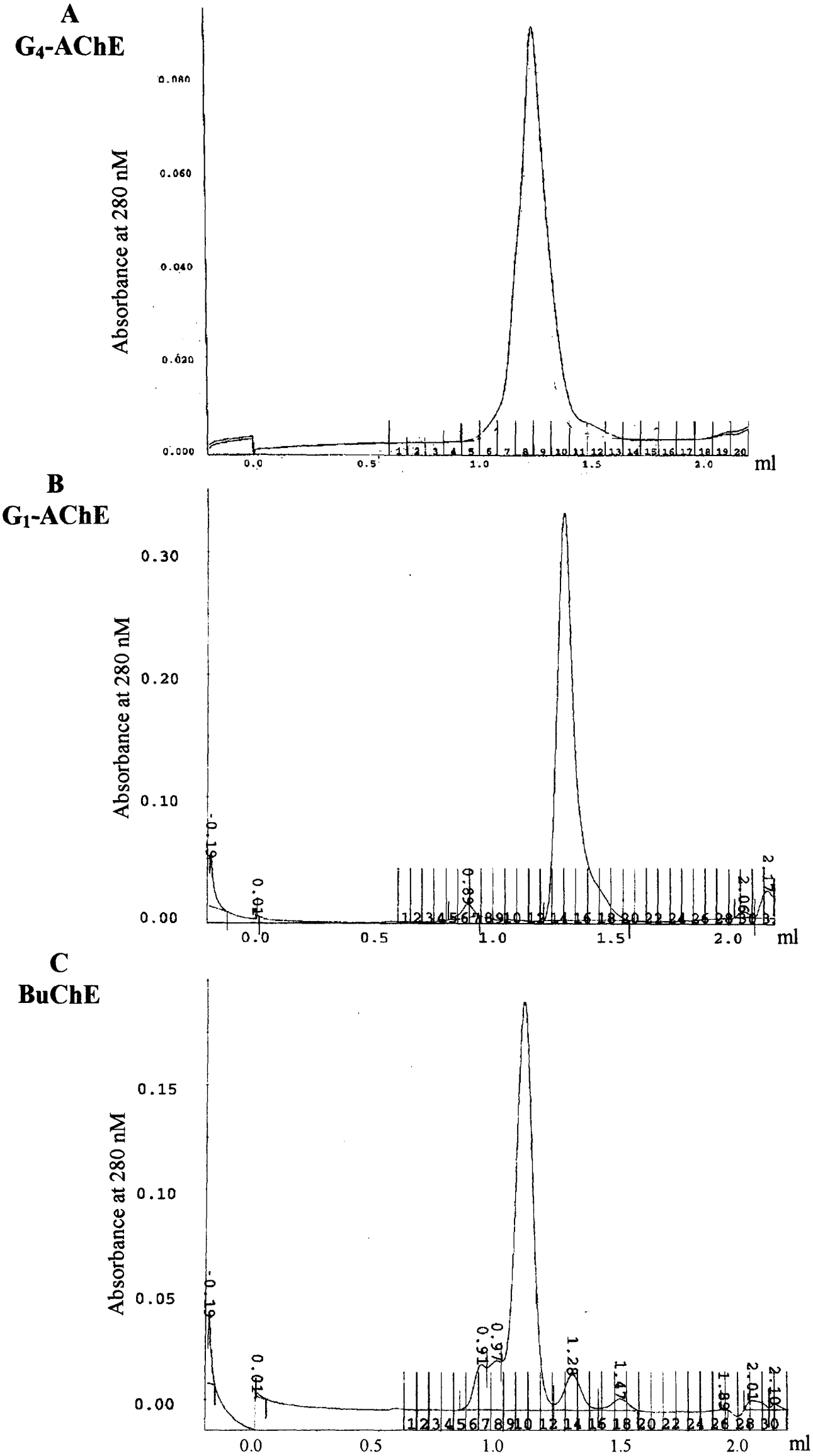

Figure 3. 3 Assessment of cholinesterase purity by gel filtration with UV detection (SMART^{TM} machine)

(A) Electrophorus electricus G_4-AChE (B) T548-mouse recombinant G_1-AChE, (C) Equine BuChE

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3.2.4 Experimental design

Cultures were chronically treated for 1, 3 or 7 days with media supplemented with BuChE, G_4 or G_1-AChE. BuChE served as a control for the hydrolytic actions of AChE. In order to compare any action of AChE against a more conventional growth factor, a set of cultures were incubated with recombinant human bFGF, a polypeptide known to increase survival of dopaminergic cells in culture (Morrison *et al.*, 1988; Silani *et al.*, 1994). To control for the possibility of any action of AChE being a result of non-specific protein, bovine serum albumin (BSA) (Sigma-Aldrich company Ltd, Poole, UK) was applied to another subset of cultures. The concentrations of test substances used are summarised in Table 3.1. Experiments were repeated a minimum of three times using foetuses from different mothers. Each treatment group contained a minimum of 4 chamber wells, giving a total of at least 12 cultures for each treatment group ($n \geq 12$).

Test Substance	Concentration
BuChE	0.3, 3 U/ml
G_4-AChE	0.3, 3 U/ml
G_1-AChE	0.3, 3 U/ml
bFGF	0.01, 0.1 µg/ml
BSA	1 mg/ml

Table 3.1 Concentrations of test substances used to identify the effects of AChE and control substances on neurite outgrowth and cell survival of embryonic dopaminergic cells in dissociated cultures

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3.2.5 Collection of data and statistical analysis

3.2.5.1 Cell numbers

Total cell numbers per culture well were counted for each treatment group at each time point. For all experiments involving treatment of cultures with test substances, data was normalised to the untreated control.

3.2.5.2 Neurite length

Camera Lucida drawings were made of every TH+ve cell in the chamber-slide well for each treatment group at each time point (Chapter 2, Section 2.2.4.2). The length of each cell's longest neurite was assessed using a sports route planner and results for each treatment group were normalised to the untreated control.

For both parameters, cell numbers and neurite length, all data was collected in a blind fashion to prevent sampling bias. For both parameters values for each data group were expressed as mean ± s.e.m. Statistical significance was assessed using either analysis of variance for normally distributed data followed by a Dunnett's multiple comparison t-test, or the Kruskal-Wallis non-parametric test for non-normally distributed data.

3.2.6 Source of test substances

BuChE, G_4- and G_1-AChE were obtained as detailed in Section 3.2.3, above. BSA was obtained from Sigma-Aldrich Company Ltd. (Poole, UK), and bFGF was obtained from Promega Corporation, USA. Stock solutions of BuChE (450 U/ml), G_4- (520 U/ml) and G_1-AChE (480 U/ml) were made in 20% glycerol: 80 % PBS, aliquoted and stored at –80 °C. A stock solution of bFGF (25 μg/ml) was made in distilled water, aliquoted and frozen at –20 °C. Stock solutions of BuChE, G_4- and G_1-AChE and bFGF were diluted in culture medium immediately prior to use. Solutions of BSA (1 mg/ml) were made in culture medium immediately prior to use.

3.3 RESULTS

3.3.1 Activity of cholinesterase in the culture system

The total cholinesterase activity of culture medium was 16.4 ± 0.46 mU/ml (n = 6) (Figure 3.4). Levels of AChE (12.4 mU/ml ± 0.27; n = 6) and BuChE (6.2 mU/ml ± 0.31; n = 6) within the culture medium were obtained by addition of 100 μM iso-OMPA or BW284c51 respectively, and assessed by the Ellman assay (Figure 3.4).

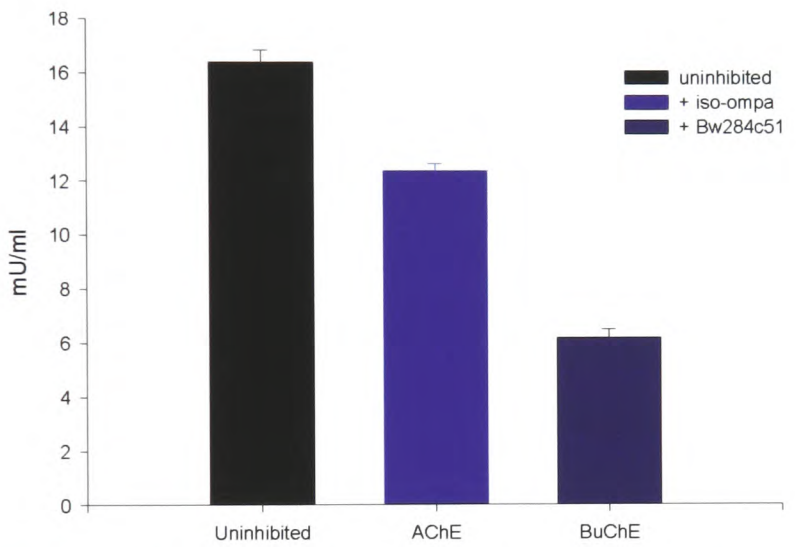

Figure 3.4 Cholinesterase levels in culture medium

Cholinesterase activity was assessed using the Ellman assay. Values shown are mean values ± s.e.m. n = 6

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3.3.2 Changes in cholinesterase activity during incubation

Decay in cholinesterase activity was observed in samples of culture medium incubated at 37 °C with either 3 U/ml AChE or 3 U/ml BuChE over a 7 day time period (Figure 3.5). The decay in AChE activity was 27.7% (from 2.96 U/ml to 2.14 U/ml; n = 6) and the decay in BuChE activity was 31.8%, (from 2.98 U/ml to 2.03 U/ml; n = 6)

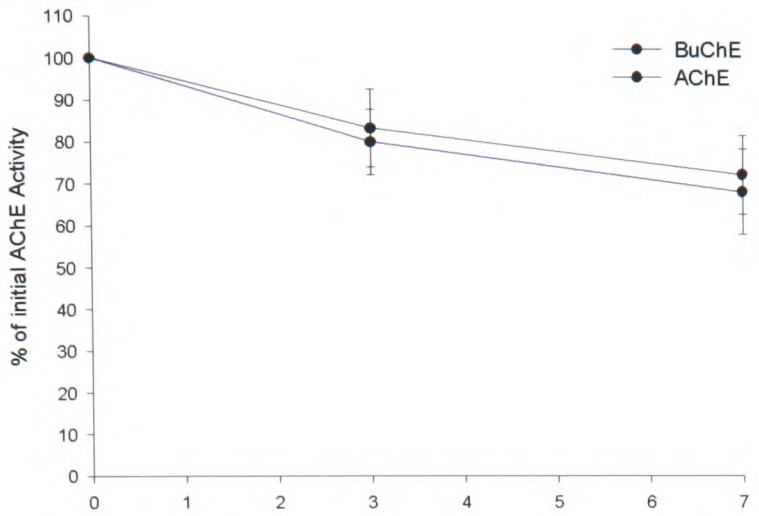

Figure 3.5 The decay in cholinesterase activity after incubation at 37°C

Incubation of AChE and BuChE resulted in decay in enzymatic activity. The graph shows the decay in samples with an initial activity of 3 U/ml of cholinesterase, diluted in culture medium. Activity was assessed using the Ellman assay at days 3 and 7 after incubation. Values shown are mean values ± s.e.m. expressed as a percentage of the original activity. n = 6.

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3.3.3 The effect of cholinesterases and controls on TH+ve neurons in dissociated embryonic cultures of the VM

The data obtained for the effects of treatment on cell survival and neurite outgrowth of dopaminergic cells at days 1, 3 and 7 *in vitro,* normalised to the no treatment control, is shown in Table 3.2

Treatment	Number of TH+ve cells (normalised to control)			Neurite length (normalised to control)		
	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7
Untreated control	1.0 ± 0.17	1.0 ± 0.18	1.0 ± 0.09	1.0 ± 0.05	1.0 ± 0.06	1.0 ± 0.13
0.3U/ml BuChE	0.99 ± 0.21	1.35 ± 0.22	0.98 ± 0.12	1.15 ±0.14	0.92 ± 0.03	1.12 ±0.24
3 U/ml BuChE	1.06 ± 0.12	1.34 ± 0.23	1.08 ± 0.11	1.39 ± 0.11**	0.83 ± 0.04	1.29 ±0.19
0.3U/ml G_4 AChE	1.09 ± 0.17	1.52 ± 0.28	1.22 ± 0.19	1.31 ± 0.11	1.47 ± 0.23**	1.04 ±0.13
3 U/ml G_4 AChE	1.0 ± 0.13	1.54 ± 0.22	1.32 ± 0.17	1.31 ± 0.12	1.54 ± 0.04**	1.58 ± 0.19*
0.3U/ml G_1 AChE	0.85 ± 0.09	0.98 ± 0.11	1.17 ± 0.09	1.68 ± 0.27*	0.57 ± 0.12	1.09 ± 0.22
3 U/ml G_1 AChE	1.0 ± 0.08	0.89 ± 0.1	0.9 ± 0.13	1.79 ± 0.27**	1.5 ± 0.26*	1.16 ±0.18
0.01 µg/ml bFGF	1.12 ± 0.19	1.26 ± 0.18	1.33 ± 0.16	1.49 ± 0.17*	1.34 ±0.19	1.04 ± 0.19
0.1 µg/ml bFGF	1.06 ± 0.14	1.31 ± 0.44	1.35 ± 0.11	1.69 ±0.24**	1.03 0.11	0.88 + 0.07
1 mg/ml BSA	0.84 ± 0.17	0.87 ± 0.12	0.86 ± 0.12	1.06 ± 0.09	1.05 ± 0.1	0.96 ± 0.12

* denotes $P < 0.05$
** denotes $P < 0.01$

Table 3.2 The effect of cholinesterases and controls on TH+ve neurons in dissociated embryonic cultures of the VM

Values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. Statistical significance was tested for using a one-way ANOVA followed by a Dunnett's multiple comparison test. n ≥ 12.

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3.3.3.1 Survival of TH+ve cells

3.3.3.1.a Cell death under control (untreated) conditions

Figure 3.6 illustrates the TH+ve cell death that occurs under control (untreated) conditions over the 7 day culture period. At day 1, the average number of cells per culture was 123.6 ± 12.8, (mean ± s.e.m. n = 14) at day 3 the average number of cells was 83.4 ± 7.6 (mean ± s.e.m. n = 12) which is 67.5% of the initial TH+ve cell number. At day 7 the average number of cells was 69.8 ± 6.4 (mean ± s.e.m. n = 15) which is 56.5% of the initial TH+ve cell number.

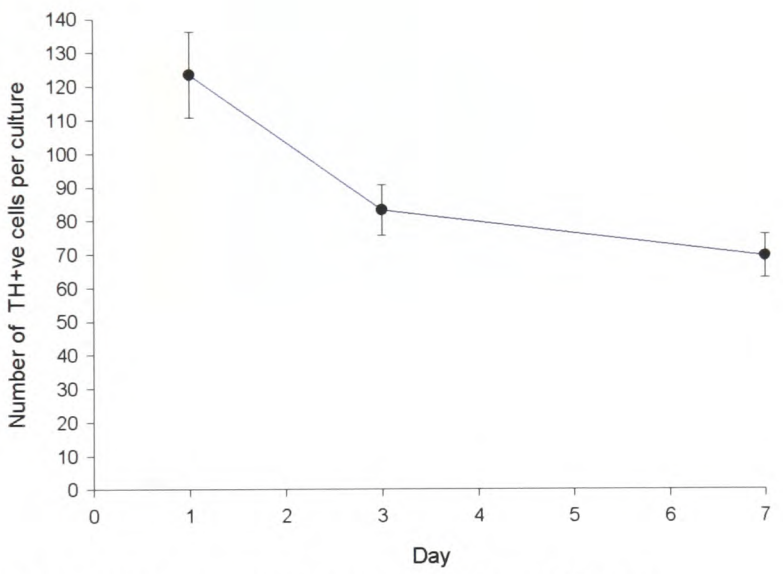

Figure 3.6 Cell death under control (untreated) conditions

This graph shows the decrease in the number of TH+ve cells per culture over the 7 day culture period. Values shown are mean value ± s.e.m. The number of cultures used for each time point was between 12-15.

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3.3.3.1.b Effect of test substances on cell numbers

There were no significant changes in total TH+ve cell number per culture from the untreated control with any of the treatments, at any of the time points (Figure 3.7 A, B and C).

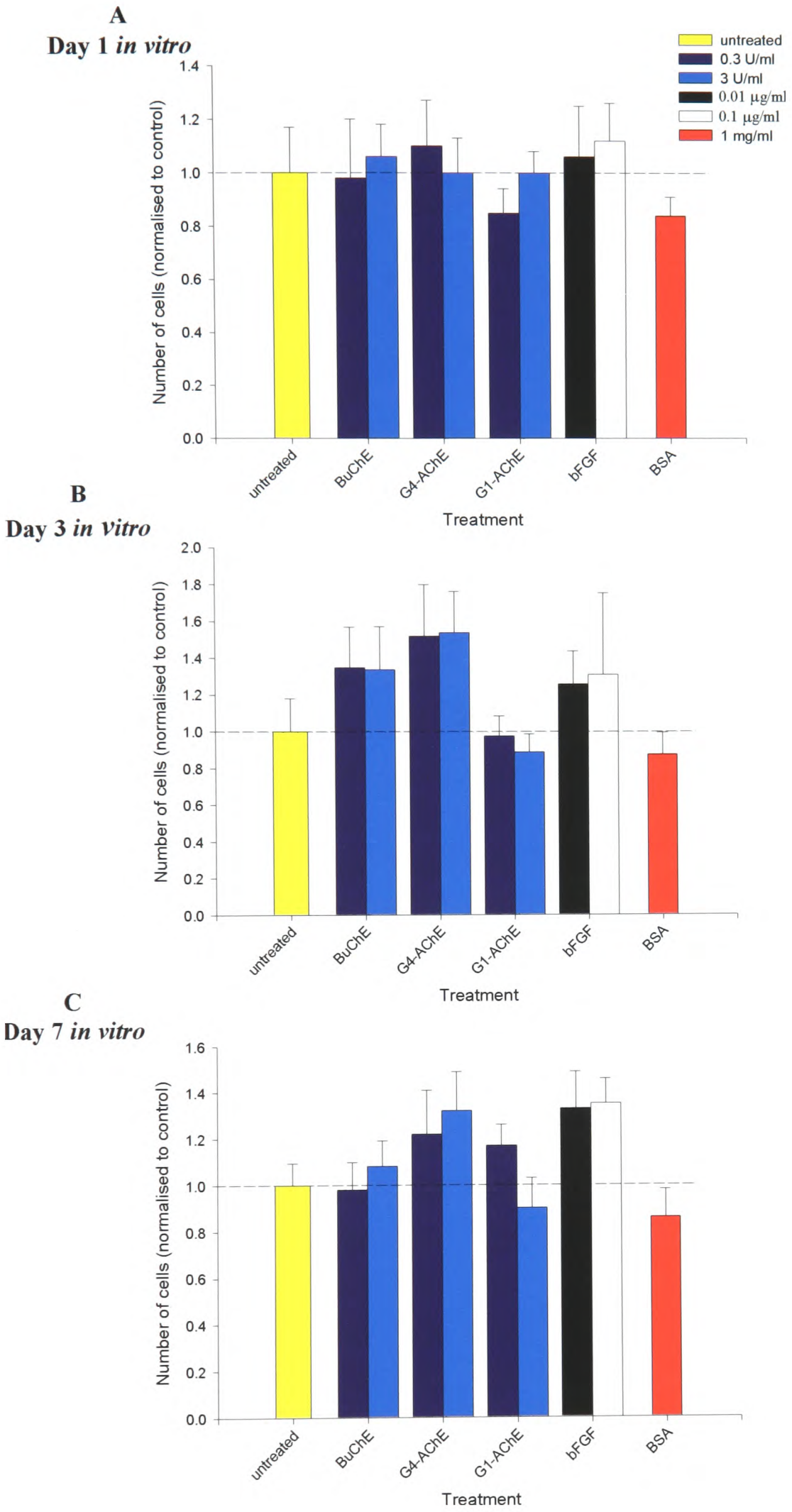


Figure 3.7 Effects of cholinesterases and controls on Th+ve cell numbers

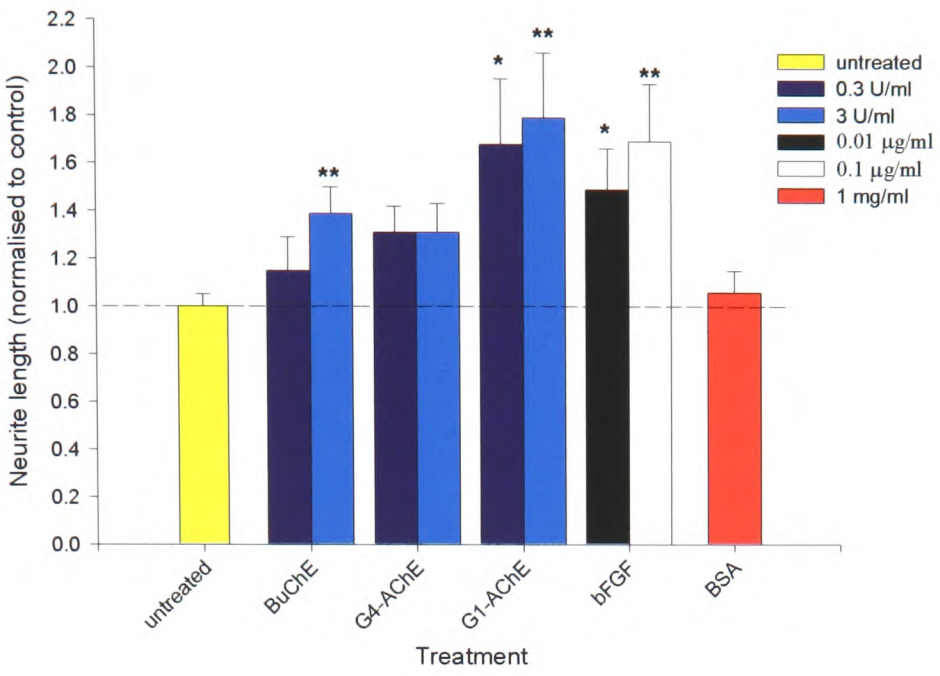
There were no significant changes in total TH+ve cell number per culture from the untreated control with any of the treatment at any of the time points examined. Statistical significance was tested for using a one-way ANOVA followed by a Dunnett's multiple comparison test. Values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. $n \geq 12$.

******

3.3.3.2 Neurite outgrowth of TH+ve cells

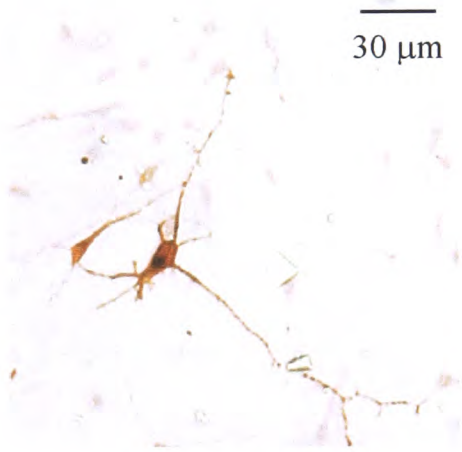
Figure 3.8A illustrates that at day 1 *in vitro* (24 hour exposure to test substance), addition of 3 U/ml of BuChE (protein content 4.98 μg/ml), 0.3 U/ml (protein content 0.13 μg/ml) and 3 U/ml (protein content 1.34 μg/ml) G_1-AChE and 0.01 and 0.1 μg/ml bFGF had significantly increased neurite outgrowth in comparison to the untreated control. Addition of 1 mg/ml BSA had no effect upon neurite outgrowth in comparison to the untreated control. At day 3 *in vitro* (72 hour exposure to test substance) Figure 3.8B, a significant increase in outgrowth compared to the untreated control was seen with 0.3 U/ml (protein content 0.52 μg/ml) and 3 U/ml (protein content 5.2 μg/ml) G_4-AChE and 3 U/ml G_1-AChE. By day 7 *in vitro* (168 hour exposure to test substance) Figure 3.8C, only exposure to 3 U/ml G_4-AChE had enhanced neurite outgrowth in comparison to the untreated control. Photographic representations of the stimulatory effects upon neurite outgrowth of the various treatments are shown alongside relevant figures.

A
Day 1 *in vitro*

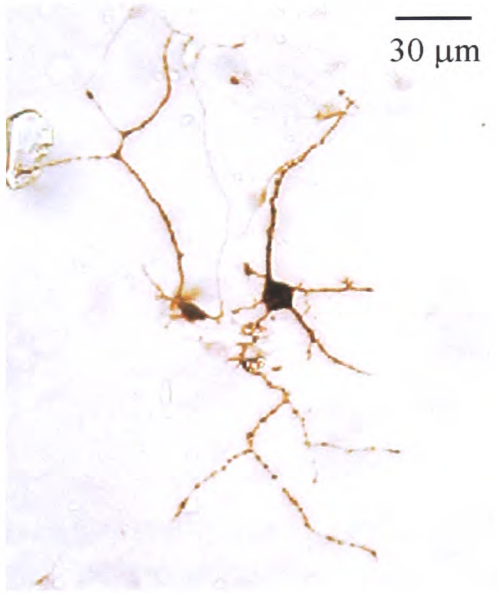




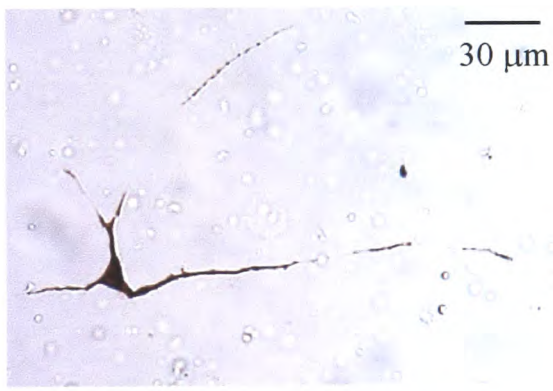
Untreated



3 U/ml BuChE



0.1 µg/ml bFGF



3 U/ml G_1-AChE

Figure 3.8A The effect of cholinesterases and controls on neurite outgrowth of TH+ve cells. Day 1 *in vitro* (24 hour exposure to test substances)

Addition of 3 U/ml of BuChE, 0.3 U/ml and 3 U/ml G_1-AChE and 0.01 and 0.1 µg/ml bFGF had significantly increased neurite outgrowth in comparison to the untreated control (denotes $P < 0.05$, ** denotes $P < 0.01$, Dunnett's multiple comparison test). Graphical values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. $n \geq 12$.*

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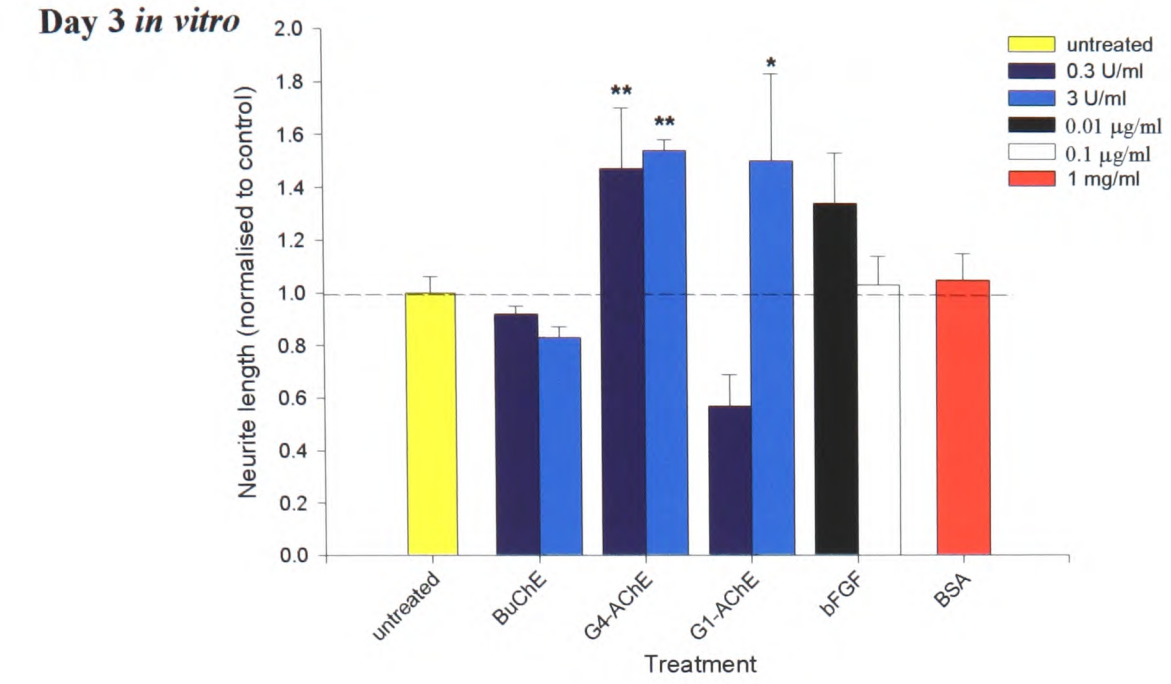

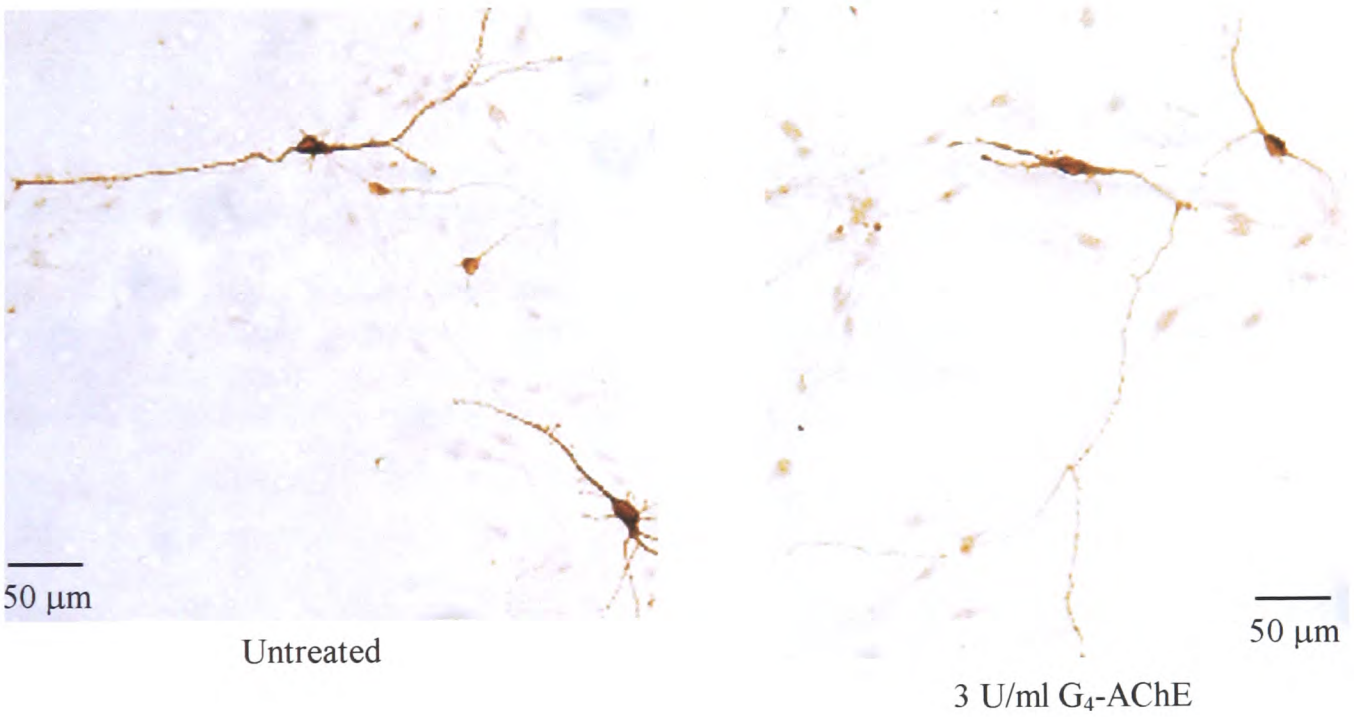

Figure 3.8B The effect of cholinesterases and controls on neurite outgrowth of TH+ve cells. Day 3 *in vitro* (72 hour exposure to test substances)

Addition of G_4-AChE at a concentration of 0.3 and 3 U/ml and G_1-AChE at a concentration of 3 U/ml had significantly increased outgrowth compared to the untreated control (denotes $P < 0.05$, ** denotes $P < 0.01$; Dunnett's multiple comparison test). Addition of BuChE, bFGF or BSA had no effect upon this parameter at this time point. Graphical values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. $n \geq 12$.*

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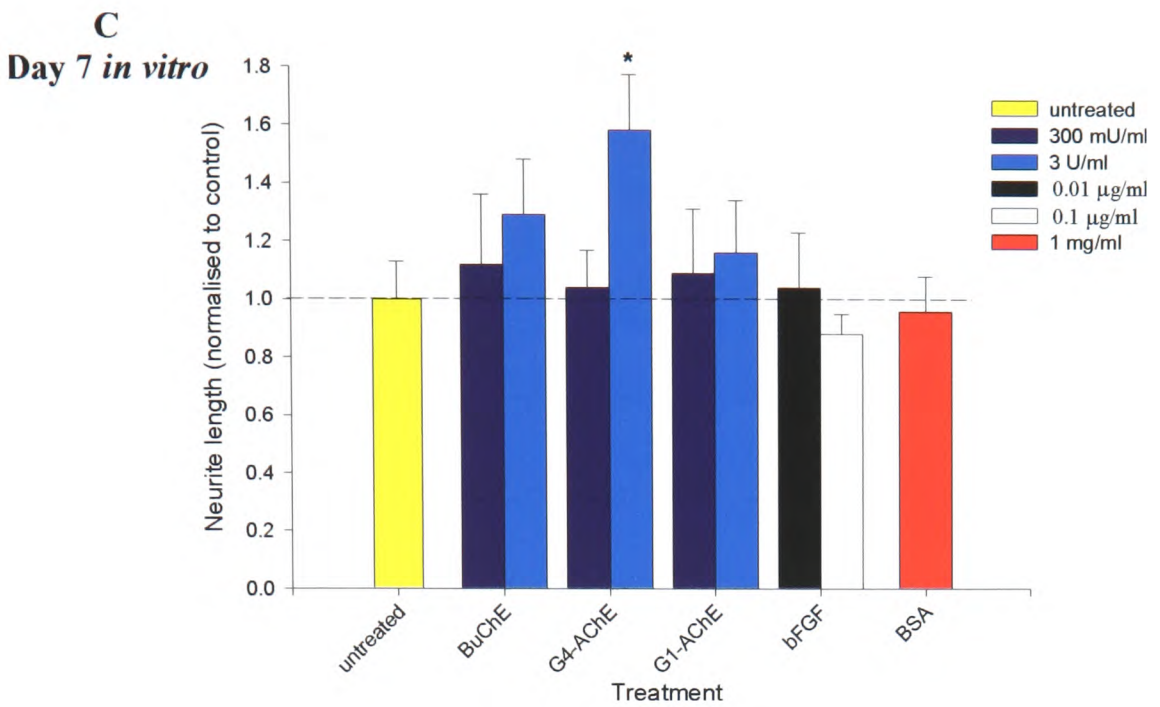

Figure 3.8C The effect of cholinesterases and controls on neurite outgrowth of TH+ve cells. Day 7 *in vitro* (168 hour exposure to test substances)

Addition of 3 U/ml G_4-AChE had significantly increased outgrowth compared to the untreated control (denotes $P < 0.05$; Dunnett's multiple comparison test). Addition of BuChE, G_1-AChE, bFGF or BSA had no effect upon this parameter at this time point. Graphical values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. $n \geq 12$.*

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3.4 DISCUSSION

The aim of the studies in this chapter was to investigate a putative non-cholinergic trophic role for AChE on developing dopamine cells. For this purpose, dissociated embryonic cultures of rat ventral mesencephalon were maintained for up to 7 days under control conditions, or in the presence of AChE or appropriate cholinergic or trophic controls. Two parameters of health, neurite outgrowth and total number of cells per culture were assessed.

3.4.1 Cholinesterases within the culture system

Assessment of the levels of cholinesterase present in culture medium illustrated that some activity was present (16.4 mU/ml ± 0.46), of which most was attributable to AChE (12.4 mU/ml ± 0.27) (Figure 3.4). The likeliest source of this is the foetal calf serum added to the media to improve cell survival (Blair *et al.*, 1999) which has been reported to be high in AChE (Mendelson *et al.*, 1998). However, in comparison to the amounts of BuChE and G_1- and G_4-AChE that the experiments in this chapter have illustrated are required to produce a significant increase in neurite outgrowth, the background levels of cholinesterase present in culture media are negligible.

Analysis of the decay of cholinesterase in culture medium due to incubation at 37 °C revealed that although the activity of both AChE and BuChE did decay, active AChE and BuChE was still present after 7 days incubation (Figure 3.5). Since 150 µl of treated culture medium was replaced every 48 hours, it can be concluded that cells in cultures that were incubated with media supplemented with BuChE or AChE were constantly exposed to cholinesterase.

3.4.2 Survival of TH+ve cells

3.4.2.1 Cell death under control (untreated) conditions

Considerable dopamine cell death occurred under control (untreated) conditions during the 7 day culture period, as illustrated by Figure 3.6. Similar levels of cell death over a 7 day period in dissociated VM cultures have been documented elsewhere (Fawcett *et al.*, 1995; Branton *et al.*, 1998). Cell death within such preparations is of particular interest as this is the method of preparing a suspension for use in foetal transplantation work, both in animal models and in humans. It is obvious that a certain amount of cell death will be attributable to traumatisation during the preparation of the suspension and by the fact that culture conditions are not precisely parallel to *in vivo* conditions (see Chapter 2 Section 2.1.3). It is also true however, that cells die during normal development of the nervous system. Most regions of the central nervous system, including the substantia nigra (Janec and Burke, 1993, Oo and Burke, 1997), undergo a distinct phase during which significant proportions of the constituent cells die and are rapidly removed by the surrounding glial cells (see Linden, 1994). It is thought that such cell death may be due to limited availability of neurotrophic factors within the developing nervous system, as a result of which neurons receiving inadequate amounts of neurotrophic factor die. Several reasons have been postulated for such 'naturally occurring' cell death including the possibilities that it is a mechanism for matching the size of the neuronal population to the magnitude of its target field, or a procedure for eliminating erroneous projections (Cowan *et al.*, 1984; Linden 1994). The phenomenon of naturally occurring cell death will be discussed in greater detail in Chapter 6.

3.4.2.2 Effect of test substances on cell numbers

The number of embryonic dopamine cells per culture was not affected by addition of BuChE, G_1- or G_4-AChE, bFGF or BSA. It is interesting that none of the factors decreased cell death over the observed time period, and yet AChE, BuChE and bFGF increased neurite outgrowth and therefore may be considered to have a trophic role in this system. It could be postulated that upon application of the trophic factor, two hours after plating, the mechanisms causing cell death, whether a result of natural occurrence or due to traumatisation, are sufficiently advanced to prevent reversal by addition of a trophic factor.

3.4.3 A neurotrophic role for cholinesterase

The addition of BuChE, G_1- and G_4-AChE and bFGF significantly enhanced neurite outgrowth of embryonic dopamine cells in comparison to the untreated control. The possibility that these increases were due to a non-specific protein effect is unlikely as no effect upon outgrowth was seen with BSA. The trophic action of bFGF upon neurite outgrowth is well documented (Morrisson *et al.*, 1988; Silani *et al.*, 1994, Engele and Bohn, 1991; Knusel *et al.*, 1990). The positive effect that bFGF had upon neurite outgrowth confirms that this culture system is a viable assay for investigating the effects of potentially trophic test substances.

3.4.4 Possible mechanisms for the growth promoting function of cholinesterase

3.4.4.1 Cholinergic actions

The possibility of the trophic effects of BuChE and G_4- and G_1-AChE being a result of cholinergic activity cannot be discounted. A preparation of the ventral mesencephalon comprises several brain structures that give rise to a heterogenous cell population (Chapter 2,

Section 2.1.3.1). As discussed in Chapter 1 (Section 1.1.2.3) the SNpc receives cholinergic afferentation from the pedunculopontine nucleus, and as such, cultures used in the experiments presented in this chapter will contain both cholinergic neurons from the pedunculopontine nucleus and cholinoceptive neurons from the SNpc. Since the classical role of cholinesterase is termination of cholinergic transmission, the most immediate explanation for the stimulatory role of both BuChE and AChE seen in these experiments is that it is a function of the hydrolysis of acetylcholine (ACh). Indeed, Lipton *et al.* (1988) demonstrated that addition of nicotinic antagonists (*d*-tubocurarine and mecamylamine) enhanced neurite outgrowth from retinal ganglion cells, suggesting that ACh could be an inhibitory growth signal. Moreover, Small *et al.* (1995) found that acetylcholine and the cholinergic agonist carbamylcholine (carbachol) inhibited neurite outgrowth of cultured chick sympathetic neurons. However, a non-cholinergic role of AChE in neurite outgrowth is well documented (Chapter 1, Section 1.3.4.1.iii). Numerous *in vitro* studies have shown that inhibition of the hydrolytic ability of AChE has no effect on neurite outgrowth (Layer *et al.*, 1993; Dupree and Bigbee, 1994; Jones *et al.*, 1995; Srivatsan and Peretz, 1997). A study by Sternfeld *et al.* (1998) demonstrated that neurites of cultured spinal cells from *Xenopus* laevis embryos that had been microinjected with human AChE cDNA vectors grew three times faster than control neurons. Moreover, in the same study, insertion of AChE-protein mutated to be incapable of hydrolysis had the same neuritogenic effect as microinjection of active enzyme cDNA. Furthermore, Layer *et al.* (1993) has shown, using drugs that are known to inhibit hydrolytic activity, that both BuChE and AChE can promote neurite outgrowth of chick tectal cells independently of substrate hydrolysis. The findings of these studies strongly suggest a non-cholinergic role for AChE, and BuChE in neurite extension.

The involvement of the ability of BuChE and AChE to hydrolyse ACh in neurite outgrowth of embryonic dopaminergic cells is investigated in Chapter 4 of this thesis.

3.4.4.2 Cell adhesion molecule properties

As discussed in Chapter 1 (Section 1.3.4.1.3.iii), it has been proposed that the trophic effect of BuChE and AChE may be due to a cell adhesion molecule (CAM) property of these proteins (Layer and Kaulich, 1991 Layer *et al.*, 1993; Sharma and Bigbee, 1998). Both BuChE and AChE carry the HNK-1 epitope expressed by CAMs. However, CAMs function as membrane bound molecules and it would be unusual for a carbohydrate moiety on soluble, exogenous AChE to modulate neurite outgrowth. In support of this, AChE but not BuChE was found to be trophic in the work by Holmes *et al.* (1997), who used an early postnatal nigral organotypic preparation.

A further role of AChE in cell-adhesion processes is postulated by Grifman *et al.* (1998). In this study, antisense suppression of AChE in PC12 cells resulted in impaired neurite outgrowth and neurexin expression. Overexpression of neuroligin-1 restored both neurite extension and neurexin expression. As discussed in Chapter 1 (Section 1.3.4.1.3.iii) it is thought that binding of neuroligin to neurexin is important in inter-neuronal recognition pathways regulating axon pathfinding (Ichtchenko *et al.*, 1996; Song *et al.*, 1999; Scheiffele *et al.*, 2000; for review see Rao *et al.*, 2000). Studies have revealed a homologous extracellular domain in neuroligin 1, 2 and 3 and AChE (Ichtchenko *et al.*, 1995 and 1996). Furthermore, Tsigelny *et al.* (2000) uncovered a common EF-hand motif in BuChE, AChE and neuroligins. The findings of Grifman *et al.* (1998) suggest that AChE may compete with neuroligins for extracellular binding to neurexins, and so induce intracellular signals leading

to neurite outgrowth. In addition, a study by Andres *et al.* (1997) associated the overexpression of AChE in embryonic spinal cord neurons with suppression in the level of neurexin Iβ mRNA, suggestive of a functional AChE-neurexin relationship.

Similarities between cholinesterases and neuroligin, taken with the findings of Grifman *et al.* (1998) and Andres *et al.* (1997) suggest that a CAM property of AChE and BuChE might be involved in the trophic actions of these substances. However, again, the lack of effect of BuChE on neurite outgrowth in the study of Holmes *et al.* (1997) does not concur with this postulation.

An alternative suggestion is that the response of neurites to cholinesterases is age-dependent. Thus, the trophic effects of both BuChE and AChE on embryonic TH+ve cells presented in this chapter, in contrast to the trophic effect of AChE, but not BuChE on postnatal TH+ve cells in the study by Holmes *et al.* (1997), may be a result of a developmental effect of cholinesterases.

3.4.4.3 A developmental role for cholinesterases

BuChE and AChE are transiently expressed at early stages of embryogenesis (Drews 1975; Layer, 1983; see Chapter 1 Section 1.3.4.1.3.i). BuChE expression is known to spatially foreshadow AChE expression (Layer *et al.*, 1987; Layer *et al.*, 1988, Willbold and Layer, 1992). Furthermore, the molecular composition of embryonic AChE in many animals comprises high levels of G_1 with a low G_4: G_1 ratio (Chapter 1 Section 1.3.4.1.3.ii). This ratio increases during development in many species including the chick (Layer *et al.*, 1987), rat (Rieger and Vigny, 1976), mouse (Inestrosa *et al.*, 1994) and human (Arendt *et al.*, 1992)

with the predominate form of AChE in the adult brain being G_4. It is interesting that in the studies presented in this chapter, the stimulatory effects on neurite outgrowth of BuChE and G_1-AChE precede a trophic response to G_4-AChE. This could correspond to the *in vivo* situation, whereby the 'windows' of response to factors *in vitro* parallel the sequential expression of the same factors *in vivo.* Also, in the experiments presented in this chapter, all test substances which increased neurite outgrowth at day 1 or day 3 *in vitro*, with the exception of 3 U/ml G_4-AChE, lost effect over the remainder of the 7 day incubation period. This may again parallel the *in vivo* situation, whereby cells are exposed, and may respond to the transient expression of stimulatory factors. Prolonged exposure *in vitro* may be of little consequence if the cells have such a 'window' of response to particular factors.

In conclusion, the viability of this culture system as a means of detecting effects of trophic compounds was confirmed by the significant increase in neurite outgrowth upon addition of bFGF to culture medium. The stimulatory effects of BuChE, G_1- and G_4-AChE upon neurite outgrowth of embryonic dopamine cells are consistent with a trophic role for these molecules in development and lend support to other evidence of cholinesterases performing a similar function.

The next chapter aims to examine whether or not the trophic effects of BuChE and AChE on embryonic dissociated dopaminergic cells in the preparation used in this thesis are attributable to the hydrolytic activity of these enzymes, using cholinesterase inhibitors. As discussed in Section 3.4.4.1 of this chapter, above, numerous studies, in various cell preparations, have documented that inhibitors of the hydrolytic action of AChE have no

effect upon neurite outgrowth. However, the effects of such inhibitors on developing embryonic dopamine cells have not been examined.

CHAPTER 4

The effect of AChE inhibitors on survival and outgrowth of embryonic dopaminergic cells in dissociated cultures of the VM

4.1 INTRODUCTION

Chapter 3 illustrated that BuChE and G_4- and G_1-AChE increase neurite outgrowth of developing embryonic TH+ve cells. The demonstration of a trophic effect of AChE on neurite outgrowth is considered to contribute to the evidence for a non-classical property of AChE (Holmes *et al.*, 1997; Srivatsan and Peretz, 1997; Chapter 1, Section 1.3.4). However, a non-cholinergic basis for the trophic effect of BuChE and AChE on embryonic dopaminergic cells cannot be established from the experiments presented in Chapter 3 of this thesis alone. An alternative approach to investigating a non-cholinergic role for AChE in developing neurons has been used by other outgrowth studies (Layer *et al.*, 1993; Dupree and Bigbee, 1994; Jones *et al.*, 1995; Srivatsan and Peretz, 1997). These studies used inhibitors of AChE to illustrate that irreversible inhibition of the active site of cholinesterase has no detrimental effect upon neurite outgrowth, but that inhibition of both the active and peripheral anionic site (PAS) of acetylcholinesterase results in significantly reduced neurite outgrowth. Of particular relevance to this thesis, are the studies of Jones *et al.*, (1995) which used postnatal dopaminergic cells, and Layer *et al.* (1993) which used embryonic neuronal cultures of avian origin. Both of these studies demonstrated that BW284c51, an inhibitor of both the active and PAS of acetylcholinesterase, reduced neurite outgrowth, whilst the active site inhibitor echothiophate had no effect. In both studies it was concluded that the results were indicative of a trophic role of cholinesterases, which was mediated through a non-cholinergic mechanism, potentially associated with the PAS.

This chapter aims to examine the effect of cholinesterase inhibitors on embryonic TH+ve cells in cultures, using inhibitors BW284c51 and echothiophate as tools to investigate the

importance of the hydrolytic ability of AChE and BuChE, and the PAS of the AChE molecule to dopaminergic cell survival and neurite outgrowth.

(i) BW284c51 (1,5-bis(4-allydimethylammoniumphenyl)-pentan-3-one dibromide)

BW284c51 is a bisquarternary ammonium compound that inhibits the active and peripheral anionic sites of the AChE (Figure 4.1). BW284c51 has previously been shown to decrease neurite outgrowth of postnatal dopamine cells (Jones *et al.*, 1995), chick tectal cells (Layer *et al.*, 1993), dorsal root ganglion cells (Dupree and Bigbee, 1994) and chick sympathetic neurons (Postuma *et al.*, 1999).

(ii) Echothiophate

Echothiophate, an organophosphate, is a non-specific irreversible cholinesterase inhibitor that forms a covalent bond with the Serine residue of the esteratic site within the catalytic gorge of cholinesterases. There is currently no published crystal structure for the interaction of echothiophate with AChE. No effect upon neurite outgrowth has been found on cultured chick tectal neurons (Layer *et al.*, 1993) or postnatal dopamine neurons (Jones *et al.*, 1995). Srivatsan and Peretz (1997) found that echothiophate was not capable of blocking the neurotrophic effect of AChE in *Aplysia* hemolymph.



Figure 4.1 Representation of *Torpedo californica* AChE complexed with BW284c51.

This figure shows a monomer of AChE (blue) and indicates the binding site for BW284c52 (green). Yellow is the active serine (Ser-200) of AChE. Courtesy of Dr. S. Emmett; based on the structure by Felder, Harel, Silman and Sussman. PDB: 1E3Q.

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4.2 EXPERIMENTAL PROCEDURES

4.2.1 Dissociated cultures of the VM: preparation and staining

Suspensions of ventral mesencephalon cells from E14 rats were prepared as described in Chapter 2 (Section 2.2.2). Cultures were chronically treated for 1, 3 or 7 days with media supplemented with either BW284c51 or echothiophate. Untreated cultures served as controls. Every 48 hours 150 µl of media was removed and replaced with 150 µl of fresh media containing, where appropriate the test substance. Cultures were fixed and processed for TH immunoreactivity as described in Chapter 2 (Section 2.2.3) at days 1, 3 and 7 *in vitro.*

4.2.2 Experimental design

Cultures were chronically treated for 1, 3 or 7 days with media supplemented with BW284c51 or echothiophate. The concentrations of test substances used have been shown to inhibit cholinesterase activity of the level that has been identified in culture medium in Chapter 3, Section 3.3.1 (Postuma *et al.*, 1999; Jones *et al.*, 1995; Holmes, 1996) and are summarised in Table 4.1. Experiments were repeated a minimum of three times using foetuses from different mothers. Each treatment group contained a minimum of 4 chamber wells, giving a total of at least 12 cultures for each treatment group ($n \geq 12$).

Test Substance	Concentration
BW284c51	10 µM, 100 µM
Echothiophate	100 µM

Table 4.1 Concentrations of test substances used to inhibit the active site of cholinesterase (echothiophate) or the active and PAS of AChE (BW284c51)

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4.2.3 Collection of data and statistical analysis

4.2.3.1 Cell numbers

Total cell numbers per culture well were counted for each treatment group at each time point. For all experiments involving treatment of cultures with test substances, data was normalised to the untreated control.

4.2.3.2 Neurite length

Camera Lucida drawings were made of every TH+ve cell in the chamber-slide well for each treatment group at each time point (Chapter 2, Section 2.2.4.2). The length of each cell's longest neurite was assessed using a sports route planner and results for each treatment group were normalised to the untreated control.

For both parameters, cell numbers and neurite length, all data was collected in a blind fashion to prevent sampling bias. For both parameters, values for each data group were expressed as mean ± s.e.m. Statistical significance was assessed using either analysis of variance (ANOVA) for normally distributed data followed by a Dunnett's multiple comparison t-test, or the Kruskal-Wallis non-parametric test for non-normally distributed data.

4.2.4 Source of test chemicals

BW284C51 was obtained from Sigma-Aldrich Company Ltd. (Poole, UK). Echothiophate was a kind gift from Wyeth Laboratories (Taplow, UK). Stock solutions of BW284c51 (10 mM) and echothiophate (10 mM) were made in distilled water, aliquoted and frozen at –20°C. Stock solutions of BW284c51 and echothiophate were diluted in culture medium immediately prior to use in experiments.

4.3 RESULTS

4.3.1 The effect of AChE inhibitors on dopaminergic neurons in dissociated embryonic cultures of the VM

The data obtained for the effects of treatment on cell survival and neurite outgrowth of dopaminergic cells at days 1, 3 and 7 *in vitro,* normalised to the no treatment control, is shown in Table 4.2

Treatment	Number of TH+ve cells (normalised to untreated control)			Neurite length (normalised to untreated control)		
	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7
Untreated control	1.0 ± 0.17	1.0 ± 0.18	1.0 ± 0.09	1.0 ± 0.05	1.0 ± 0.05	1.0 ± 0.07
10 μM BW284c51	0.80 ± 0.08	0.96 ± 0.16	0.24 ±0.05***	0.91 ± 0.06	0.76 ±0.05*	0.6 ± 0.12*
100 μM BW284c51	0.84 ± 0.12	0.1± 0.05***	0.03 ±0.02***	0.72± 0.05**	0.31 ±0.03***	0.16± 0.02***
100 μM Echothiophate	0.72 ± 0.13	0.76 ± 0.1	0.9 ± 0.16	0.93 ± 0.11	1.02 ± 0.09	1.6 ± 0.25*

* denotes $P < 0.05$
** denotes $P < 0.01$
*** denotes $P < 0.001$

Table 4.2 The effect of cholinesterase inhibitors on TH+ve neurons in dissociated embryonic cultures of the VM.

Values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. Statistical significance was assessed by the Kruskal-Wallis non-parametric test. $n \geq 12$.

******

4.3.1.1 The effect of AChE inhibitors on TH+ve cell numbers

At day 1 *in vitro* (24 hour exposure to test substances) Figure 4.2A, there were no significant changes in total TH+ve cell number from the untreated control. At day 3 *in vitro* (72 hour exposure to test substance) Figure 4.2B, 100 μM BW284c51 had significantly decreased the total number of TH+ve cells in culture in comparison to the untreated control ($P < 0.001$; Kruskal-Wallis non-parametric test). There were no effects upon cell numbers with 10 μM

BW284c51 or echothiophate at this time point. By day 7 *in vitro* (168 hour exposure to test substances) Figure 4.2 C, both 10 μM and 100 μM BW284c51 had significantly decreased the total number of TH+ve cells per culture in comparison to the untreated control ($P < 0.001$; Kruskal-Wallis non-parametric test). There were no effects on cell numbers of incubation with echothiophate at this time point.

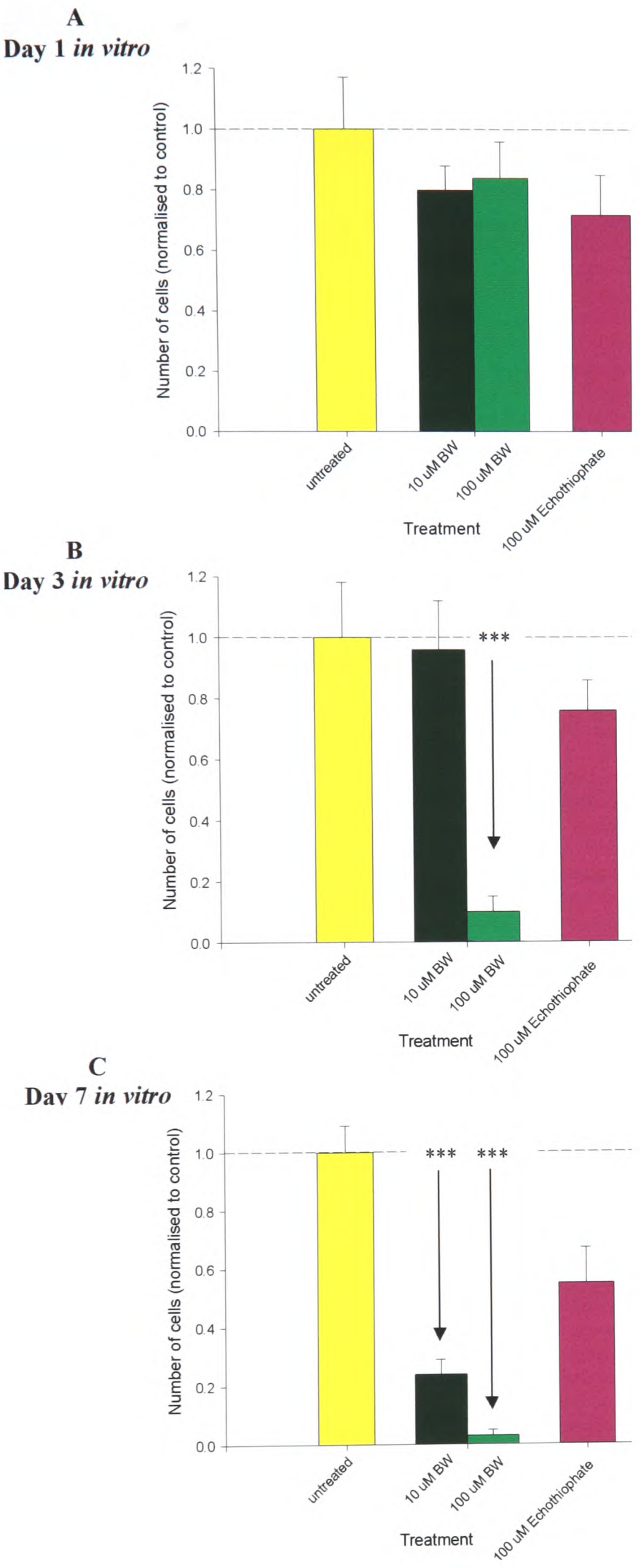

Figure 4.2 Effect of AChE inhibitors on TH+ve cell numbers

At day 1 in vitro (24 hour exposure to test substances) ***A****, there were no significant changes in total TH+ve cell number per culture from the untreated control. At day 3 in vitro (72 hour exposure to test substance)* ***B****, 100 μM BW284c51 had significantly decreased the total number of TH+ve cells in culture in comparison to the untreated control (*** denotes $P < 0.001$; Kruskal-Wallis non-parametric test). By day 7 in vitro (168 hour exposure to test substances)* ***C****, both 10 μM and 100 μM BW284c51 had significantly decreased the total number of TH+ve cells per culture ($P < 0.001$ for both; Kruskal-Wallis non-parametric test). Values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. BW = BW284c51. $n \geq 12$.*

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4.3.1.2 The effect of AChE inhibitors on TH+ve neurite outgrowth

At day 1 *in vitro* (24 hour exposure to test substances) Figure 4.3A, addition of 100 μM BW284c51 to the culture medium had significantly decreased dopaminergic neurite outgrowth in comparison to the untreated control ($P < 0.01$; Kruskal-Wallis non-parametric test). No effect upon outgrowth was seen with echothiophate. At day 3 *in vitro* (72 hour exposure to test substance) Figure 4.3B, both 10 μM and 100 μM BW284c51 had significantly decreased neurite outgrowth in comparison to the untreated control ($P < 0.05$ and $P < 0.001$ respectively; Kruskal-Wallis non-parametric test). There was no effect upon outgrowth with echothiophate at this time point. At day 7 *in vitro* (168 hour exposure to test substances) Figure 4.3C, addition of 10 μM and 100 μM BW284c51 had significantly decreased neurite outgrowth in comparison to the untreated control ($P < 0.05$ and $P < 0.001$ respectively; Kruskal-Wallis non-parametric test). Echothiophate at 100 μM had significantly increased neurite outgrowth at this time point ($P < 0.05$; Kruskal-Wallis non-parametric test).

Photographic representations of various treatments are shown alongside relevant figures.

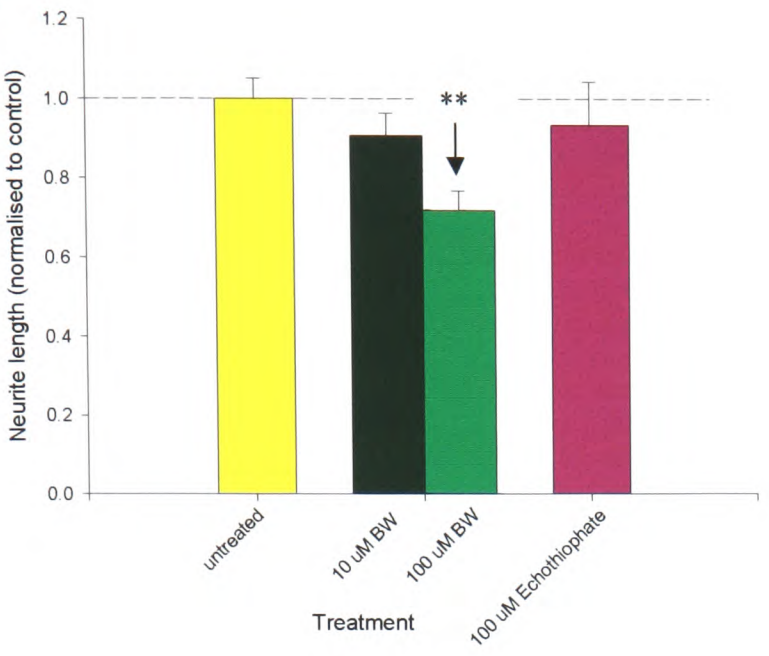





Figure 4.3A The effect of AChE inhibitors on neurite outgrowth of TH+ve cells. Day 1 *in vitro* (24 hour exposure to test substances).

*Addition of 100 µM BW284c51 had significantly decreased neurite outgrowth in comparison to the untreated control (** denotes $P < 0.01$; Kruskal-Wallis non-parametric test). No effect upon outgrowth was seen with echothiophate. Graphical values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. BW = BW284c51. $n \geq 12$.*

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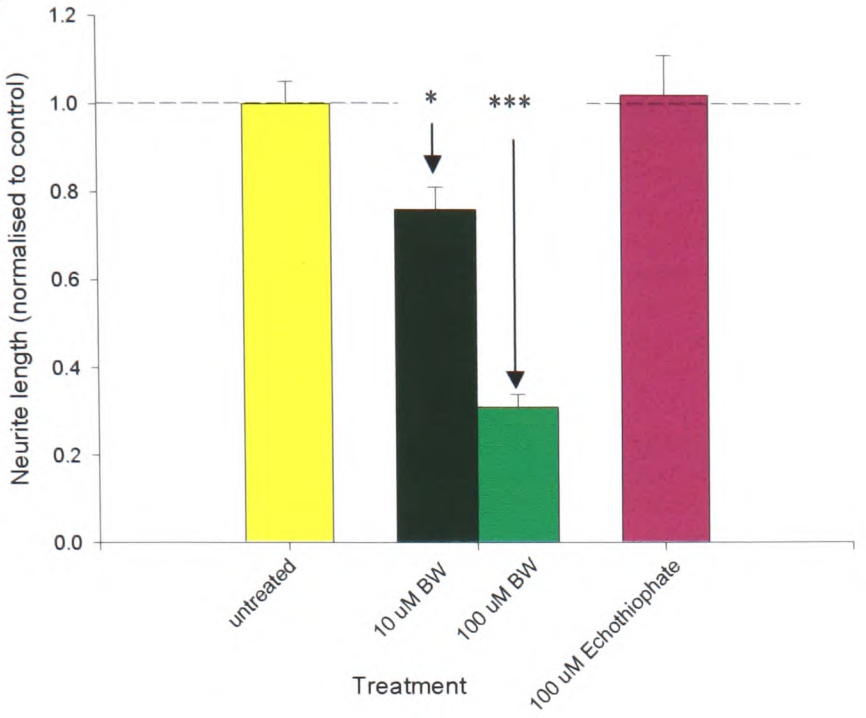
B
Day 3 *in vitro*



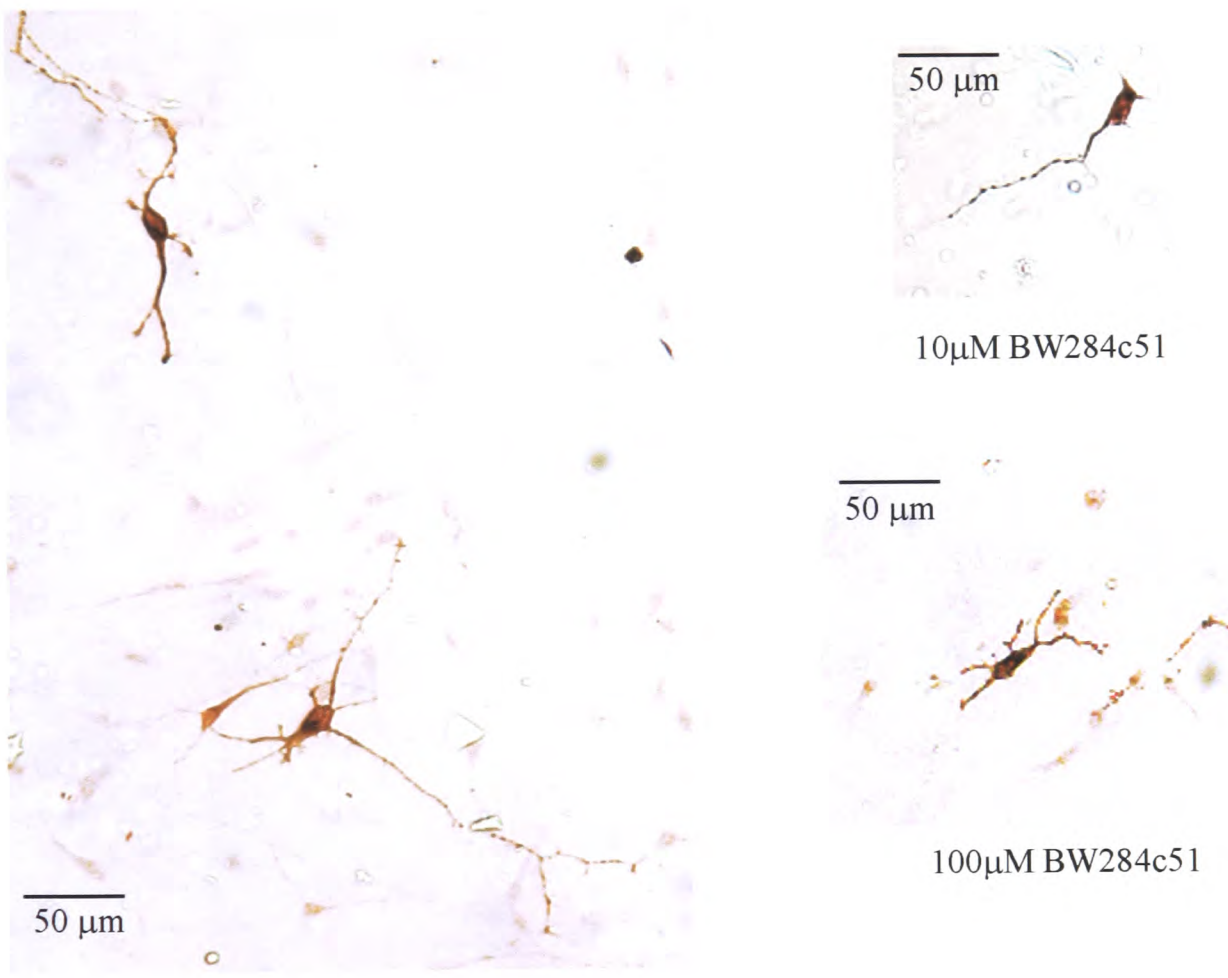


Figure 4.3B The effect of AChE inhibitors on neurite outgrowth of TH+ve cells. Day 3 *in vitro* (72 hour exposure to test substances).

Addition of 10 µM and 100 µM BW284c51 had significantly decreased dopamine cell neurite outgrowth in comparison to the untreated control (denotes $P < 0.05$ and *** denotes $P < 0.001$ respectively; Kruskal-Wallis non-parametric test). There were no effects upon outgrowth with echothiophate at this time point. Graphical values shown are mean ±s.e.m. and have been normalised to the untreated control for each time point. BW = BW284c51. $n \geq 12$*

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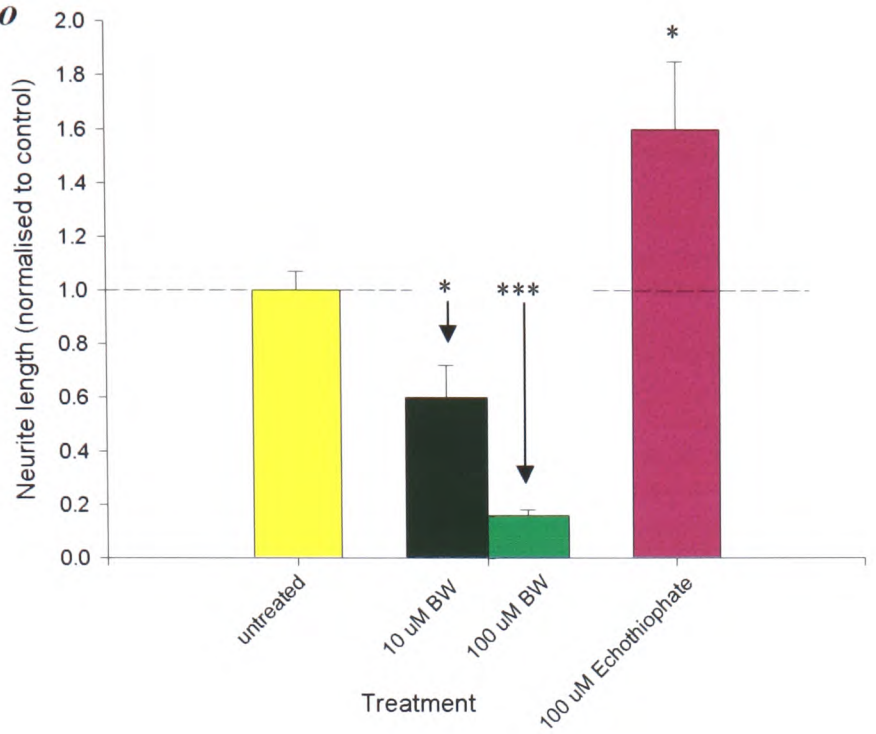
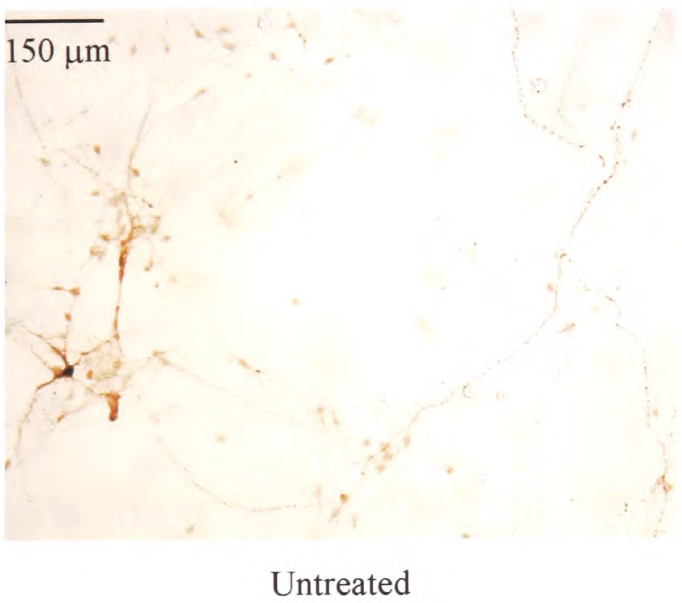
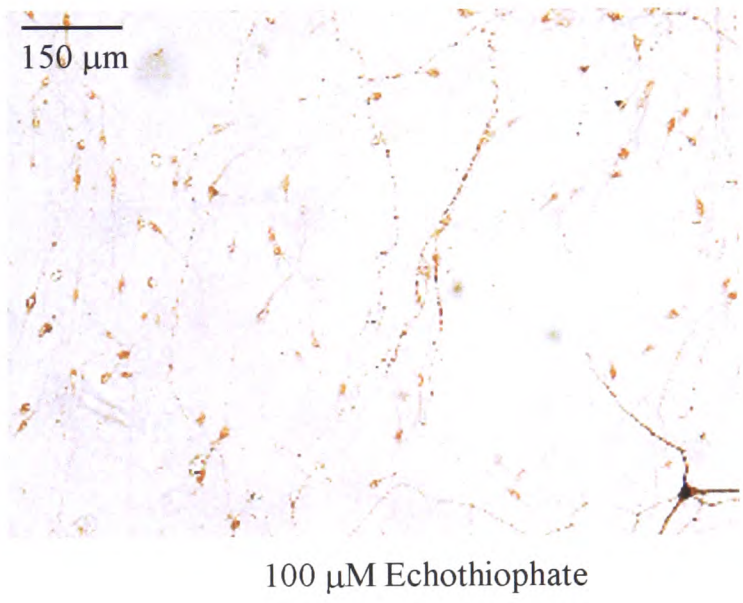
C

Day 7 *in vitro*





Untreated



100 µM Echothiophate



10 µM BW284c51



100 µM BW284c51

Figure 4.3C The effect of AChE inhibitors on neurite outgrowth of TH+ve cells. Day 7 (168 hour exposure to test substances).

Addition of 10 µM and 100 µM BW284c51 had significantly decreased dopamine cell neurite outgrowth in comparison to the untreated control (denotes $P < 0.05$ and *** denotes $P < 0.001$ respectively; Kruskal-Wallis non-parametric test). Echothiophate at 100 µM had significantly increased neurite outgrowth at this time point ($P < 0.05$; Kruskal-Wallis non-parametric test). Graphical values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. BW = BW284c51. $n \geq 12$*

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4.4 DISCUSSION

Chapter 3 of this thesis demonstrated that BuChE and G_4- and G_1-AChE increase neurite outgrowth of embryonic TH+ve cells in culture. A trophic role for AChE in neurite outgrowth of developing cells has been attributed to a non-cholinergic property of the molecule (Holmes *et al.*, 1997; Srivatsan and Peretz, 1997; Chapter 1, Section 1.3.4.1.3.iii). However, the demonstration of trophic effects of both BuChE and AChE on embryonic TH+ve cells in the studies presented in Chapter 3 suggest that these effects could be attributable to the ability of both BuChE and AChE to hydrolyse ACh. To investigate whether the effects of BuChE and AChE on neurite outgrowth seen in Chapter 3 were due to their hydrolytic capacity, the experiments presented in this chapter used an inhibitor of the active site of cholinesterase, echothiophate. Previous studies have found that addition of echothiophate has no effect upon neurite outgrowth or cell survival (Dupree and Bigbee, 1994; Layer *et al.*, 1993; Srivatsan and Peretz, 1997; Jones *et al.*, 1995), suggesting that the effects of AChE and BuChE on neurite outgrowth are not dependent upon their ability to hydrolyse ACh. Further to this, addition of the active site and PAS inhibitor of AChE, BW284c5 has been shown to reduce cell survival and neurite outgrowth (Layer 1993; Dupree and Bigbee, 1994; Jones *et al.*, 1995; Srivatsan and Peretz, 1997), suggesting that the PAS of AChE may be fundamental to these parameters. The aim of this chapter was therefore to examine the effects of echothiophate and BW284c51 on cell survival and neurite outgrowth of embryonic dopaminergic cells in dissociated cultures of the VM. These inhibitors were used in order to explore whether BuChE and AChE have a non-cholinergic role in neurite outgrowth of these cells, and also to investigate the importance of the PAS of the AChE molecule to embryonic TH+ve cell survival and outgrowth.

4.4.1 A non-cholinergic role for BuChE and AChE

Addition of 100 μM echothiophate, an inhibitor of the active site of both BuChE and AChE to the culture medium of dissociated embryonic VM cells had no effect upon dopaminergic cell survival, and no detrimental effect upon neurite outgrowth of dopaminergic cells. The presence of both BuChE and AChE within the culture system was established in Chapter 3 (Section 3.3.1). It can therefore be concluded from these studies that inhibition of the active site of endogenous BuChE and AChE has no effect upon cell survival or neurite outgrowth of embryonic dopaminergic cells in dissociated VM cultures. These findings are in agreement with those of other groups (Layer 1993; Dupree and Bigbee, 1994; Jones *et al.*, 1995; Srivatsan and Peretz, 1997) and contribute to the abundance of literature documenting a non-cholinergic role of cholinesterase in morphogenesis. Further to this, a previously undocumented result is the trophic effect on TH+ve neurite outgrowth of chronic (7days; 168 hour) exposure of cultured cells to echothiophate. It is possible that echothiophate may have inhibited other hydrolases, or proteases present within the culture medium. For example, small quantities of trypsin, which may have persisted following its use in the culture procedure. Trypsin is a proteolytic enzyme, which cleaves polypeptide chains on the carboxyl side of arginine and lysine residues. Its presence within cultures may have disrupted growth of constituent cells. Inhibition of trypsin by echothiophate may therefore have enhanced neurite outgrowth.

4.4.2 Involvement of the PAS

Addition of the active and peripheral anionic site inhibitor of AChE, BW284c51 (10 μM and 100 μM) decreased TH+ve neurite outgrowth over the 7 day time period, with the higher

concentration having a detrimental effect upon neurite length within 24 hours (Figure 4.3A). BW284c51 also decreased TH+ve cell survival at both concentrations by day 7 *in vitro* (Figure 4.2). These results are consistent with the findings of other studies on cultured cells that have demonstrated a detrimental effect of BW284c51 on outgrowth and survival (Layer *et al.*, 1993; Dupree and Bigbee, 1994; Jones *et al.*, 1995; Small *et al.*, 1995; Srivatsan and Peretz, 1997).

Postuma *et al.* (1999) suggested that the effects caused by BW284c51 might be due to non-specific toxicity. This was postulated following their findings that a concentration of 500 μM BW284c51 caused toxicity and inhibited neurite outgrowth of chick sympathetic neurons, but that 50 μM BW284c51 had no effect upon neurite outgrowth even though this was an adequate concentration to inhibit all cholinergic activity in their system. The demonstration in this chapter that 10 μM BW284c51 significantly decreased neurite outgrowth and cell survival of embryonic dopamine cells are not consistent with the findings of Postuma *et al.* (1999), and as such suggest against a non-specific toxic effect of BW284c51. The dramatic effects of BW284c51 on cell health, in comparison to echothiophate, are thus suggestive of a fundamental role of the PAS in cell survival and neurite outgrowth.

The peripheral anionic site of AChE is situated at the rim of the catalytic gorge and is composed of several overlapping subsites. It serves as an initial binding site for substrate entry to the gorge (Rosenberry *et al.*, 1999; Szegletes *et al.*, 1999) and is thought to be involved in the substrate inhibition exhibited by AChE (Radíc *et al.*, 1990; Chapter 1,

Section 1.3.3.3). Several studies, in which differences in effect between echothiophate and BW284c51 were observed, postulated that the detrimental effects of BW284c51 on outgrowth and survival were due to interaction of BW284c51 with a site distinct from the active site (Layer 1993; Jones 1995; Srivatsan and Peretz; 1997). The identification, by Johnson and Moore (1999) of an adhesion site at the PAS of AChE supported the potential of the PAS to be involved in mediating the stimulatory actions of AChE on cell outgrowth. However, before adopting this theory as an explanation for the results seen in this chapter, the trophic effects of both BuChE and AChE, as seen in Chapter 3 of this thesis, must be considered. BuChE lacks a PAS, and as such, it is hard to see how the trophic effects of AChE on neurite outgrowth may be mediated solely through the PAS when BuChE, which enhances neurite outgrowth under identical culture conditions, has no PAS. Two possibilities arise. Firstly, that BuChE and AChE mediate neurite outgrowth through different mechanisms, with the PAS involved in the actions of AChE, and an alternative site on BuChE involved in mediating neurite extension. Alternatively, AChE and BuChE could affect neurite outgrowth through a similar mechanism, which is not associated with the PAS. In the latter situation, the toxic effects of BW284c51 may be due to a secondary effect on AChE's mechanism of action caused by the interaction of BW284c51 with the PAS of AChE. The study by Layer *et al.* (1993), which demonstrated that inhibition of BuChE by the selective inhibitor ethopropazine resulted in a similar decrease in neurite outgrowth to that seen following inhibition of AChE by BW284c51, proposed a scheme to support a non-PAS-mediated mechanism of action similar to both AChE and BuChE. This scheme suggested that the trophic actions of both BuChE and AChE were mediated through a CAM-like region of each molecule, which is distinct from both the active site and PAS. It was postulated that binding of echothiophate to the active site would have no effect upon the distinct CAM-like

region, but binding of BW284c51 to the PAS may result in a steric interaction with the CAM property of AChE or BuChE, with repercussions on cell adhesion, and consequently neurite outgrowth. This scheme does not account, however, for the lack of effect of BuChE seen in the study on early postnatal tissue by Holmes *et al.* (1997).

To definitively answer the question of whether the trophic actions of AChE are mediated through the PAS, a specific PAS inhibitor is required. Several inhibitors are available that bind solely to the PAS but they all also affect the hydrolytic activity of AChE, usually by sterically obstructing the entrance to the catalytic gorge and therefore effectively performing the same purpose as BW284c51. One such example is fasciculin, a 61 amino acid toxin purified from green mamba (*Dendroaspis angusticeps*) snake venom that binds to the PAS, but also obstructs the narrow gorge leading to the active site (Bourne, 1997; Harel, 1997). As a specific inhibitor of the PAS is not currently available, the next chapter of this thesis examines the question of PAS inhibition from an alternative perspective. Experiments in Chapter 5 aim to demonstrate that BW284c51 toxicity is not due to elevated acetylcholine levels having a detrimental effect upon acetylcholine receptors.

CHAPTER 5

Investigation of the mechanism of BW284c51 toxicity

5.1 INTRODUCTION

In Chapter 4 it was shown that 10 μM and 100 μM BW284c51 markedly reduced cell numbers and neurite outgrowth of embryonic TH+ve cells in culture, whilst 100 μM echothiophate had no detrimental effect upon either cell number or neurite outgrowth. Both inhibitors prevent catalytic activity of AChE, but only BW284c51 inhibits the peripheral anionic site of AChE, an area that has been proposed to be the site of the non-cholinergic, trophic property of AChE (Jones 1995; Johnson and Moore, 1999). Chapter 3 of this thesis illustrated that addition of AChE to cultures increased neurite outgrowth of embryonic TH+ve cells, but had no effect upon cell numbers, yet addition of BW284c51 caused toxicity, as demonstrated by Chapter 4. This poses the question as to whether the toxicity caused by BW284c51 is due to direct inhibition of an essential property of the AChE molecule (potentially the PAS) or whether its actions are causing a separate effect that is detrimental to cell survival and outgrowth. The latter postulation has two possibilities. Firstly, that the toxicity of BW284c51 may be a non-specific toxic effect. This idea has been proposed by Postuma *et al.* (1999), following findings that 500 μM BW284c51 causes toxicity and inhibits neurite outgrowth of chick sympathetic neurons, but that 50 μM BW284c51 has no effect upon neurite outgrowth, despite being an adequate concentration to inhibit all AChE in the system used. An alternative suggestion is that inhibition of AChE by BW284c51 may result in elevated acetylcholine levels that, through an effect upon acetylcholine receptors (AChR), may ultimately result in a decrease in cell numbers and neurite outgrowth. Lipton *et al.* (1988) suggested that ACh could be an inhibitory growth signal after demonstrating that addition of nicotinic antagonists *d*-tubocurarine and mecamylamine enhanced neurite outgrowth from retinal ganglion cells. Furthermore, Small *et al.* (1995) found that ACh and

the cholinergic agonist carbachol inhibited neurite outgrowth of cultured chick sympathetic neurons.

This chapter aims to address the question of the toxicity of BW284c51 by investigating the suggestion that toxicity may be caused by elevated ACh levels and mediated through AChR. This hypothesis is addressed in two ways. Firstly, incubation of cultures with AChR agonists nicotine (a nicotinic ACh receptor agonist) or carbachol (a muscarinic and nicotinic ACh receptor agonist) in order to mimic the effects that elevated ACh may have. Secondly, inhibition of nicotinic acetylcholine receptors (nAChR) using a mixture of nicotinic receptor antagonists consisting of methyllcaconitine (an α_7 nAChR antagonist), mecamylamine (an α_3/β_2 nAChR antagonist) and di-hydro-β-erythroidine (an α_4/β_2 nAChR antagonist), in combination with BW284c51 application. The aim of this is to prevent the effects that elevated ACh may have upon nAChR and examine whether this alleviates BW284c51 toxicity.

5.1.1 Acetylcholine receptors

ACh activates two classes of membrane receptor that are distinguished pharmacologically by the preferential effects of nicotine and muscarine.

Nicotinic acetylcholine receptors (nAChR) are ionotropic pentameric membrane proteins (Changeux *et al.*, 1998; Lukas *et al.*, 1999; Figure 5.1A). Muscle-type nAChR are heteromeric and formed by four subunits, α1, β1, γ (or ε) and δ. Neuronal nAChR are composed of two distinct receptor sub-units, the α-like subunits and the β-like subunits. Eight α (α2-α9) subunits and three β subunits (β2-β4) have so far been identified (Arias,

1998; McIntosh *et al.*, 1999). These subunits may make up heteromeric or homomeric receptors, (for example, $\alpha_4\beta_2$ or α_7) and subunits confer distinctive functional and structural properties to the nAChR that they form (Changeux *et al.*, 1998; Lukas *et al.*, 1999).

Muscarinic acetylcholine receptors (mAChR) are members of the G-protein coupled receptor family. mAChR are seven-helix transmembrane proteins homologous to the adrenergic receptor. The mAChR is so called because of its sensitivity to muscarine. Upon agonist activation mAChR produce a slow excitatory postsynaptic potential (EPSP) that lasts for several seconds and is mediated through a slowly inactivating voltage-gated M-type (muscarine-sensitive) potassium channel. Initially it was thought that there were just two subtypes of muscarinic receptors, M1 and M2, defined primarily on the basis of their binding affinities for certain antagonists, notably pirenzepine (Natsuk and Graybiel, 1991). It is now recognised that there are more than two subtypes; five related genes encoding muscarinic receptors have been cloned and sequenced (see Bonner, 1989). These five muscarinic AChR subtypes, referred to as m1-5, have similar predicted membrane topographies, and are homologous primarily in their membrane-spanning regions (Figure 5.1B).

5.1.2 Presence of acetylcholine receptors in the VM

Naeff *et al.* (1992) used autoradiography to investigate the expression of nicotinic binding sites in the developing rat brain. This study illustrated that nicotinic binding sites could be identified within the VM by embryonic day 13, and the density of labelling tended to increase during gestation. The mesencephalon showed maximal binding by the early postnatal stage, with the SNpc labelled most intensively. By postnatal day 27 the distribution of nicotinic binding sites throughout the brain was found to be very similar to the adult

pattern, with an intermediate level of binding observed in the substantia nigra and mesencephalon in general.

Muscarinic receptor density in the midbrain is significantly lower than in other regions of the brain, such as the hippocampus, cingulate cortex or striatum (Kuhar and Yamamura, 1975). Numerous autoradiography studies have documented low levels of mAChR expression within the VM, and in particular within the SN (Cortés *et al.*, 1984; Cortés *et al.*, 1986; Cortés and Palacios, 1986; Spencer *et al.*, 1986; Miyoshi *et al.*, 1989). Within the SN, mAChR binding is documented as being more prominent in the SNpr than the SNpc (Natsuk and Graybiel, 1991).



Figure 5.1 Schematics showing transmembrane topology of (A) nicotinic AChR subunit and (B) muscarinic AChR subunit assembly.

(A) *illustrates the existence of an extensive, extracellular N-terminal region, four transmembrane segments, a cytoplasmic loop and an extracellular C terminus for a single subunit of the pentameric nAChR. Adapted from Lukas et al. (1999).* ***(B)*** *illustrates the seven transmembrane-spanning regions of the muscarinic AChR, with, again, an extracellular N-terminus, a large cytoplasmic domain between the fifth and sixth membrane-spanning regions and an intracellular C terminus. Adapted from Hosey, (1992).*

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5.2 EXPERIMENTAL PROCEDURES

5.2.1 Dissociated cultures of the VM: preparation and staining

Suspensions of ventral mesencephalon cells from E14 rats were prepared as described in Chapter 2 (Section 2.2.2). Cultures were chronically treated for 1, 3 or 7 days with media supplemented with test substances. Untreated cultures served as controls. Every 48 hours 150 µl of media was removed and replaced with 150 µl of fresh media containing, where appropriate the test substance. Cultures were fixed and processed for TH immunoreactivity as described in Chapter 2 (Section 2.2.3) at days 1, 3 and 7 *in vitro.*

5.2.2 Experimental design

Two separate groups of experiments were carried out in which the culture medium was supplemented with (1) nicotine or carbachol, or (2) BW284c51, a mixture of nAChR antagonists consisting of methyllcaconitine, mecamylamine and di-hydro-β-erythroidine ('mixture'), or a combination of BW284c51 and mixture. Concentrations of test substance used are given in Tables 5.1. Experiments were repeated a minimum of three times using foetuses from different mothers. Each treatment group contained a minimum of 4 chamber wells, giving a total of at least 12 cultures for each treatment group ($n \geq 12$).

5.2.2.1 Experiment 1:

Test substance	Concentration
Nicotine	0.1 µM, 10 µM
Carbachol	0.1 µM, 10 µM

5.2.2.2 Experiment 2:

Test Substance		Concentration
BW284c51		10 μM, 100 μM
Mixture {	Methyllcaconitine	10 nM
	Mecamylamine	10 nM
	Di-hydro-β-erythroidine	100 nM
Mixture + BW284c51		Concentrations of nAChR antagonists in mixture as above + 10 μM or 100 μM BW284c51

Tables 5.1 Concentrations of test substances used in the two experiments

Experiment 1: Nicotine and carbachol were used in order to mimic the effects that elevated ACh levels may have upon neurite outgrowth and cell survival of embryonic dopaminergic cells in dissociated cultures.

Experiment 2: A mixture of nAChR antagonists was used to investigate whether inhibition of nAChR alleviated the toxic effects of BW284c51 on embryonic dopaminergic cells in dissociated cultures.

5.2.3 Collection of data and statistical analysis

5.2.3.1 Cell numbers

Total cell numbers per culture well were counted for each treatment group at each time point. For all experiments involving treatment of cultures with test substances, data was normalised to the untreated control.

5.2.3.2 Neurite length

Camera Lucida drawings were made of every TH+ve cell in the chamber-slide well for each treatment group at each time point (Chapter 2, Section 2.2.4.2). Length of each cell's longest neurite was assessed using a sports route planner and results for each treatment group were normalised to the untreated control.

For both parameters, cell numbers and neurite length, all data was collected in a blind fashion to prevent sampling bias. For both parameters, values for each data group were expressed as mean ± s.e.m. Statistical significance was assessed using either analysis of variance (ANOVA) for normally distributed data followed by a Dunnett's multiple comparison t-test, or the Kruskal-Wallis non-parametric test for non-normally distributed data.

5.2.4 Source of test chemicals

BW284C51, carbachol, methyllcaconitine citrate salt, mecamylamine hydrochloride and (-)-nicotine were obtained from Sigma-Aldrich Company Ltd. (Poole, UK). Di-hydro-β-erythroidine was obtained from Research Biochemicals International (USA). Stock solutions of all test substances (10 mM) were made in distilled water, aliquoted and frozen at -20°C. All test substances were diluted in culture medium immediately prior to use.

5.3 RESULTS

5.3.1 Experiment 1: The effect of nicotine and carbachol on TH+ve neurons in dissociated embryonic cultures of the VM

The data obtained for the effects of treatment with nicotine and carbachol on cell survival and neurite outgrowth of dopaminergic cells at days 1, 3 and 7 *in vitro*, normalised to the no treatment control, is shown in Table 5.2

Treatment	Number of TH+ve cells (normalised to untreated control)			Neurite length (normalised to untreated control)		
	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7
Untreated control	1.0 ± 0.16	1.0 ± 0.08	1.0 ± 0.15	1.0 ± 0.05	1.0 ± 0.05	1.0 ± 0.09
0.1 μM Nicotine	1.18 ± 0.17	0.73 ± 0.09	0.97 ± 0.15	1.13 ± 0.17	0.94 ± 0.08	1.35 ± 0.13
10 μM Nicotine	1.22 ± 0.17	0.79 ± 0.10	1.44 ± 0.18	1.03 ± 0.11	0.90 ± 0.09	1.19 ± 0.1
0.1 μM Carbachol	0.73 ± 0.11	0.77 ± 0.09	1.14 ± 0.14	1.11 ± 0.19	0.93 ± 0.14	1.24 ± 0.14
10 μM Carbachol	1.21 ±0.18	0.89 ± 0.11	0.72 ± 0.11	1.44 ± 0.14	1.33 ± 0.15	0.89 ± 0.14

Table 5.2 Experiment 1: The effect of nicotine and carbachol on TH+ve neurons in dissociated embryonic cultures of the VM.

Values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. Statistical significance was assessed by the Kruskal-Wallis non-parametric test. $n \geq 12$.

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5.3.1.1 The effect of nicotine and carbachol on TH+ve cell numbers

There were no significant changes in total TH+ve cell number per culture from the untreated control with nicotine or carbachol at any of the time points (Figure 5.2 A, B and C).

5.3.1.2 The effect of nicotine and carbachol on TH+ve neurite outgrowth

There were no significant changes in TH+ve neurite outgrowth from the untreated control following incubation of embryonic dissociated VM cultures with nicotine or carbachol at any of the time points (Figure 5.3 A, B and C).

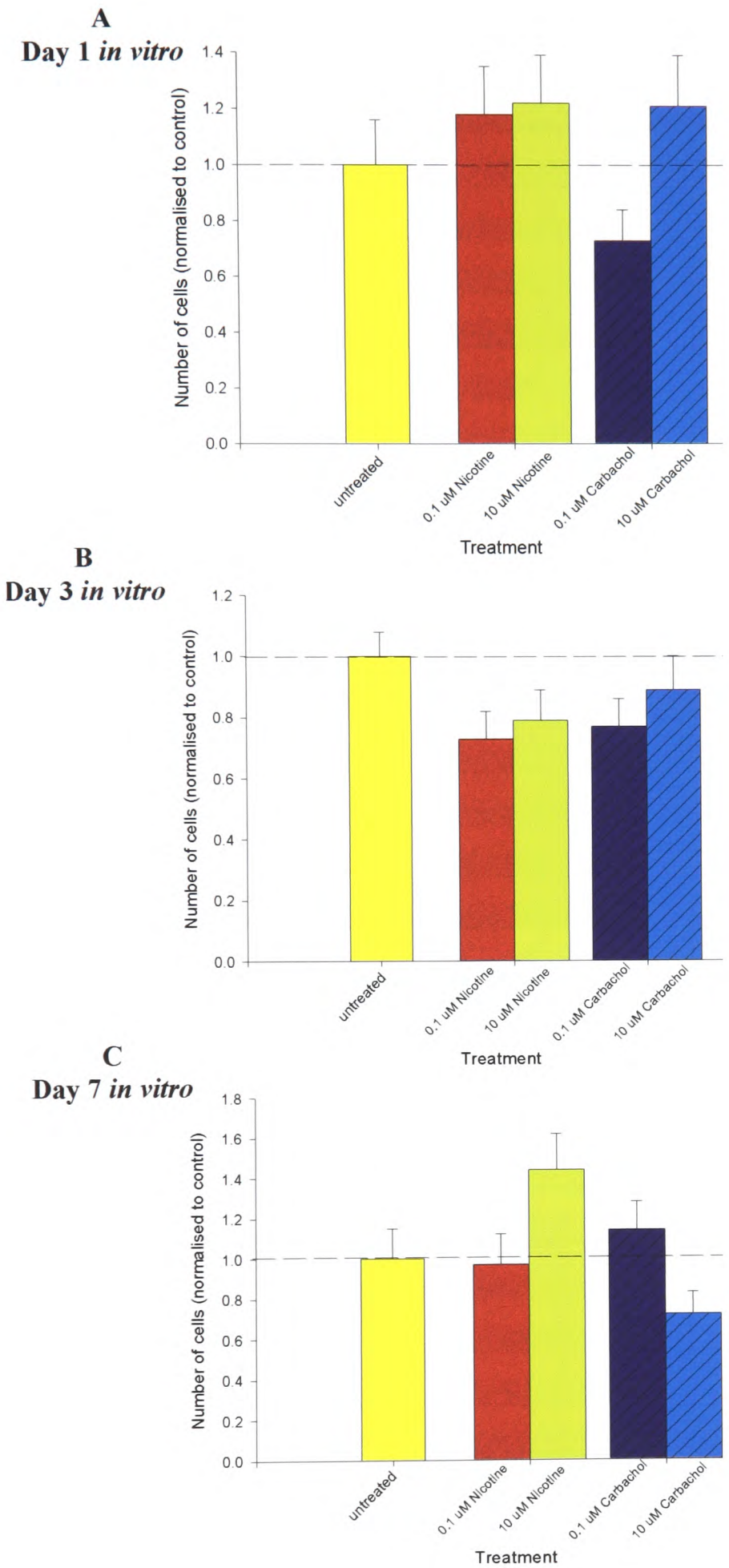

Figure 5.2 Effects of nicotine and carbachol on TH+ve cell numbers.

There were no significant changes in total TH+ve cell number from the untreated control with either nicotine or carbachol at any of the time points examine. Statistical significance was tested for using the Kruskal-Wallis non-parametric test. Values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. $n \geq 12$.

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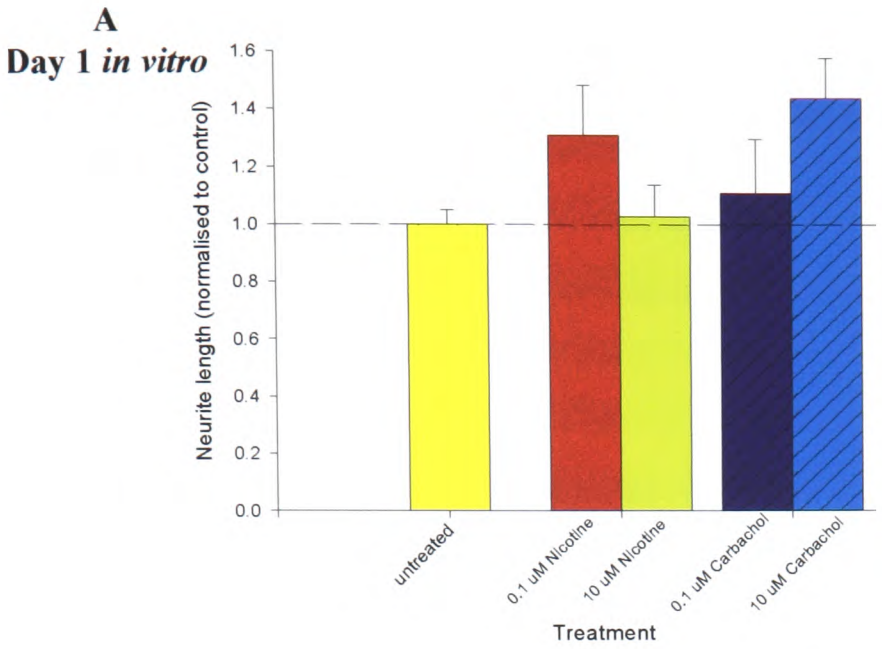

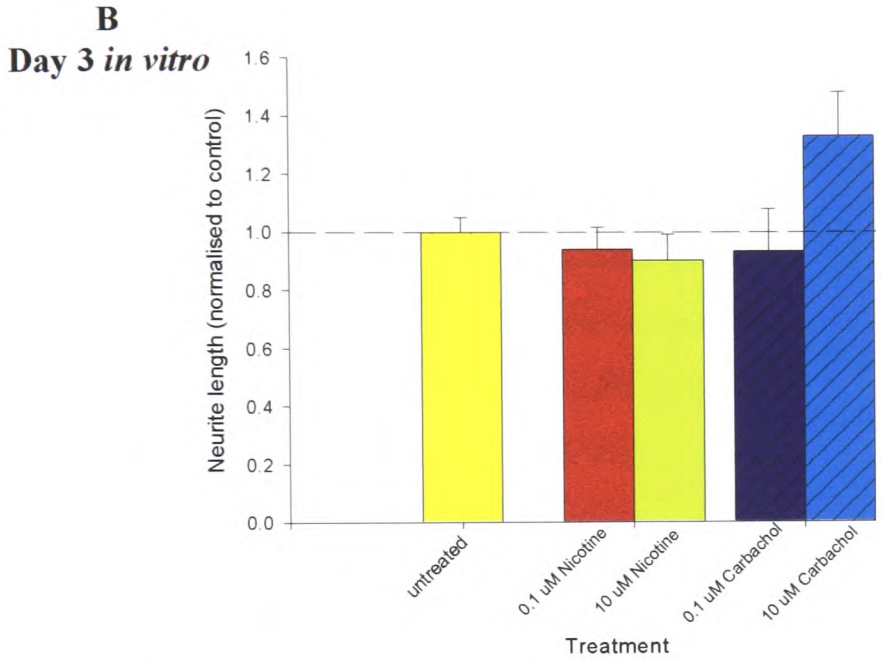

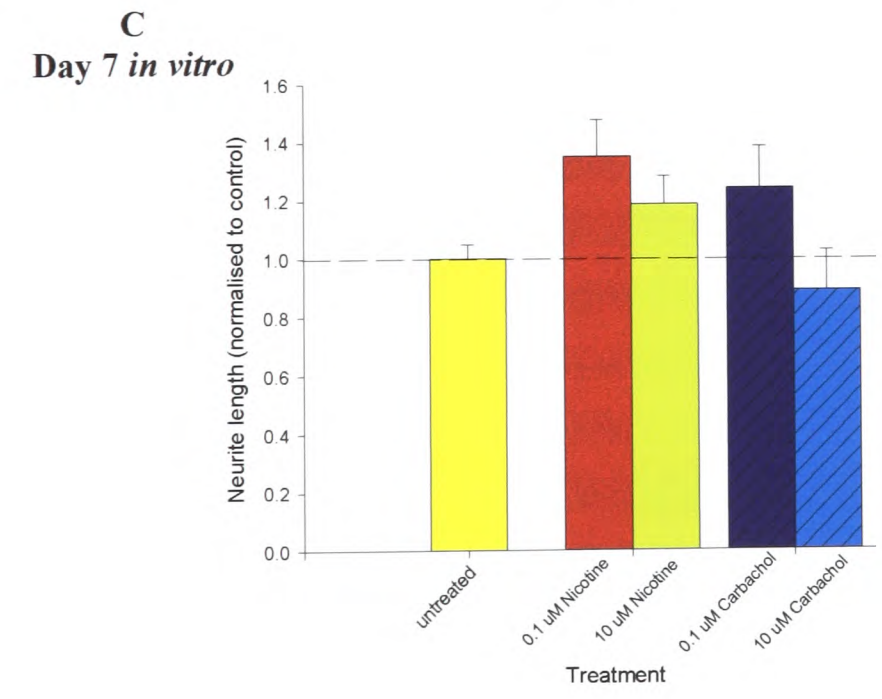

Figure 5.3 Effects of nicotine and carbachol on neurite outgrowth of TH+ve cells.

There were no significant changes in neurite outgrowth from the untreated control with either nicotine or carbachol at any of the time points examine. Statistical significance was tested for using the Kruskal-Wallis non-parametric test. Values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. $n \geq 12$.

******

5.3.2 Experiment 2: The effect of a mixture of nAChR antagonists on the toxicity caused by BW284c51 to TH+ve neurons in dissociated embryonic cultures of the VM

The data obtained for the effects of treatment with BW284c51, the mixture of nAChR antagonists alone, or the mixture in conjunction with BW284c51, on cell survival and neurite outgrowth of dopaminergic cells at days 1, 3 and 7 *in vitro,* normalised to the untreated control, is shown in Table 5.3

Treatment	Number of TH+ve cells (normalised to untreated control)			Neurite length (normalised to untreated control)		
	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7
Untreated control	1.0 ± 0.13	1.0 ± 0.17	1.0 ± 0.13	1.0 ± 0.05	1.0 ± 0.07	1.0 ± 0.06
10 μM BW284c51	0.89 ±0.09	0.57 ± 0.07	0.25 ± 0.09***	0.91 ± 0.06	0.73 ± 0.06*	0.58 ± 0.09*
100 μM BW284c51	0.85 ± 0.07	0.34 ± 0.06***	0.16 ± 0.05***	0.71 ± 0.05**	0.34 ± 0.04***	0.11 ± 0.08***
Mixture	1.03 ±0.11	1.03 ± 0.12	0.83 ± 0.14	1.12 ± 0.08	1.0 ± 0.06	1.08 ± 0.1
10 μM BW284c51 + Mixture	1.05 ±0.16	0.82 ± 0.06	0.48 ± 0.09**	1.03 ± 0.07	1.19 ± 0.11	0.93 ± 0.14
100 μM BW284c51 + Mixture	0.95 ±0.17	0.73 ± 0.07	0.02 ± 0.02***	0.91 ± 0.07	1.05 ± 0.17	0.14 ± 0.06***

* denotes $P < 0.05$
** denotes $P < 0.01$
*** denotes $P < 0.001$

Table 5.3 Experiment 2: The effect of a mixture of nAChR antagonists upon the toxicity caused by BW284c51 application to TH+ve neurons in dissociated embryonic cultures of the VM.

'Mixture' consisted of methyllcaconitine (an α_7 nAChR antagonist; 10 nM), mecamylamine (an α_3/β_2 nAChR antagonist; 10 nM), and di-hydro-β-erythroidine (an α_4/β_2 nAChR antagonist; 100 nM). Values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. Statistical significance was assessed by the Kruskal-Wallis non-parametric test. $n \geq 12$.

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5.3.2.1 The effect of BW284c51, mixture, and BW284c51 + mixture on TH+ve cell numbers

At day 1 *in vitro* (24 hour exposure to test substances) Figure 5.4A, there were no significant changes in total TH+ve cell number from the untreated control. At day 3 *in vitro* (72 hour exposure to test substance) Figure 5.4B, 100 μM BW284c51 had significantly decreased the total number of TH+ve cells in culture in comparison to the untreated control ($P < 0.001$; Kruskal-Wallis non-parametric test). There were no effects upon cell numbers with the other test substances at this time point. By day 7 *in vitro* (168 hour exposure to test substances) Figure 5.4C, both 10 μM and 100 μM BW284c51 had significantly decreased the total number of TH+ve cells per culture ($P < 0.001$ for both; Kruskal-Wallis non-parametric test). The mixture had no effect upon cell numbers, but incubation of cultures with mixture and 10 μM and 100 μM BW284c51 had both reduced cell numbers ($P < 0.01$ and $P < 0.001$ respectively; Kruskal-Wallis non-parametric test).

5.3.2.2 The effect of BW284c51, mixture, and BW284c51 + mixture on TH+ve neurite outgrowth

At day 1 *in vitro* (24 hour exposure to test substances) Figure 5.5A, addition of 100 μM BW284c51 to the culture medium had significantly decreased dopamine cell neurite outgrowth in comparison to the untreated control ($P < 0.01$; Kruskal-Wallis non-parametric test). No effect upon outgrowth was seen with 10 μM BW284c51, mixture, or a combination of BW284c51 and mixture. At day 3 *in vitro* (72 hour exposure to test substance) Figure 5.5B, both 10 μM and 100 μM BW284c51 had significantly decreased neurite outgrowth in comparison to the untreated control ($P < 0.05$ and $P < 0.001$ respectively; Kruskal-Wallis

non-parametric test). There were no effects upon neurite outgrowth with 10 μM BW284c51, mixture or a combination of BW284c51 and mixture at this time point. At day 7 *in vitro* (168 hour exposure to test substances) Figure 5.5C, addition of 10 μM and 100 μM BW284c51 had significantly decreased neurite outgrowth in comparison to the untreated control ($P < 0.05$ and $P < 0.001$ respectively; Kruskal-Wallis non-parametric test). No effect upon outgrowth was seen upon addition of mixture or 10 μM BW284c51 when co-applied with mixture. 100 μM BW284c51 co-applied with mixture had significantly decreased neurite outgrowth ($P < 0.001$; Kruskal-Wallis non-parametric test). Photographic representations of various treatments are shown alongside relevant figures.

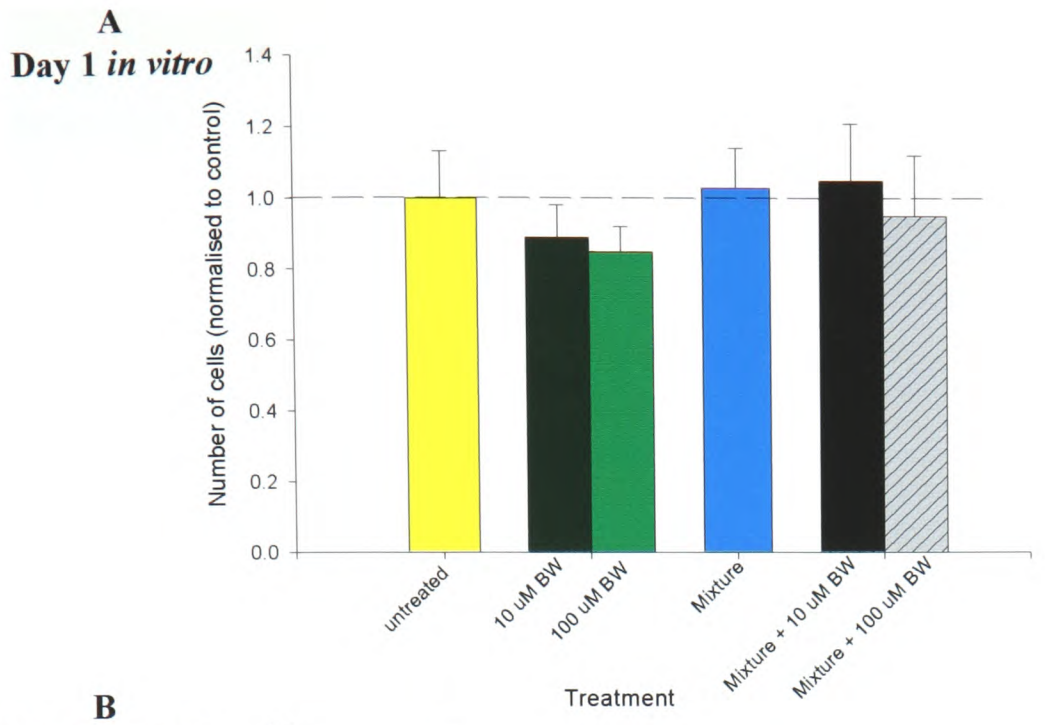

Figure 5.4 The effect of mixture and BW284c51 + mixture on TH+ve cell numbers.

At day 1 in vitro (24 hour exposure to test substances) ***A****, there were no significant changes in total TH+ve cell number per culture from the untreated control. At day 3 in vitro (72 hour exposure to test substance)* ***B****, 100 μM BW284c51 had significantly decreased the total number of TH+ve cells in culture in comparison to the untreated control (*** denotes $P < 0.001$; Kruskal-Wallis non-parametric test). By day 7 in vitro (168 hour exposure to test substances)* ***C****, 10 μM and 100 μM BW284c51 had significantly decreased the total number of TH+ve cells per culture ($P < 0.001$ for both; Kruskal-Wallis non-parametric test). Incubation of cultures with mixture and 10 μM and 100 μM BW284c51 had also reduced cell numbers (**denotes $P < 0.01$, and $P < 0.001$ respectively; Kruskal-Wallis non-parametric test). Mixture alone had no effect upon cell numbers at any of the time points. Values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. BW = BW284c51. Mixture = methyllcaconitine (10 nM), mecamylamine (10 nM), and di- hydro-β-erythroidine (100 nM). $n \geq 12$.*

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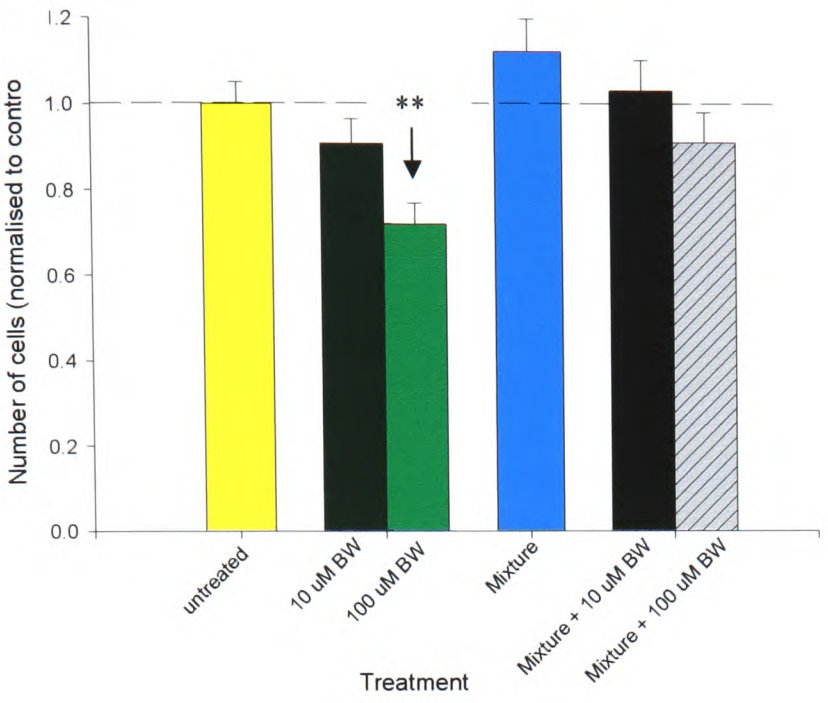
A
Day 1 *in vitro*



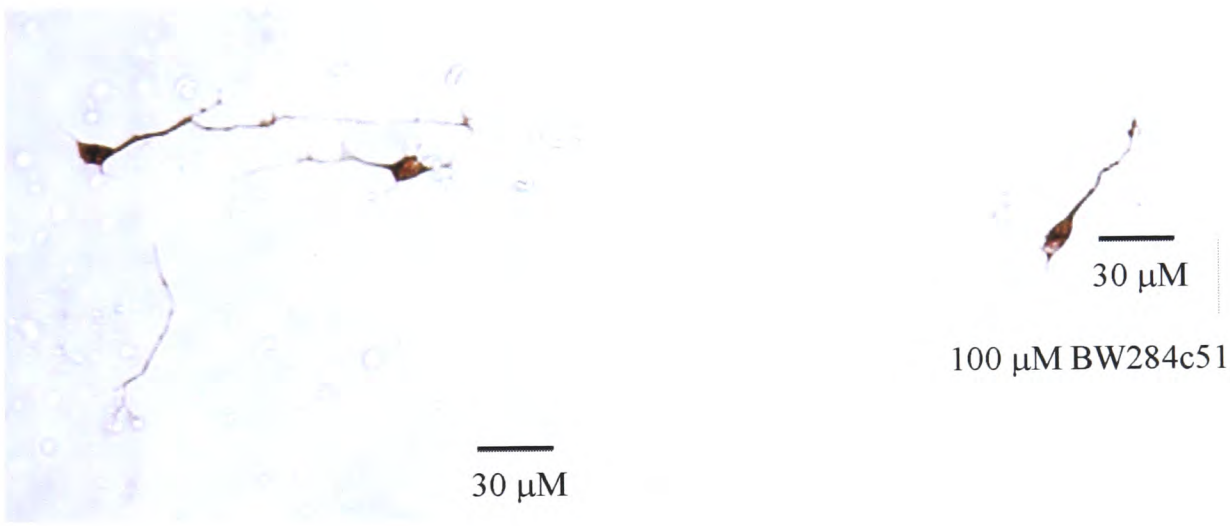


Figure 5.5A The effect of mixture and BW284c51 + mixture on neurite outgrowth of TH+ve cells. Day 1 *in vitro* (24 hour exposure to test substances).

*Addition of 100 μM BW284c51 had significantly decreased neurite outgrowth in comparison to the untreated control (** denotes $P < 0.01$; Kruskal-Wallis non-parametric test). No effect upon outgrowth was seen with 10 μM BW284c51, mixture or a combination of BW284c51 and mixture. Graphical values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. BW = BW284c51. Mixture = methyllcaconitine (10 nM), mecamylamine (10 nM), and di- hydro-β-erythroidine (100 nM). $n \geq 12$.*

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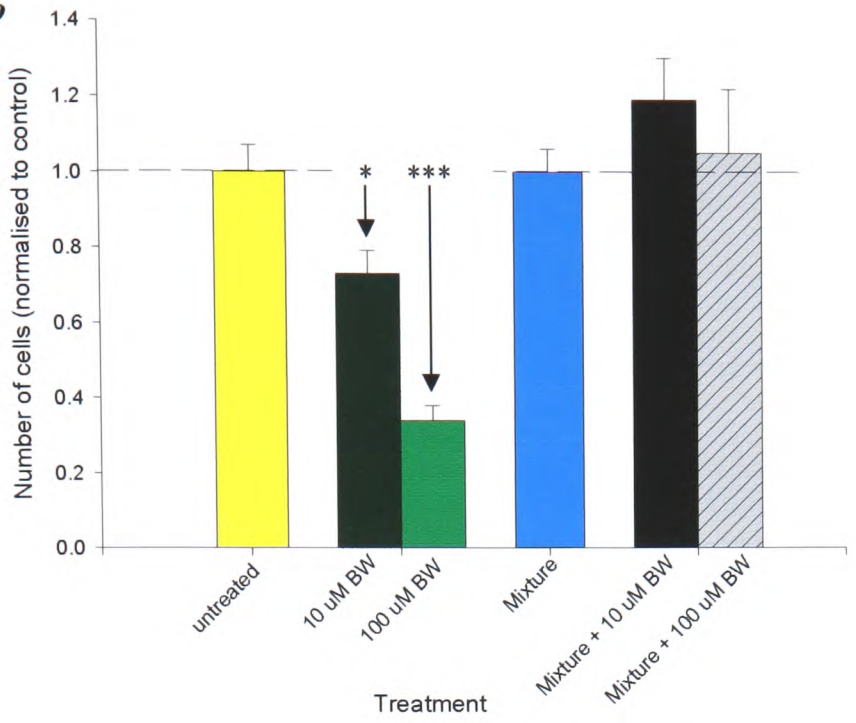
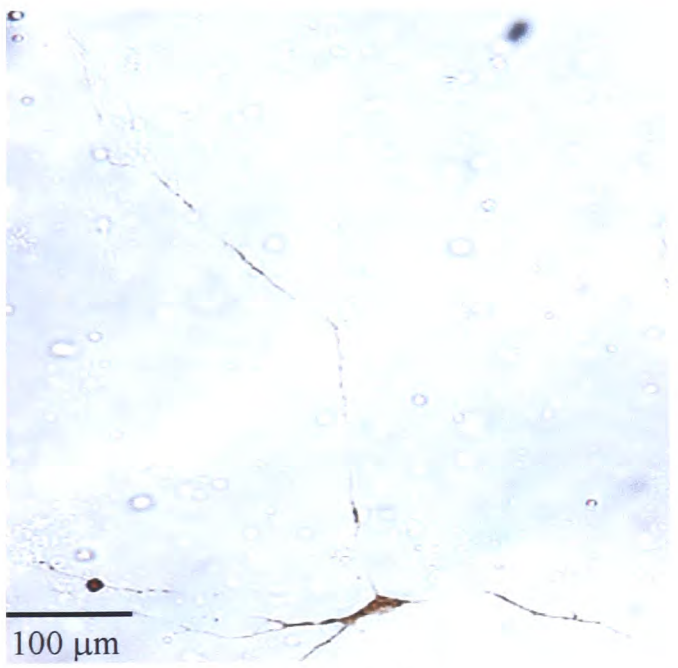
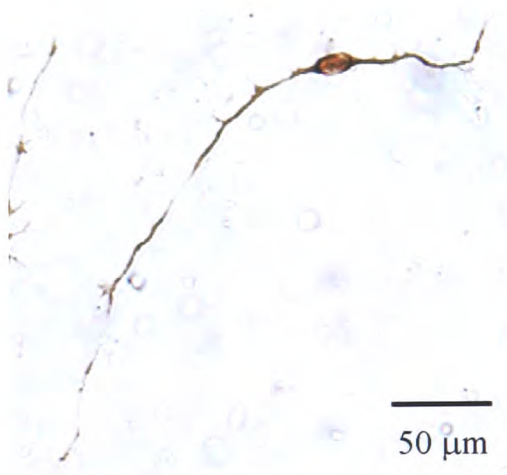
B
Day 3 *in vitro*

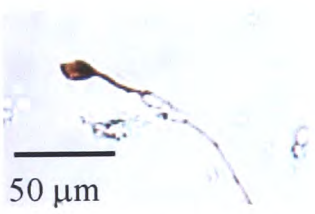




Untreated



10 µM BW284c51



100 µM BW284c51

Figure 5.5B The effect of mixture and BW284c51 + mixture on neurite outgrowth of TH+ve cells. Day 3 *in vitro* (72 hour exposure to test substances).

Addition of 10 µM and 100 µM BW284c51 had significantly decreased dopamine cell neurite outgrowth in comparison to the untreated control (denotes $P < 0.05$ and *** denotes $P < 0.001$ respectively Kruskal-Wallis non-parametric test). There were no effects upon outgrowth with 10 µM BW284c51, mixture or a combination of BW284c51 and mixture at this time point. Graphical values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. BW = BW284c51. Mixture = methyllcaconitine (10 nM), mecamylamine (10 nM), and di- hydro-β-erythroidine (100 nM). $n \geq 12$.*

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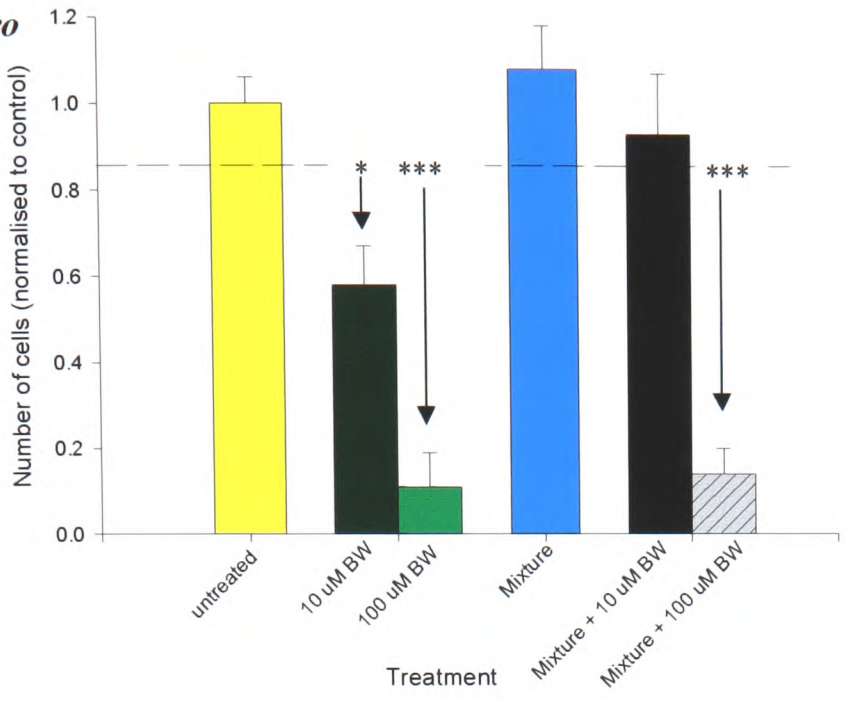
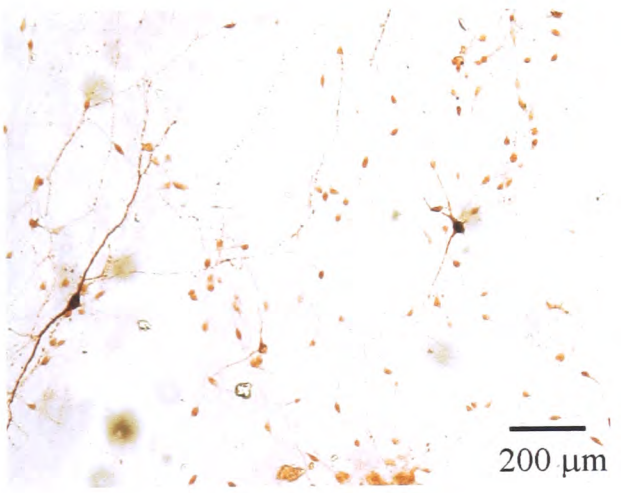
C

Day 7 *in vitro*

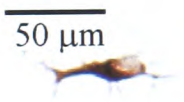




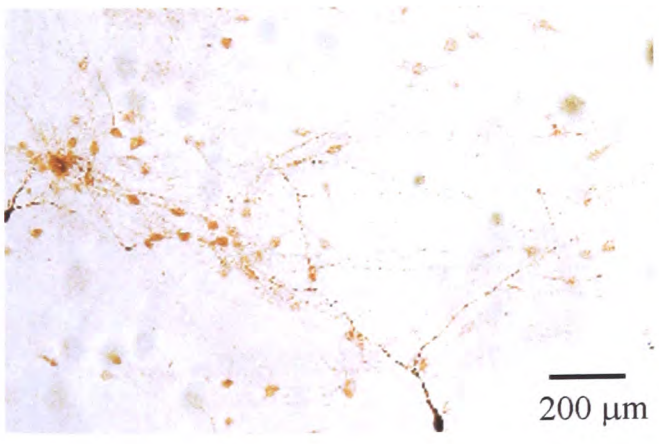
Untreated



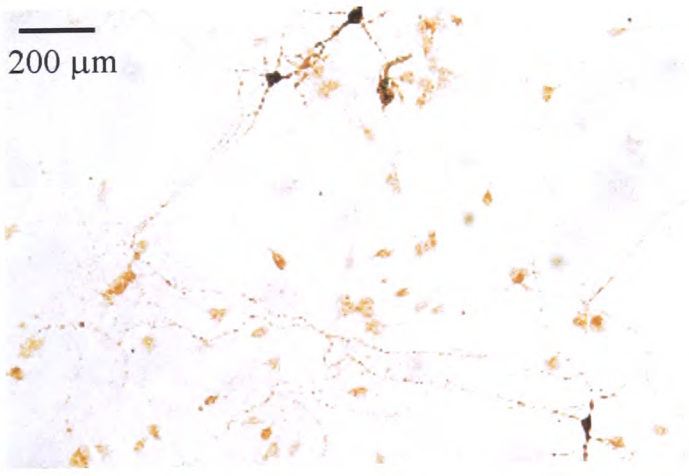
10 µM BW284c51



100 µM BW284c51



Mixture



10 µM BW284c51 + mixture



100 µM BW284c51
+ mixture

Figure 5.5C The effect of mixture and BW284c51 + mixture on neurite outgrowth of TH+ve cells. Day 7 *in vitro* (168 hour exposure to test substances)

Addition of 10 µM and 100 µM BW284c51 had significantly decreased dopamine cell neurite outgrowth in comparison to the untreated control (denotes $P < 0.05$ and *** denotes $P < 0.001$ respectively Kruskal-Wallis non-parametric test). No effect upon outgrowth was seen upon addition of mixture or 10 µM BW284c51 when co-applied with mixture. 100 µM BW284c51 co-applied with mixture significantly decreased neurite outgrowth ($P < 0.001$). Graphical values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. BW = BW284c51. Mixture = methyllcaconitine (10 nM), mecamylamine (10 nM), and di- hydro-β-erythroidine (100 nM). $n \geq 12$.*

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5.4 DISCUSSION

The detrimental effects of BW284c51, an inhibitor of the active site and PAS of AChE on neurite outgrowth and cell survival, when active site inhibitor echothiophate had no effect upon either parameter, implicates the PAS in the non-cholinergic effects of AChE (Jones *et al.*, 1995; Johnson and Moore, 1999). However, the severity of the effects of BW284c51 on cell survival and neurite outgrowth leads to the question of whether the toxicity caused by BW284c51 is due to direct inhibition of an essential property of the AChE molecule (potentially the PAS) or whether its actions are causing a separate effect that is detrimental to cell survival and outgrowth. The latter proposal presents two possibilities. Firstly, the suggestion by Postuma *et al.* (1999), that the toxicity of BW284c51 may be a non-specific toxic effect, or secondly, that the inhibition of AChE by BW284c51 may result in elevated ACh levels that, through an effect upon AChR results in a decrease in cell numbers and neurite outgrowth.

As discussed in Chapter 4, to definitively investigate the involvement of the PAS of AChE in neurite outgrowth and survival, a specific PAS inhibitor that does not affect catalytic activity is required, and, as yet, is not available. The demonstration by experiments in Chapter 4 that a low concentration (10 μM) of BW284c51 markedly reduces cell survival and neurite outgrowth suggests against a non-specific toxic effect of BW284c51. Thus, this chapter addresses the toxicity of BW284c51 by adopting an alternative approach; attempting to demonstrate that the detrimental effects of BW284c51 on cell survival and neurite outgrowth are not due to elevated ACh levels.

The obvious repercussion of inhibiting endogenous AChE is an increase in the amount of ACh, which will then act upon nicotinic and muscarinic AChR. Several studies have suggested that ACh can act as an inhibitory growth signal, including Lipton *et al.* (1988) who demonstrated that addition of various nicotinic antagonists enhanced neurite outgrowth of retinal ganglion cells, and Small *et al.* (1995) who found that acetylcholine and carbachol inhibited neurite outgrowth. As an excitatory neurotransmitter, immoderate levels of ACh also have the potential to cause cell death through excitotoxicity. As discussed in Chapter 1 (Section 1.2.3.3.ii), excitotoxicity occurs when excessive levels of an excitatory neurotransmitter cause overstimulation of its receptors. This results in an increase in intracellular calcium, which can activate calcium-dependent enzymes leading to cell death. Such a role for ACh is understood to underlie the highly toxic effect of the nerve gas Soman (pinacolymethylphosphonofluoridate) which irreversibly inhibits AChE (Sterling *et al.*, 1988; Zimmer *et al.*, 1998). It has also been shown that ACh potentiates glutamate-induced neurodegeneration in cultured hippocampal neurons (Mattson, 1989).

As discussed in Chapter 1 (Section 1.1.2.3), the SN receives cholinergic afferentation from the pedunculopontine nucleus. The presence of nAChR in the SN (Naeff *et al.*, 1992), albeit at low levels, suggests that dopaminergic cells of the SN are responsive to ACh. In accordance with this, intravenous administration of nicotine during *in vivo* extracellular recordings in the SN has been shown to increase firing rate of dopaminergic cells of the SNpc (Clarke *et al.*, 1985). As the VM preparation described in this thesis comprises several brain structures including the pedunculopontine nucleus, cultures used in the studies presented in this thesis will contain both cholinergic and cholinoceptive neurons (Chapter 2, Section 2.1.3.1). It is therefore feasible that the effects of BW284c51 on neurite outgrowth

and cell survival seen in Chapter 4 may have been a result of elevated ACh levels due to inhibition of AChE by BW284c51.

Thus, the first experiment presented in this chapter investigated the effects of AChR agonists nicotine and carbachol on neurite outgrowth and cell survival in order to mimic the effects that elevated ACh may have. Secondly, nAChR were inhibited using a 'mixture' of nicotinic receptor antagonists consisting of methyllcaconitine, mecamylamine and di-hydro-β-erythroidine in combination with BW284c51 application. The aim of this was to prevent the effects that elevated ACh may have upon nAChR and examine whether this alleviated BW284c51 toxicity.

5.4.1 Nicotine and carbachol

Addition of 0.1 μM or 10 μM nicotine or 0.1 μM or 10 μM carbachol had no detrimental effect upon cell numbers or neurite outgrowth, suggesting that AChR stimulation is not the mechanism of BW284c51 toxicity. However, it is known that both muscarinic and nicotinic AChR desensitise very rapidly (Hosey, 1992; Small and Reed, 1995; Holroyd *et al.*, 1999; Reitsetter *et al.*, 1999). Desensitisation is a phenomenon that was first described by Katz and Thesleff in 1957 and results from prolonged exposure of a receptor to an agonist, which produces an inactive state so that the receptor no longer permits ion passage across the channel. This means that the lack of effect upon parameters of cell health seen in experiment 1 of this chapter may be attributable to desensitisation of AChR, thus preventing excessive stimulation by ACh, which would lead to inhibition of neurite outgrowth and / or cell death.

5.4.2 A mixture of nicotinic receptor antagonists

As an alternative means of addressing the question of BW284c51 toxicity, a mixture of nAChR antagonists, in combination with BW284c51 was added to culture medium. It was decided to use antagonists that inhibit nAChRs due to the presence of these receptor subtypes upon TH+ve cells of the substantia nigra (Deneris *et al.*, 1991; Naeff *et al.*, 1992; Del Toro *et al.*, 1994). In contrast to this, the substantia nigra of many species has been determined by autoradiography to have a low level of mAChR (Cortés *et al.*, 1986; Cortés and Palacios, 1986; Spencer *et al.*, 1986; Peacock *et al.*, 1988; Miyoshi *et al.*, 1989) most of which have been localised to the pars reticulata, particularly during development (Natsuk and Graybiel, 1991). In support of this, Levin *et al.* (1994) illustrated that muscarinic antagonists had no effect on choice accuracy when infused into the substantia nigra of rats, but nicotinic antagonists impaired memory performance. It was therefore considered appropriate to inhibit nAChR, as they would constitute the principal AChR subtype on dopamine cells within the preparation.

The results presented in this chapter demonstrate that incubation of cultures with the mixture of nAChR antagonists by itself had no effect upon neurite outgrowth or cell survival. However, co-incubation of cultures with BW284c51 and the mixture of nAChR antagonists ameliorated the deleterious effects of 24 and 72 hour exposure to 100 μM BW284c51 on neurite outgrowth, and the detrimental effects on neurite outgrowth of exposure to 10 μM BW284c51 for 72 and 168 hours. Similarly, co-incubation of BW284c51 with the mixture of nAChR antagonists prevented the reduction in cell numbers by 100 μM BW284c51 at day 3 *in vitro*. These data suggest that detrimental effects of short-term (24-72 hour) incubation

with 100 μM of BW284c51 and chronic incubation with 10 μM BW284c51 are repercussions of elevated ACh.

Conversely, the mixture failed to prevent the chronic (7 day; 168 hour exposure) effects of 100 μM BW284c51 on neurite outgrowth or cell numbers. Similarly, co-incubation of BW284c51 with the mixture of nAChR antagonists for 168 hours (day 7 *in vitro*) reduced the loss in cell number caused by 10 μM BW284c51 by only one level of significance. These findings suggest that the chronic detrimental effects of both 10 μM and 100 μM BW284c51 may not be a result of elevated ACh.

However, certain weaknesses in the mixture experiments described in this chapter must be considered. The antagonists used in the mixture, mecamylamine, methyllcaconitine and di-hydro-β-erythroidine are competitive antagonists of nAChRs. It is possible that as time in culture progressed and ACh levels increased, competition for binding sites between ACh and inhibitors occurred, and a sufficient amount of ACh reached receptor sites to cause excitotoxicity. Furthermore, as muscarinic AChR were not antagonised by the inhibitors used, the possibility that excitotoxicity was mediated by mAChR, that were situated either on dopamine cells, or on non-TH+ve cells in the culture which then had detrimental effects upon the TH+ve population, cannot be discounted.

Nevertheless, given the differences in effect on short-term and long-term exposure of cultured embryonic TH+ve cells to BW284c51 presented in this chapter, it could be

postulated that the effects of BW284c51 are a result of a synergy between elevated ACh levels and interference with a site on the AChE molecule, potentially the PAS.

As discussed in Chapter 4, the definitive answer to the question of whether the trophic actions of AChE and the toxic actions of BW284c51 are mediated solely through the PAS requires a specific PAS inhibitor that does not affect catalytic activity. As yet, there are no such inhibitors available, although work is currently being carried out by Professor S. Greenfield's group to modify the snake toxin fasciculin, so that it will bind to the PAS without obstructing the catalytic gorge (Dr. S. Emmett, personal communication). If this should come to fruition, it will serve as a very useful tool for further investigating the mechanism of AChE's non-cholinergic actions.

The following chapters return to the question of the trophic actions of BuChE and AChE upon embryonic dopaminergic cells, as demonstrated in Chapter 3 of this thesis. As discussed in Chapter 4, it is hard to see how the trophic effects of AChE on neurite outgrowth may be mediated solely through the PAS when BuChE, which has been shown to enhance neurite outgrowth under identical culture conditions, has no PAS. A novel mechanism of action of BuChE and AChE is thus proposed, based upon the findings of Jia *et al.* (1999), who demonstrated an acute mechanism of action of the well-known trophic agent, NGF, on voltage-dependent calcium channels (VDCC) of PC12 cells. These findings inspired the hypothesis that BuChE and AChE may increase neurite outgrowth by modulating intracellular calcium levels through VDCC. Consequently, a whole-cell patch-clamp technique was established in order to investigate the effects of BuChE and AChE upon voltage-dependent calcium currents elicited from dissociated cells in embryonic VM cultures

being used in this thesis. An introduction to the role of calcium in neurite outgrowth and VDCC is provided in the next chapter, followed by details of the characterisation of VM cells by whole-cell patch clamp and the subsequent effects of application of BuChE and G_4- and G_1-AChE.

CHAPTER 6

Introduction to Chapters 7, 8 and 9: VDCC, and the role of calcium, and VDCC in development

OVERVIEW

Numerous lines of evidence support a non-cholinergic, trophic role for AChE and BuChE in neurite outgrowth (Layer *et al.* 1993; Dupree and Bigbee, 1994; Jones *et al.*, 1995; Holmes *et al.*, 1997; Srivatsan and Peretz, 1997; Sharma and Bigbee, 1998). However, the mechanism of action of these trophic effects has not been definitively elucidated. As discussed previously (Chapter 1, Section 1.3.4.1.3.iii), a CAM-mediated action of BuChE and AChE has been proposed as being responsible (Layer and Kaulich 1991, Layer *et al.*, 1993; Grifman *et al.* 1998; Sharma and Bigbee, 1998). However, this is not consistent with the data of Holmes *et al.* (1997) which demonstrated that BuChE had no effect upon neurite outgrowth in organotypic preparations of the SN. A novel mechanism of action for the trophic effects of AChE and BuChE on neurite outgrowth of embryonic dopaminergic cells is thus suggested. It has been shown in studies on various cell types that the growth factors NGF and bFGF affect conductance through voltage-dependent calcium channels (VDCC) (Koike *et al.* 1989; Rogers and Hendry, 1990; Nikodijevic and Guroff, 1992; Levine *et al.*, 1995; Shitaka *et al.*, 1996; Collings *et al.*, 1999; Jia *et al.*, 1999). Modification of VDCC conductance results in modulation of intracellular calcium concentration $[Ca]_i$. $[Ca]_i$ is fundamental to many developmental processes including those governing neurite outgrowth and cell survival. Thus, alteration of $[Ca]_i$ by the actions of NGF and bFGF on VDCC is thought to be at least partly responsible for the trophic effects of these substances on cell survival and neurite outgrowth (Koike *et al.* 1989; Rogers and Hendry, 1990; Nikodijevic and Guroff, 1992; Levine *et al.*, 1995; Shitaka *et al.*, 1996; Collings *et al.*, 1999; Jia *et al.*, 1999). It is therefore proposed that cholinesterases may affect neurite outgrowth of developing cells through a similar mechanism, wherein AChE and BuChE alter $[Ca]_i$ through modulation of VDCC conductance. This hypothesis forms the basis for the investigations

presented in Chapters 7, 8 and 9. This chapter, therefore, provides an overview firstly of the structure and function of VDCC, secondly of the involvement of calcium and VDCC in neuronal development. Finally the evidence supporting a role for trophic factors in VDCC modulation will be detailed.

6.1 VOLTAGE-DEPENDENT CALCIUM CHANNELS (VDCC)

Voltage dependent calcium channels provide the primary route of calcium entry into the cell in response to depolarisation (Miller, 1987; Bean 1989). Six types of neuronal VDCC have been classified according to their electrophysiological and pharmacological properties; T, L, N, P, Q, and R (Hess, 1990; Zhang *et al.*, 1993; Tsien *et al.*, 1995). T-type channels are low-voltage activated (LVA) and are thought to be important in neuronal and cardiac pacemaker activity (Soong *et al.*, 1993). T-type channels are activated and inactivated more rapidly and at more negative membrane potentials than other calcium current types; the high-voltage activated calcium channels (HVA), L, N, P, Q and R require greater depolarisation for activation.

6.1.1 VDCC composition

Neuronal VDCCs are heteromeric complexes composed of three main subunits; α_1 (150-250 kDa), β (52-78 kDa), and $\alpha_2\delta$ (140-170 kDa) (Walker and De Waard, 1998; Hofmann *et al.*, 1999; Catterall, 2000; Figure 6.1A). The α_1 subunit shares topology with the pore-forming element of sodium and potassium channels and is important in determining channel type. It consists of four homologous transmembrane domains, each composed of six transmembrane spanning segments linked by variable cytoplasmic loops. The fourth transmembrane

segment of each domain has positively charged residues at every third amino acid of its α helix, which are sensitive to changes in voltage and thus important in channel gating (Hofmann *et al.*, 1999; Catterall, 2000). The pore is formed by the extracellular loops between transmembrane segments 5 and 6 of each domain, with each loop briefly entering the membrane to line the channel mouth (Figure 6.1B). Molecular cloning has identified eight neuronal α_1 subunit genes: α_{1A} - α_{1E},, which encode the HVA channels, and α_{1G} - α_{1I} which encode the LVA T-type channel (Hofmann *et al.*, 1999). As the T-type channel is inactivated at the voltages required to detect HVA calcium channels, it will not be considered further in this thesis.

The β subunit is entirely cytoplasmic. It forms a tight association with the cytoplasmic domain of the α_1 subunit. Four β subtypes, β_1-β_4 have been cloned and sequenced individually for the mammalian brain, and this heterogeneity is augmented by further splicing of the beta transcripts (Williams *et al.*, 1992; Kamp and Hell, 2000). Co-expression of a β subunit with various α_1 subunits increases peak current (Stea *et al.*, 1993; Kamp *et al.*, 1996). This is thought to occur by an increase in the number of functional surface membrane channels and by facilitation of channel pore opening (Neely *et al.*, 1993; Kamp *et al.*, 1996).

The highly glycosylated α_2-δ subunit consists of two disulphide-linked peptides encoded for by the same gene (Catterall, 1995; Hofmann *et al.*, 2000). It has been postulated that the α_2-δ subunit may facilitate or stabilise plasma membrane incorporation of the calcium channel complex and may play a functional role in channel regulation (Hofmann *et al.*, 1999).



Figure 6.1 Structural organisation of voltage-dependent calcium channels (VDCC).

A shows the topological organisation of the core subunits common to all VDCC types and the interactions between them. Sites of interaction between subunits are indicated by double-ended arrows (dashed arrows indicate a presumed interaction). Glycosylation sites (branched structures) are predicted sites only. From Walker and De Waard, (1998). ***B*** *The α_1 subunit comprises four homologous domains, each containing six transmembrane spanning helices and a pore forming regions (indicated in purple). Adapted from Stotz and Zamponi, (2001).*

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6.1.2 VDCC classification and pharmacology

Neuronal HVA VDCC can be classified according to physiological and pharmacological criteria (Table 6.1). L-type calcium channels are sensitive to dihydropyridine (DHP) agonists such as BAYK 8644, and DHP antagonists such as nicardipine and nifedipine (Bean, 1989; Regan *et al.*, 1991; Randall and Tsien, 1995; Zamponi, 1997). L-type channels exhibit little voltage-dependent inactivation but do show calcium-dependent inactivation (Catterall, 2000).

N-type channels undergo slow voltage-dependent inactivation (Nowycky *et al.*, 1985). They are specifically inhibited by the peptide toxin obtained from the piscivorous cone snail *Conus geographus,* ω-Conotoxin GVIA (GVIA) (Regan *et al.*, 1991; Randall and Tsien, 1995) and

are insensitive to modulation by DHPs (Williams *et al.*, 1992). Evidence suggests that N-type channels are largely responsible for the voltage-dependent release of neurotransmitters in many neurons (Miller, 1987; Hirning *et al.*, 1988; Takahashi and Momiyama, 1993).

P-type channels are insensitive to DHPs and GVIA (Regan *et al.*, 1991), but show marked sensitivity to ω-Agatoxin IVA (Aga IVA), a polypeptide isolated from the venom of the funnel web spider *Agelenopsis aperta* (Mintz *et al.*, 1992) with an IC_{50} value of ~1-10 nM in cerebellar purkinje neurons (Mintz *et al.*, 1992; Mintz and Bean, 1993) and cerebellar granule neurons (Randall and Tsien, 1995). P-type channels exhibit modest voltage-dependent inactivation and are relatively sustained during depolarisation (Stotz and Zamponi, 2001).

The Q-type channel is also inhibited by Aga IVA, but at higher concentrations than are observed for the P-type calcium channel currents (IC_{50} value of 90 nM in cerebellar granule neurons (Randall and Tsien, 1995). The Q-type channel has more rapid activation / inactivation kinetics than the P-type channel (Sather *et al.*, 1993).

The R-type or 'residual' channel can be isolated following inhibition of the L-, N-, P-, and Q-type channels by nifedipine, GVIA and Aga IVA respectively. The R-type channel is partly encoded for by the α_{1E} gene (Wilson *et al.*, 2000) and this component is sensitive to the polypeptide toxin SNX-482 from the tarantula *Hysterocrates gigas* (Newcomb *et al.*, 1998)

The whole-cell calcium current can be blocked by inorganic ions such as cadmium (Cd^{2+}) or cobalt (Hagiwara and Byerly 1981; Nachshen, 1985; Lansmann *et al.*, 1986).

Channel type	α_1 subunit subtype	Specific inhibitor
L	α_{1C} α_{1D}	Dihydropyridines
N	α_{1B}	ω-Conotoxin GVIA
P	α_{1A}	ω-Agatoxin IVA
Q	α_{1A}	ω-Agatoxin IVA
R	α_{1E} / ?	SNX-482 / -

Table 6.1 Classification of voltage-dependent calcium channel types

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6.2 THE ROLE OF CALCIUM AND VDCC IN DEVELOPMENT

The development of the nervous system takes place in two main stages. Firstly, an extensive preliminary network is formed, characterised by overproduction of processes and synaptic connections. This phase is followed by a stage of network refinement during which neurites are retracted and cells die. Approximately half of all neurons produced in the developing vertebrate nervous system die before adulthood as a result of this second stage of development, which is known as naturally occurring, or pre-programmed cell death (see Harris, 1981; Franklin and Johnson, 1992; Linden 1994).

One reason for such cell death is purported to be the matching of the size of the neuronal projection to the magnitude of its target field. This was suggested by Levi-Montalcini and Hamburger in the 1940s, following experiments that demonstrated that removal of a limb from a chick embryo leads to excessive death of the sensory neurons that would have innervated the limb (see Linden, 1994). It was proposed that neuronal pruning might occur as a result of target cells secreting a limited amount of neurotrophic factors, which bind to surface receptors of approaching neurites. Neurons that do not receive adequate amounts of neurotrophic factor die (Figure 6.2). The idea of developing neurons competing with each

other for a limited supply of a neurotrophic factor provided by the target tissue was called the 'neurotrophic hypothesis'. Subsequent discovery of NGF (Chapter 1 Section 1.2.4.3) and experiments illustrating that modification of the size of the target had a marked effect upon the size of the corresponding neural centre confirmed the basic principles of this idea (see Oppenheim, 1985; Yuen *et al.*, 1996 for reviews).

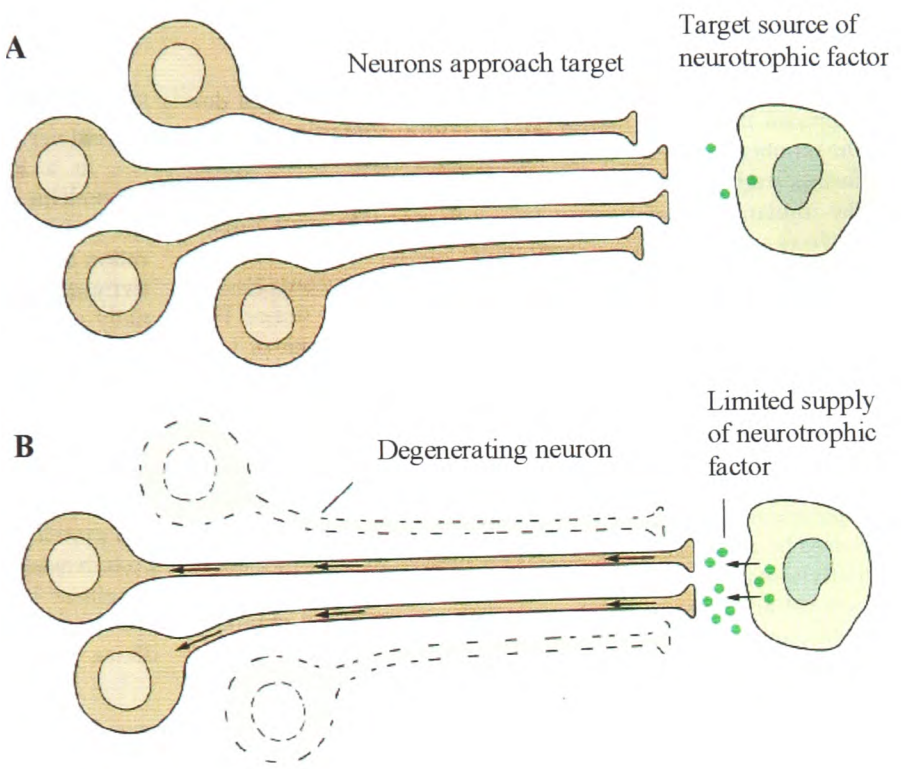

Figure 6.2 The neurotrophic factor hypothesis.

Neurons extend axons to the vicinity of target cells (A). The target cells secrete limited amounts of neurotrophic factors. Neurons that do not receive adequate amounts of neurotrophic factor die (B)

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A further governing factor in ontogenesis is electrical activity. Removal of afferent input to, or pharmacological blockade of synaptic transmission between developing neurons can cause cell death and neurite inhibition (Harris, 1981; Ramakers and Boer, 1990; Sherrard and Bower, 1998). The importance of electrical activity was first shown by Scott and Fischer three decades ago, who illustrated that the survival of embryonic chicken dorsal root ganglion neurons in culture was enhanced when they were maintained in a medium

containing elevated concentrations of potassium (K^+). The effect of elevated K^+ on cell survival is now considered to be a robust phenomenon, as it can sustain 100 % survival of some types of neurons in culture, whilst without elevated K^+, virtually all cells die (Hack *et al.*, 1993; Franklin *et al.*, 1995; Koshimura *et al.*, 1999; Chen *et al.*, 2001).

Calcium is fundamentally involved in both the electrical activity and neurite outgrowth that govern cell survival of developing neurons. Exposure of cultured neurons to elevated K^+ has been shown to cause depolarisation of constituent cells that lasts as long as elevated K^+ is present. This depolarisation results in a sustained increase in $[Ca]_i$, inhibition of which prevents the effects of K^+ on survival (Collins *et al.*, 1991, Hack *et al.*, 1993; Franklin *et al.*, 1995; Koshimura *et al.*, 1999; for review see Neely and Nicholls, 1995).

Neurite outgrowth is governed by positive or negative growth cues (for example, increasing or decreasing gradients of neurotrophic factors) which are transduced into growth signals by the growth cone; a protuberance at the end of the growing neurite (Ramon y Cajal, 1890). Such growth signals regulate the formation of the cytoskeleton. Neuronal outgrowth and guidance are critically dependent on the coupling between sensory and motor capabilities of the growth cone. Growth cone receptors are therefore coupled to numerous signalling pathways, many of which are reliant upon calcium (Kater *et al.*, 1988; Kater and Mills, 1991; Mattson, 1999; Gomez and Spitzer, 2000). These calcium-dependent pathways are frequently mediated by binding of calcium to calmodulin, a 17 kDa calcium-binding protein that can act as an intracellular calcium sensor (see Ghosh and Greenberg, 1995; Barish, 1998). Calmodulin consists of two similar globulin lobes joined by an α-helix. Each lobe contains two calcium-binding sites (Ikura *et al.*, 1992; Meador *et al.*, 1992). Binding of calcium

activates calmodulin and enables it to stimulate a variety of secondary messenger enzymes including adenylate cyclase, calcineurin and calcium/calmodulin-dependent protein kinases and protein phosphatases (see Ghosh and Greenberg, 1995). These translate the calcium signal into other intracellular signals or biochemical events such as increased production of cyclic adenosine monophosphate (cAMP) and phosphorylation or dephosphorylation of a number of protein substrates (Ghosh and Greenberg, 1995). Such events can alter the rate of assembly and disassembly of microtubules and actin filaments in the growth cone resulting in alterations in neurite extension and retraction (Bamburg and Chapman, 1986; Lankford and Letourneau, 1989; Moskowitz, 1990), or affect cellular processes such as cell differentiation (Holliday and Spitzer, 1992; Mao *et al.*, 1999), regulation of cell-substrate attachments (Lankford and Letourneau, 1989; Leavesley *et al.*, 1993) and synaptogenesis (Scholz *et al.*, 1988). Calcium-calmodulin complex- (CaM) activation of protein kinases are also known to be important for triggering long-term changes in synaptic plasticity associated with learning and memory (McGlade-McCulloh *et al.*, 1993; Bortolotto and Collingridge, 1998; Shen *et al.*, 2000).

Developing cells are known to have a permissive range of $[Ca]_i$ compatible with neuronal survival. Likewise, growth cones have a concentration of intracellular calcium at which motility is optimal. In both cases the narrow range of permissive concentrations are referred to as the 'set-point' (see Kater *et al.*, 1988; Kater and Mills, 1991; Franklin and Johnson, 1992). The set-point has been shown to be individual to cell type, and variation either above or below it leads to disruption of calcium-dependent processes (see Kater *et al.*, 1988; Rogers and Hendry 1990; Kater and Mills, 1991; Franklin and Johnson, 1992; Neely and Nicholls, 1995). Accordingly, variation from the set-point results in retardation of neurite outgrowth,

or neurite retraction according to the cellular process disrupted, or in extreme cases cell death (Chapter 1, Section 1.2.3.3.ii on excitotoxicity).

Evidence supporting the importance of the set-point phenomenon to developing cell survival and neurite outgrowth is provided by numerous studies in a range of tissue preparations that have investigated developmental cell processes with respect to the $[Ca]_i$ of growing cells. Studies have shown that neurite extension correlates to high levels of intracellular calcium in embryonic rat diencephalon (Connor, 1986) and neonatal mouse cerebella cells (Komuro and Rakic, 1996). In studies by Collins and Lile (1989) and Collins *et al.* (1991) media supplemented with 40 mM K^+ caused a 2.4 fold increase in $[Ca]_i$ of embryonic chick ciliary ganglia neurons and improved cell survival. It has also been demonstrated that removal of calcium from the media of growing cells results in a decrease in outgrowth and causes changes in growth cone shape (Lankford and Letourneau, 1989). Other studies however, have demonstrated that an increase in $[Ca]_i$ suppresses growth cone motility and neurite elongation in *Helisoma* neurons (Mattson and Kater, 1987; Mattson *et al.*, 1988a) and *Xenopus* embryonic cells (Holliday and Spitzer, 1992). Furthermore, Randall and Thayer, (1992) demonstrated that cell death of embryonic rat hippocampal neurons in cultures exposed to glutamate correlated significantly with increases in $[Ca]_i$. Whilst the results of these studies initially appear antithetical, they actually all lend support to the notion that the optimal concentration of intracellular calcium for neurite outgrowth and cell survival is limited, and is specific to cell type.

VDCC play a fundamental role in the modulation of neurite outgrowth and cell survival during morphogenesis. Antagonists of the L-type VDCC have been shown to inhibit cell

migration (Moran, 1991) and neurite outgrowth (Tolkovsky *et al.*, 1990). The studies by Collins and Lile (1989) and Collins *et al.* (1991) that demonstrated that elevated K^+ levels improved ciliary ganglia cell survival (above) also reported that DHPs completely inhibited K^+-mediated neuronal survival. In contrast, a study by Robson and Burgoyne (1989) illustrated that the presence of 45 mM K^+ in cultured rat dorsal root ganglion cell media inhibited neurite outgrowth, but that this inhibition was abolished by addition of nifedipine. Similarly, the study by Mattson *et al.* (1988b) using cultured hippocampal pyramidal-like neurons showed that neurite outgrowth was inhibited and cell survival diminished by high concentrations of glutamate, and that the effects of glutamate were prevented by complete inhibition of VDCC by cobalt. Furthermore, a study by Porter *et al.* (1997) demonstrated that chronic treatment of hippocampal cultures with nimodipine significantly enhanced cell survival. Starikova *et al.* (1998) illustrated that addition of low concentrations (2.5 μM or 5 μM) of nifedipine to the culture medium of rat PC12 pheochromocytoma cells resulted in an increase in the total length and number of cell processes, but that addition of 10 μM nifedipine resulted in retraction of processes and prevention of proliferation activity. These studies again support the idea of a limited range of $[Ca]_i$ that are compatible with neuronal growth and survival.

6.3 TROPHIC FACTORS AND VDCC

The actions of some well-known growth factors have been shown to be mediated through VDCC. For example, NGF, a member of the neurotrophin family (Chapter 1 Section 1.2.4.3). NGF is trophic to dopaminergic cells following lesioning, and is critical for both survival and neurite extension of superior cervicular ganglia (SCG). It was shown by Nikodijevic and

Guroff, (1992) that NGF stimulates the uptake of radioactive calcium into PC12 cells, and that this stimulation was blocked by down-regulation of L-type VDCC. Rogers and Hendry (1990) demonstrated that application of 10 μM nifedipine or 100 μM cadmium to cultured SCG blocked NGF-elicited neurite outgrowth, indicating that an influx of calcium is necessary for NGF-activated neurite outgrowth and that VDCC are implicated in the effects of NGF on neuritogenesis. Koike *et al.* (1989) investigated the effects of chronic VDCC agonist and antagonist application on cultures of embryonic SCG that had been NGF deprived, but treated with high K^+, which confers protection against cell death. It was found that nifedipine and nimodipine blocked the survival-promoting effect of high K^+, and VDCC agonists augmented the protective effects of high K^+. Electrophysiological studies by Jia *et al.* (1999) illustrated that acute application of NGF to whole-cell currents elicited from rat PC12 cells increased calcium influx through L-type VDCC. Whole-cell patch-clamp electrophysiology was also used in the study by Levine *et al.* (1995) to demonstrate that chronic incubation of embryonic basal forebrain neurons with NGF increased both the L-type and N-type components of the whole-cell calcium current. Chronic application of another growth factor, bFGF (Chapter 1 Section 1.2.4.3) has also been shown by electrophysiology to increase the conductance through VDCC in cultured foetal hippocampal neurons (Shitaka *et al.*, 1996; Collings *et al.*, 1999). It has also been reported that bFGF-treated neurons displayed larger increases in $[Ca]_i$ than non-treated neurons in response to high K^+ induced depolarisation (Shitaka *et al.*, 1996; Katsuki *et al.*, 1998). This $[Ca]_i$ response could be abolished by nicardipine, thus implicating the involvement of L-type calcium channels in the effect of bFGF upon $[Ca]_i$.

It could be concluded from these studies that the effects of bFGF and NGF upon VDCC bring the $[Ca]_i$ closer to the concentration required for optimal growth cone motility (the set-point). This may result in enhanced growth cone movement and consequently increased neurite length. The effect of bFGF and NGF upon VDCC may therefore account for the stimulatory effect of these factors on neurite outgrowth both *in vitro* and *in vivo* (Chapter 1 Section 1.2.4.3).

6.4 SUMMARY

From the studies described in this chapter it can be seen that calcium, and regulation of $[Ca]_i$ through VDCC are intimately involved in the processes that regulate neurite outgrowth and cell development. Evidence has also been given for effects of two well-known trophic factors, NGF and bFGF, on VDCC conductance. These findings provide a reasonable basis for the supposition that the trophic effects of AChE and BuChE may be due to alterations in $[Ca]_i$ caused by an effect of these substances on VDCC. The aim of the work in Chapter 8 of this thesis is therefore to establish the effect of direct application of AChE and BuChE to voltage-dependent calcium currents elicited from embryonic VM cells using whole-cell patch-clamp electrophysiology. The pattern of VDCC expression in developing VM cells *in vitro* has not previously been characterised however, and this will form the aim of Chapter 7. Chapter 9 will then utilise the immunocytochemical and quantification methodology used in Chapters 3-5 to investigate the effects of some of the VDCC antagonists given in Table 6.1 upon cell survival and neurite outgrowth of embryonic dopaminergic cells in VM cultures.

CHAPTER 7

Characterisation of the expression of VDCC in developing VM cells

7.1 INTRODUCTION

The VDCC composition of embryonic midbrain dopaminergic cells is not currently known. As discussed in Chapter 6, developmental processes such as cell survival and neurite extension are dependent upon $[Ca]_i$. Furthermore, VDCC, which constitute a major route of entry of calcium into the cell, have been shown to be fundamentally important to both neurite extension and survival of developing cells (Mattson *et al.*, 1988b; Collins and Lile, 1989; Robson and Burgoyne, 1989; Tolkovsky *et al.*, 1990; Collins *et al.* 1991; Moran, 1991; Porter *et al.*, 1997). The success of foetal transplantation as a treatment for PD depends upon survival of, and innervation of the host striatum by embryonic midbrain dopaminergic neurons within the graft. Thus, investigation of the mechanisms that regulate calcium influx within cells used in foetal transplantation may be important for understanding the rationale behind survival of and neurite extension by these neurons. This chapter therefore aims to examine the pattern of VDCC expression in embryonic VM cells over a 7 day culture period using VDCC antagonists, Aga IVA, GVIA, nifedipine and cadmium to pharmacologically dissect voltage-dependent calcium currents using whole-cell patch-clamp electrophysiology.

7.2 EXPERIMENTAL PROCEDURES

7.2.1 Preparation of dissociated cultures of the VM

Suspensions of ventral mesencephalon cells from E14 rats were prepared as described in Chapter 2 Section 2.2.2 and plated onto astrocyte-covered coverslips. Cultures were used for electrophysiological recordings from day 1 to day 7 *in vitro.*

7.2.2 Electrophysiological recordings

As described in Chapter 2 (Sections 2.2.5.1 – 2.2.5.6) a culture was placed into the recording chamber and perfused with extracellular solution containing 5mM Ba^{2+}. A cell was chosen for recording based upon its morphological characteristics. The aim was to record primarily from dopaminergic cells within cultures. From the biotinylated-labelling of dopaminergic cells in the work of Chapters 3, 4 and 5, it was known that dopaminergic cells tended to have larger soma and were often pyramidal in shape. However, as cell phenotype could not be confirmed until after the experiment was terminated, data from both TH+ve and GAD+ve cells was inevitably obtained and has been analysed.

A pipette was filled with intracellular solution containing 0.2 % biocytin and a gigaseal was formed between the pipette and the chosen cell. Upon whole-cell access the cell was voltage-clamped at a holding potential (Vh) of –60 mV. Series resistance and cell capacitance were determined by analysing instantaneous membrane responses to 5 mV, 30 msec voltage steps. The mean series resistance was 5.2 ± 0.28 MΩ, and partial compensation (60-85 %) was applied.

7.2.3 Post-recording triple labelling

To identify the phenotype of the cell recorded, the biocytin fill was detected using Texas Red-conjugated streptavidin followed by labelling with fluorescent antibodies for TH (FITC fluorophore) and GAD (Cy5 fluorophore) as described in Chapter 2, Sections 2.2.5.7 and 2.2.5.8.

7.2.4 Experimental design

For the purposes of characterising the calcium currents present in developing VM cells, two stimulus protocols were used as described in Chapter 2 Section 2.2.5.6.

(1) A 'step-test' of nine, 100 msec depolarisation steps of increasing 10 mV increments was used to establish the current-voltage (IV) relationship for each cell, and thus the depolarisation step required to elicit maximal peak current (I_{max}). Peak current values for each step were normalised to I_{max}. Cell capacitance and I_{max} for each cell were compared using linear regression correlation.

(2) A single 60 mV, 100 msec depolarisation step was then applied every 30 seconds. When the current had stabilised (defined by three depolarisation steps eliciting currents within 2 picoamps (pA) of each other), a VDCC antagonist was applied.

Using protocol (2), two sets of experiments were conducted. Firstly, experiments were carried out to determine the concentration of each VDCC antagonist required to cause complete inhibition of the relevant channel subtype. This was important for the second set of

experiments described below. IC_{50} values were determined by application of the concentrations of each inhibitor given in Table 7.1.

VDCC antagonist	Concentrations
ω-Agatoxin IVA (Aga IVA; P/Q-type inhibitor)	50 nM, 100 nM, 200 nM, 250nM
ω-conotoxin GVIA (GVIA; N-type inhibitor)	0.1 μM, 0.5 μM, 1 μM, 2 μM
Nifedipine (L-type inhibitor)	1 μM, 2.5 μM, 5 μM, 10 μM

Table 7.1 Concentrations of VDCC antagonists used to determine IC_{50} values

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Secondly, experiments were carried out to investigate the VDCC composition of dissociated embryonic VM cells. VDCC antagonists were applied consecutively in the order, and at the concentrations given in Table 7.2 to cumulatively inhibit calcium channel components. Following application of specific antagonists, cadmium was applied to inhibit the remaining calcium current.

VDCC antagonist	Concentration
Aga IVA	200 nM
GVIA	1 μM
Nifedipine	5 μM
Cadmium (Cd^{2+})	20 μM

Table 7.2 Concentration of VDCC antagonists used to characterise whole-cell calcium currents in embryonic dissociated VM cells.

Drugs were applied consecutively, in the order given above.

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In both sets of experiments, each antagonist was applied until the inhibited current had stabilised. Following VDCC antagonist application, the cell was perfused with extracellular solution to increase the biocytin fill time, and the recording was ended when the current

began to deteriorate. Antagonists were applied via delivery to the bath in extracellular solution through a three-way tap system. Complete exchange of the bath solution occurred in approximately 1.5 mins.

7.2.5 Source of test chemicals

Cadmium chloride and nifedipine were obtained from Sigma-Aldrich Chemical Company Ltd. (Poole, UK), Aga IVA was obtained from Calbiochem-Novabiochem Corp., UK., and GVIA from Bachem (UK). Stock solutions of Aga IVA (100 μM), GVIA (100 μM) and cadmium chloride (100 mM) were made in distilled water. A stock solution of nifedipine (100 mM) was made in di-methyl-sulfoxide (DMSO). All stock solutions were aliquoted and frozen at -20°C. All test chemicals were diluted in extracellular solution immediately prior to use.

7.3 RESULTS

7.3.1 Current-voltage (IV) relationship

The IV relationship was examined on each day of recording (day 1 through to day 7 *in vitro*). For both cell types peak current increased with depolarisation step size, with I_{max} reached at 0 mV, and a decrease in peak current observed with further depolarisation. Test potential required to elicit I_{max} did not vary with ontogenetic age in either cell type. Data was thus pooled for each cell type (Figures 7.1 A and B). Peak current values for each depolarisation step have been normalised to I_{max}.

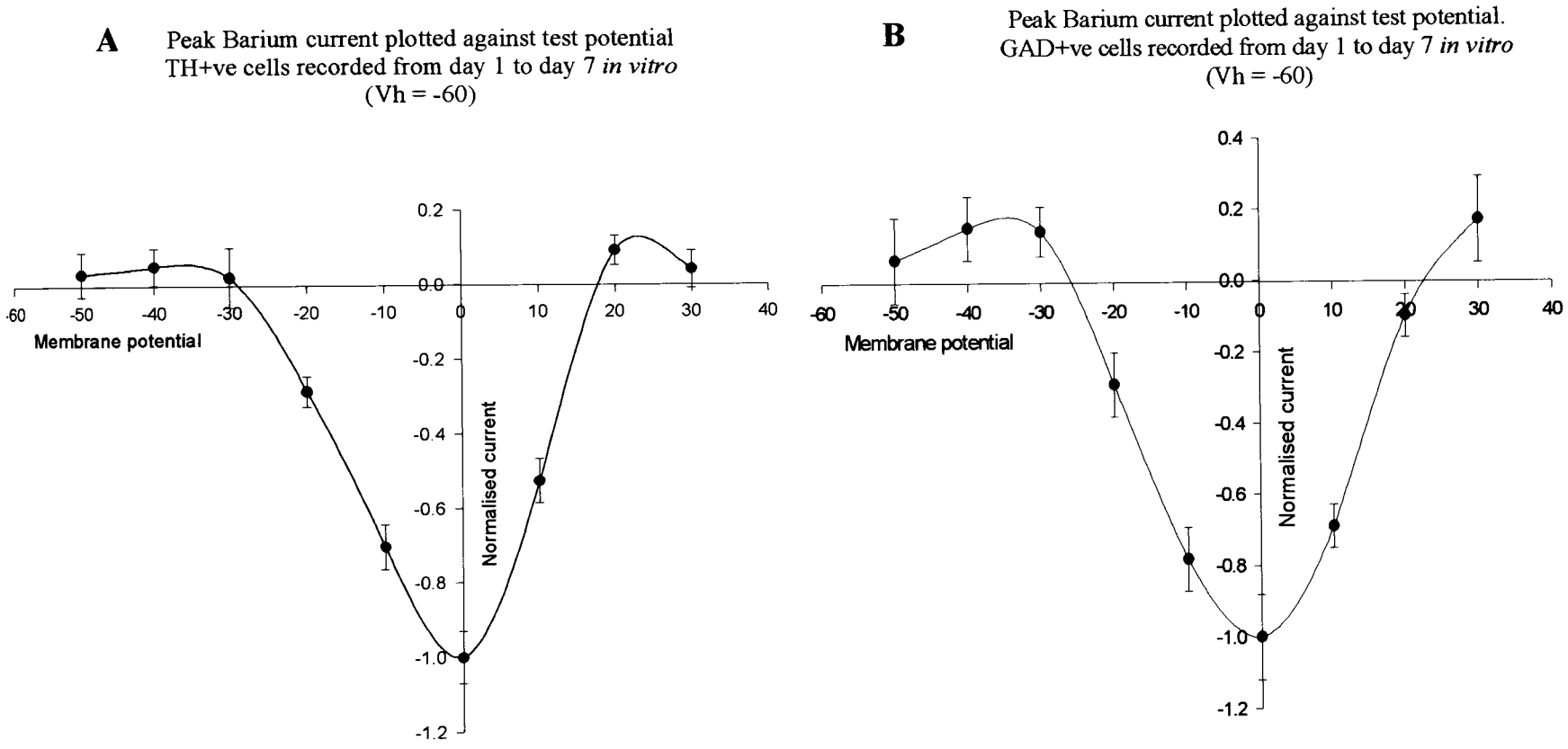

Figure 7.1 Peak barium current normalised to I_{max} plotted against test potential (Vh = -60 mV) for TH+ve cells (A) and GAD+ve cells (B), where I_{max} = maximal inward current.

For both n ≥ 67
= *5mM* Ba^{2+}

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7.3.2 Relationship between cell capacitance and current size

The relationship between cell capacitance and size of current elicited from cells voltage-clamped at –60 mV and depolarised to 0 mV for 100 msec was examined at days 1, 3 and 7

in vitro. Both TH+ve and GAD+ve cell types demonstrated a positive linear correlation between cell capacitance and current size; TH+ve cells (Figure 7.2 A) $r^2 = 0.635$; $F = 113.3$; $P < 0.0001$ ($n = 67$). GAD+ve cells (Figure 7.2 B) $r^2 = 0.623$, $F = 179$; $P < 0.0001$ ($n = 111$). The data for both cell types were also distributed according to the age in culture, with cells at day 1 *in vitro* generally having smaller currents and lower capacitance than those at day 3 *in vitro*. Similarly, cells of both phenotypes tended to have larger currents and greater capacitance at day 7 *in vitro* than at day 3 *in vitro* (Figures 7.2 A and B).

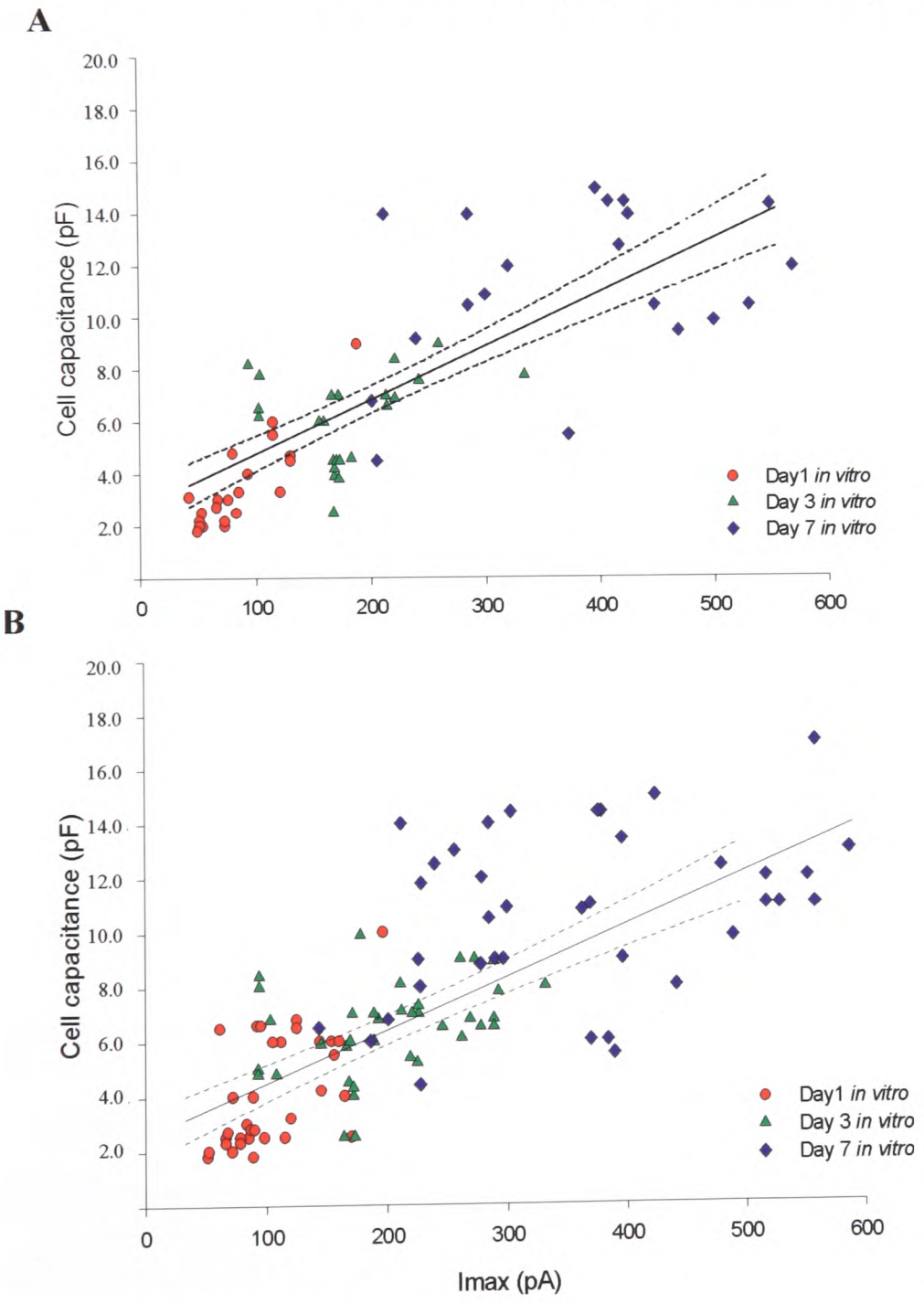

Figure 7.2 Relationship between cell capacitance and size of current elicited from cells voltage-clamped at –60 mV and depolarised to 0 mV for 100 msec at days 1, 3 and 7 *in vitro*

(A) *TH+ve cells; $r^2 = 0.635$; $F = 113.3$; $P < 0.0001$ ($n = 67$).* ***(B)*** *GAD+ve cells; $r^2 = 0.623$, $F = 179$; $P < 0.0001$ ($n = 111$)*

Lines shown in each case are goodness of fit for the entire data set (days 1, 3 and 7) ± 95% confidence interval

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7.3.3 Inhibition of voltage-dependent calcium currents by selective antagonists

IC_{50} values were determined for Aga IVA, GVIA and nifedipine to establish the concentration of each inhibitor that produced complete inhibition of the relevant VDCC subtype. Data for each antagonist were taken from a variety of days *in vitro* and pooled. (Table 7.3; for each n = 5).

VDCC inhibitor	IC_{50}
Aga IVA	89 ± 4.2 nM
GVIA	0.44 ± 0.02 μM
Nifedipine	2.32 ± 0.06 μM

Table 7. 3 Determination of IC50 values for VDCC inhibitors Aga IVA, GVIA and nifedipine. *n = 5*

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7.3.4 Characterisation of voltage-dependent calcium currents in TH+ve cells at days 1, 3 and 7 *in vitro*

The contribution of the currents from the P/Q-, L-, N-, and R-type VDCC to I_{max} of TH+ve cells, expressed as a percentage of I_{max}, are given in Table 7.4 and Figure 7.3. The actual values for the VDCC component contribution to I_{max} in pA are given in Table 7.5 and Figure 7.4.

Representative electrophysiological traces and time courses for the characterisation of the constituent VDCC of TH+ve cells at each time point are shown in Figures 7.5 A, B and C, with corresponding examples of post-recording triple fluorescent labelling. Concentrations of VDCC antagonists used are as given in Table 7.2

Day *in vitro*	Calcium channel component (% of I_{max} ± s.e.m.)			
	P / Q	N	L	R
1	11.3 ± 1.3	39.7 ± 3.8	38.5 ± 4.1	10.5 ± 1.5
3	9.0 ± 1.4	42.8 ± 4.2	36.2 ± 2.8	12.0 ± 1.5
7	8.9 ± 1.9	31.6 ± 2.6	37 ± 3.9	22.5 ± 2.5

Table 7.4 Percentage of I_{max} attributable to each VDCC component in TH+ve cells at days 1, 3 and 7 *in vitro*

Values expressed as % of I_{max} ± s.e.m. For each n ≥ 9.

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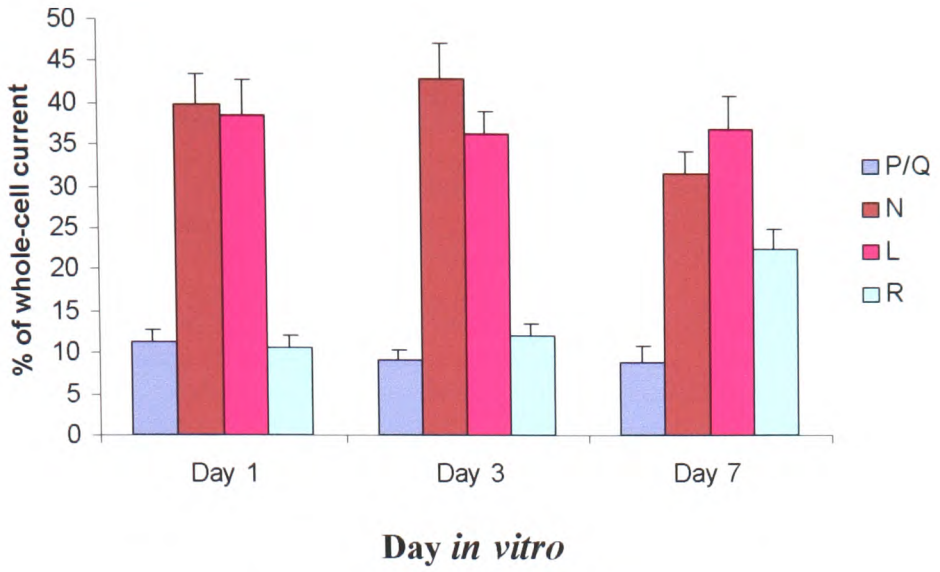

Figure 7.3 Percentage of I_{max} attributable to each VDCC component in TH+ve cells at days 1, 3 and 7 *in vitro*.

Values expressed as % of I_{max} ± s.e.m. For each n ≥ 9.

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Day *in vitro*	I_{max} (pA)	Calcium channel component (pA)			
		P / Q	N	L	R
1	106.3 ± 14.1	12.0 ± 1.5	42.2 ± 5.3	40.9 ± 4.9	11.2 ± 2.3
3	184.4 ± 12.3	16.6 ± 1.5	78.9 ± 6.2	66.8 ± 7.4	22.1 ± 6.1
7	422.5 ± 27.7	37.6 ± 6.9	133.5 ± 25.7	156.3 ± 19.7	95.1 ± 12.5

Table 7.5 Actual values for each VDCC component contribution to I_{max} of TH+ve cells at days 1, 3 and 7 *in vitro*.

Values given are the mean ± s.e.m. For each n ≥ 9

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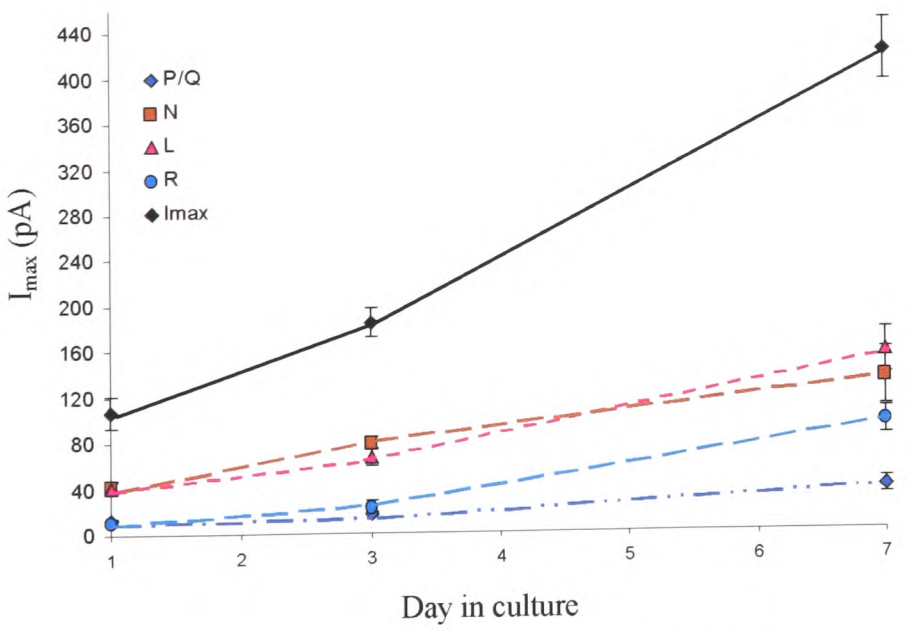

Figure 7.4 Graph to illustrate the change in the actual current value of each VDCC component of the whole-cell calcium current of TH+ve cells at days 1, 3 and 7 *in vitro*.

Values given are the mean ± s.e.m. For each n ≥ 9

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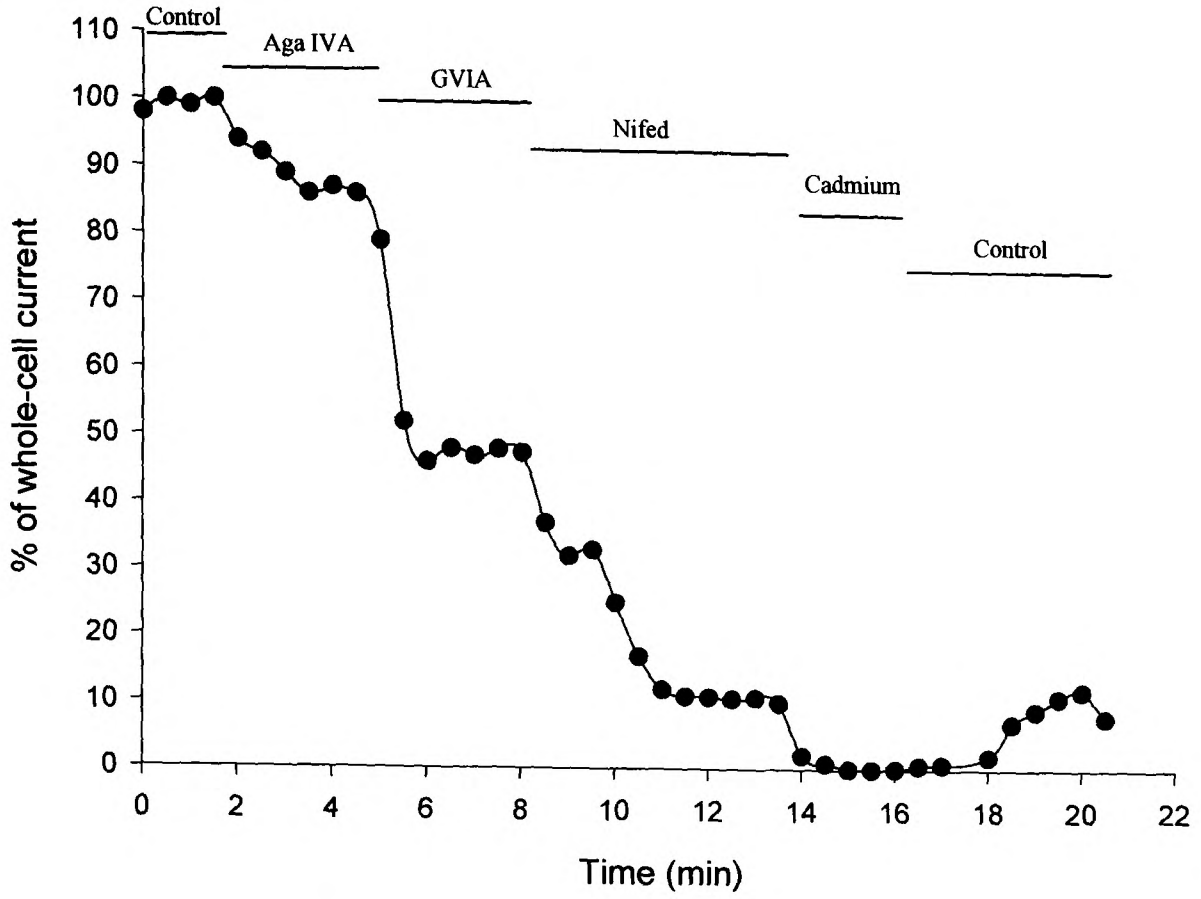

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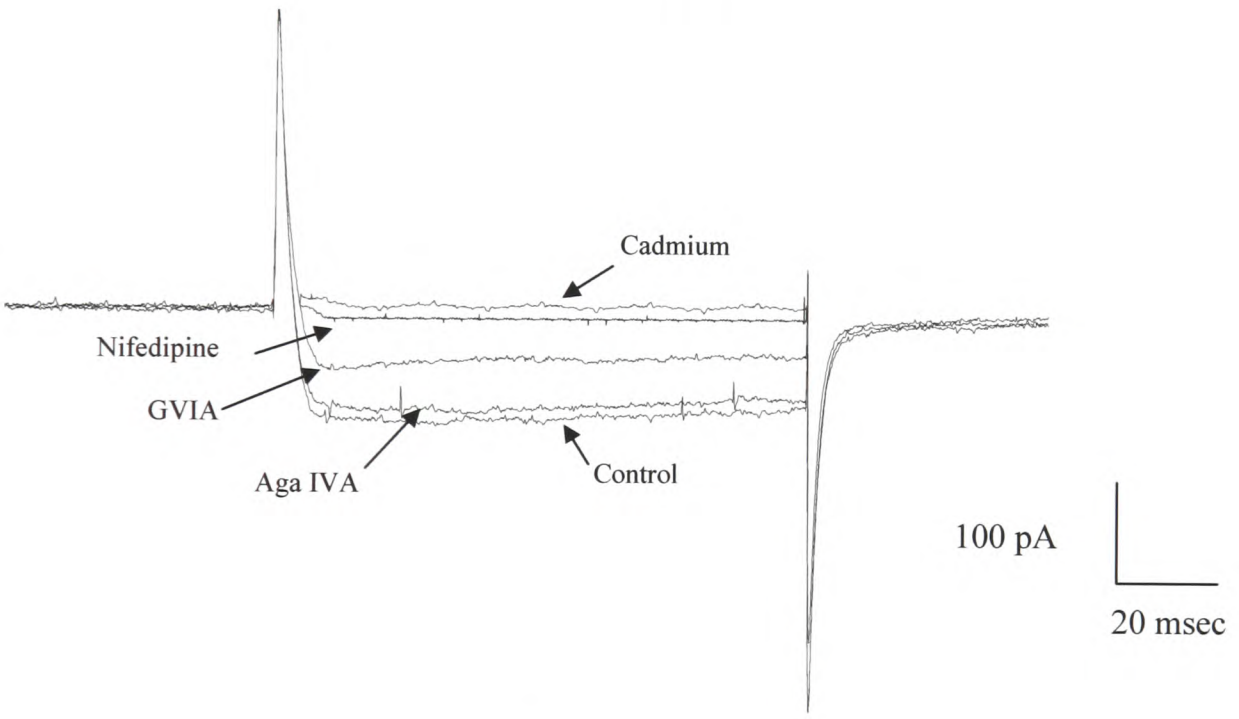

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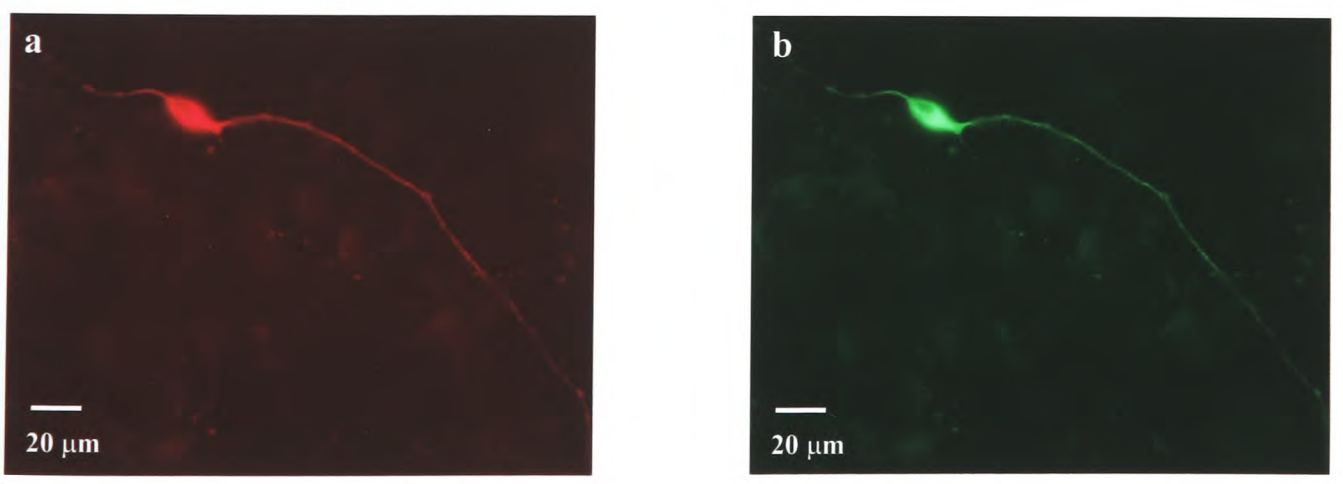

Figure 7.5 A: Time course (i) and electrophysiological traces (ii) for whole-cell calcium current characterisation of a single TH+ve cell at day 1 *in vitro*. (iii) Post-recording identification of cell phenotype: (a) Identification of the cell recorded from by streptavidin-Texas Red labelling of biocytin. (b) Co-localisation with FITC anti-TH antibody.

Nifed = nifedipine

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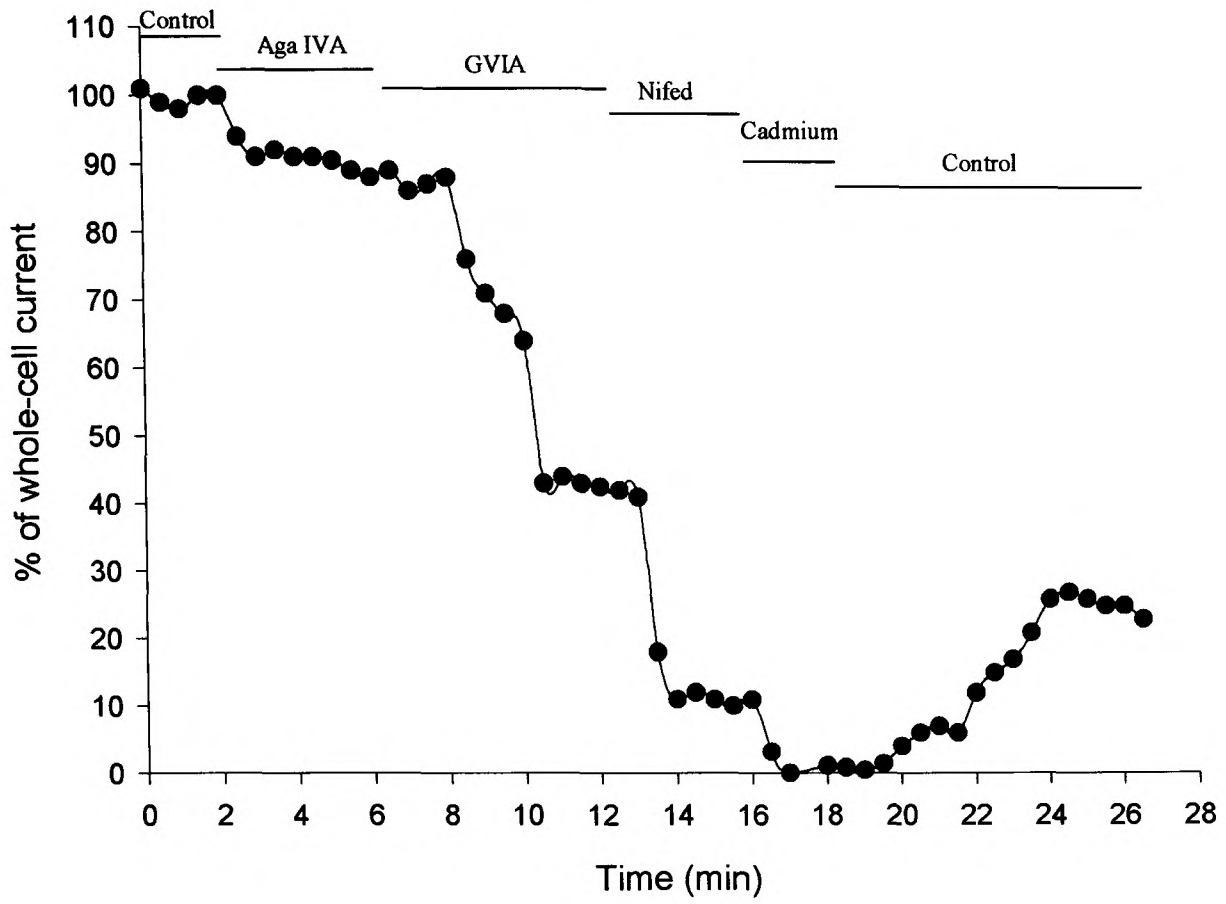

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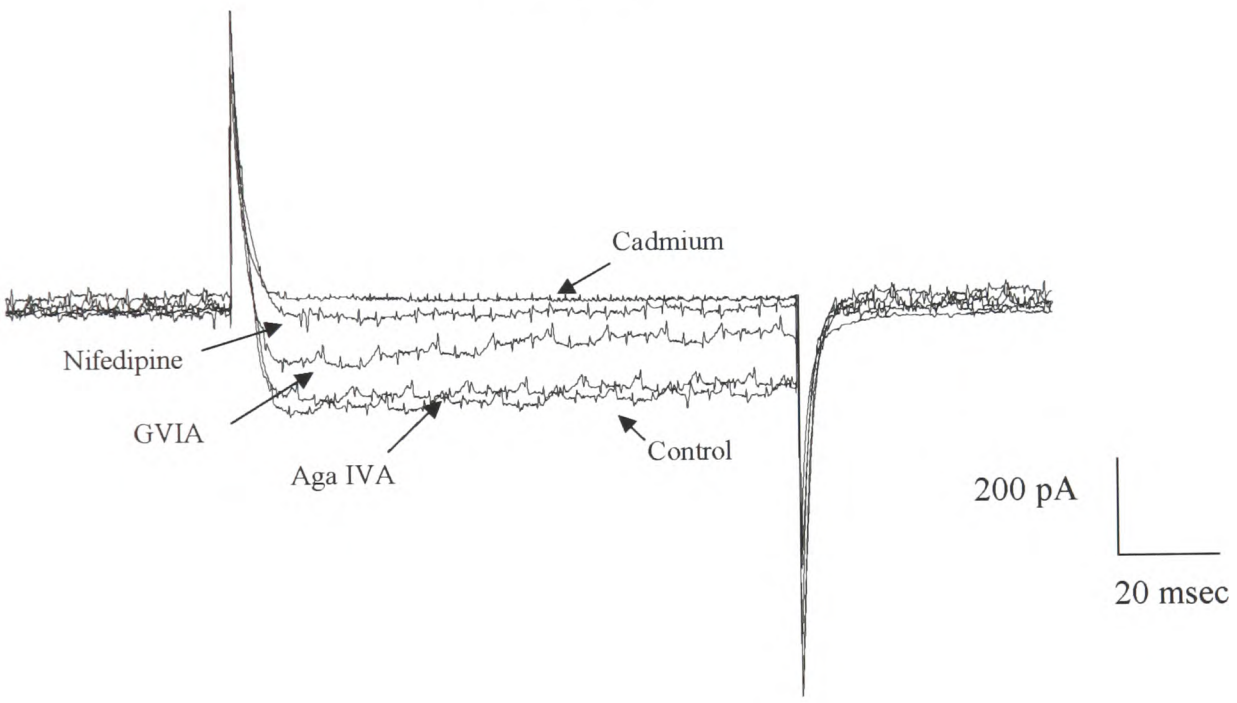

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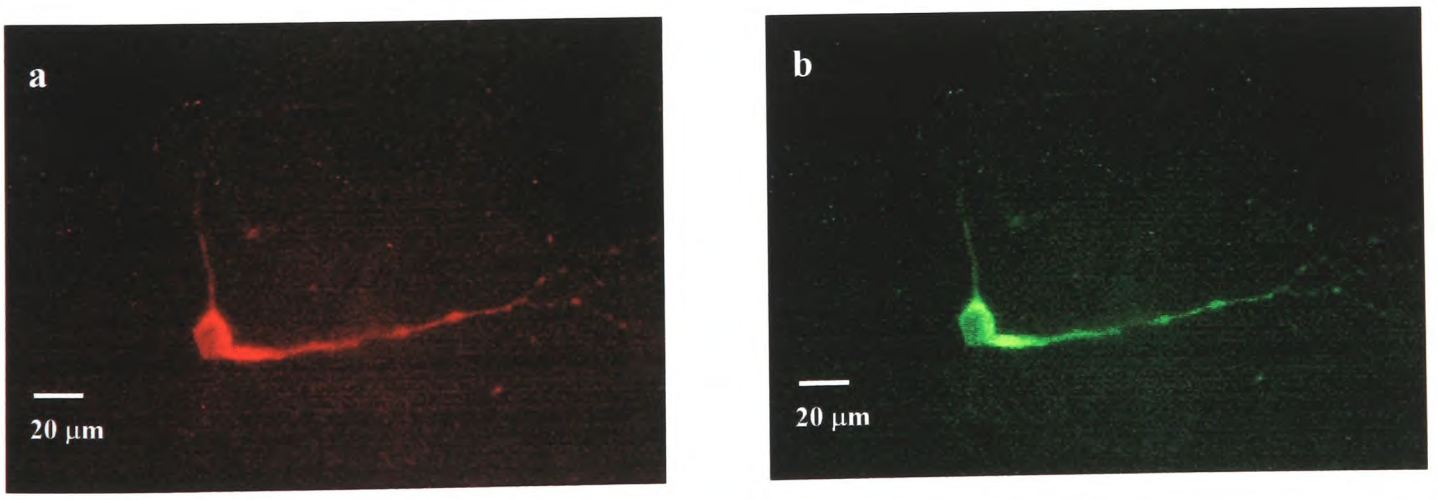

Figure 7.5 B: Time course (i) and electrophysiological traces (ii) for whole-cell calcium current characterisation in a single TH+ve cell at day 3 *in vitro*. (iii) Post-recording identification of cell phenotype: (a) Identification of the cell recorded from by streptavidin-Texas Red labelling of biocytin. (b) Co-localisation with FITC anti-TH antibody.

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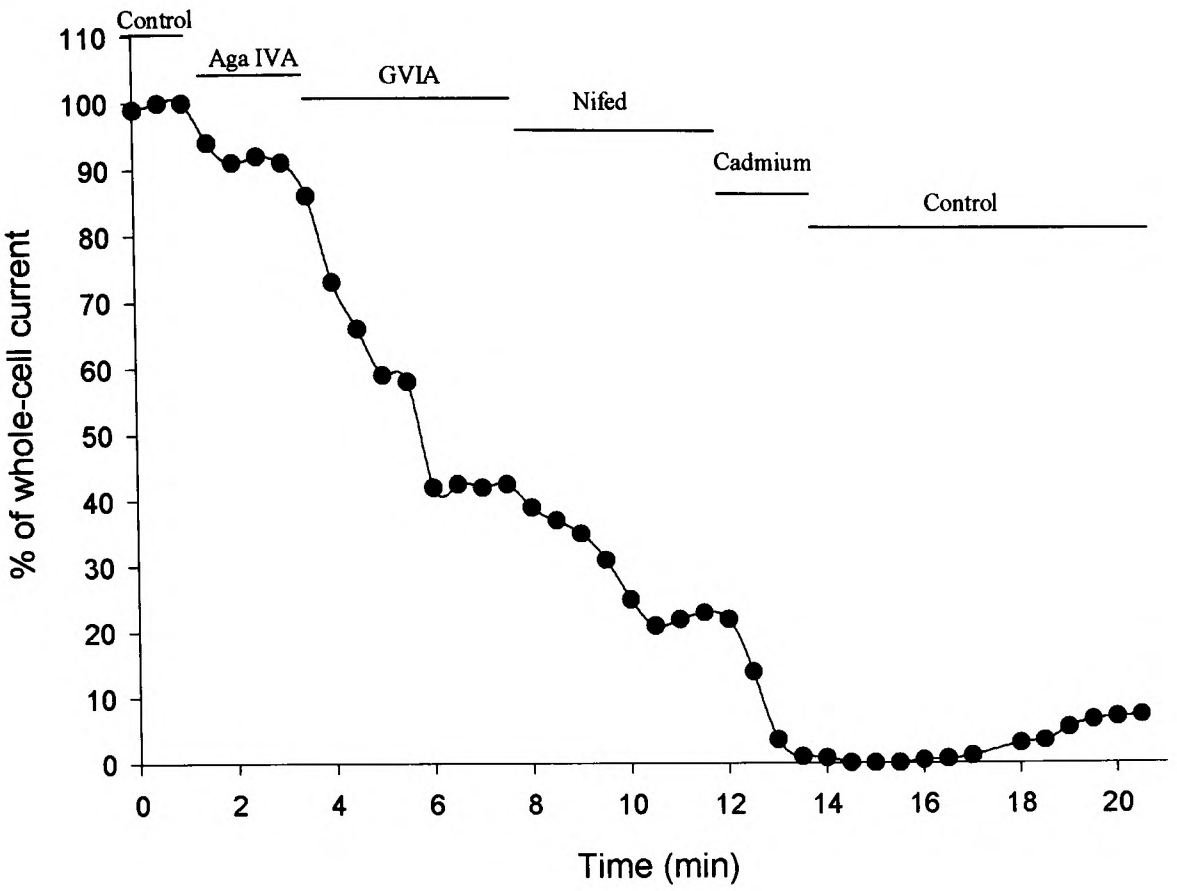

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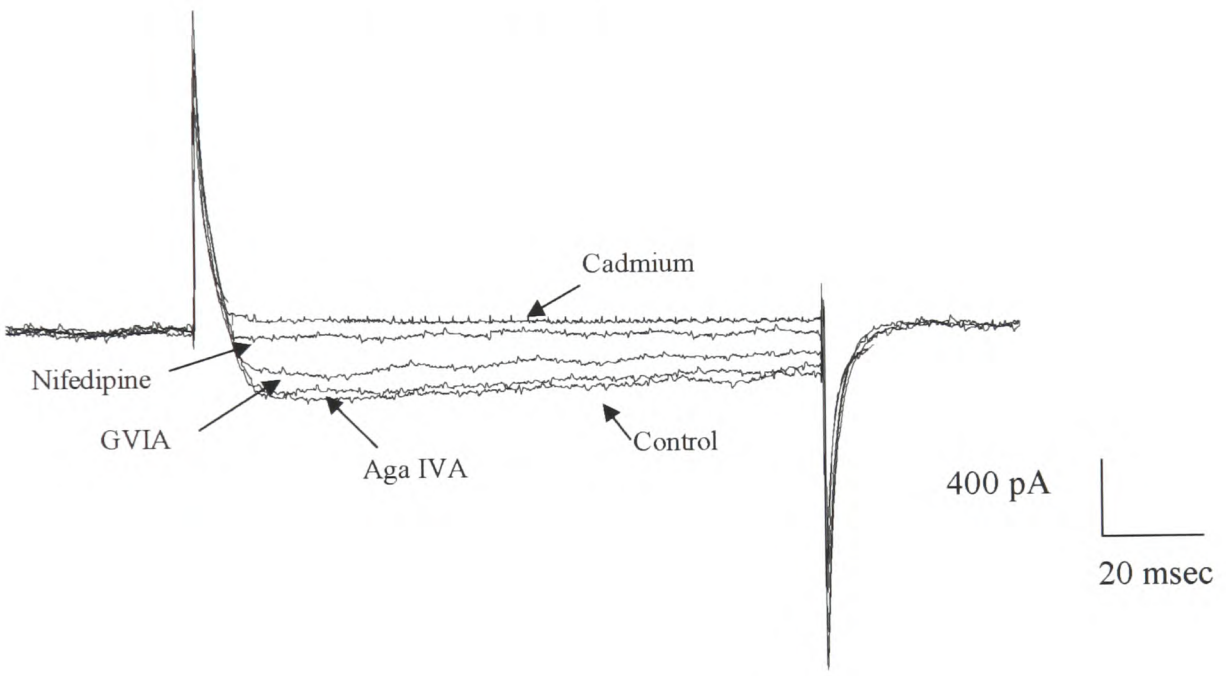

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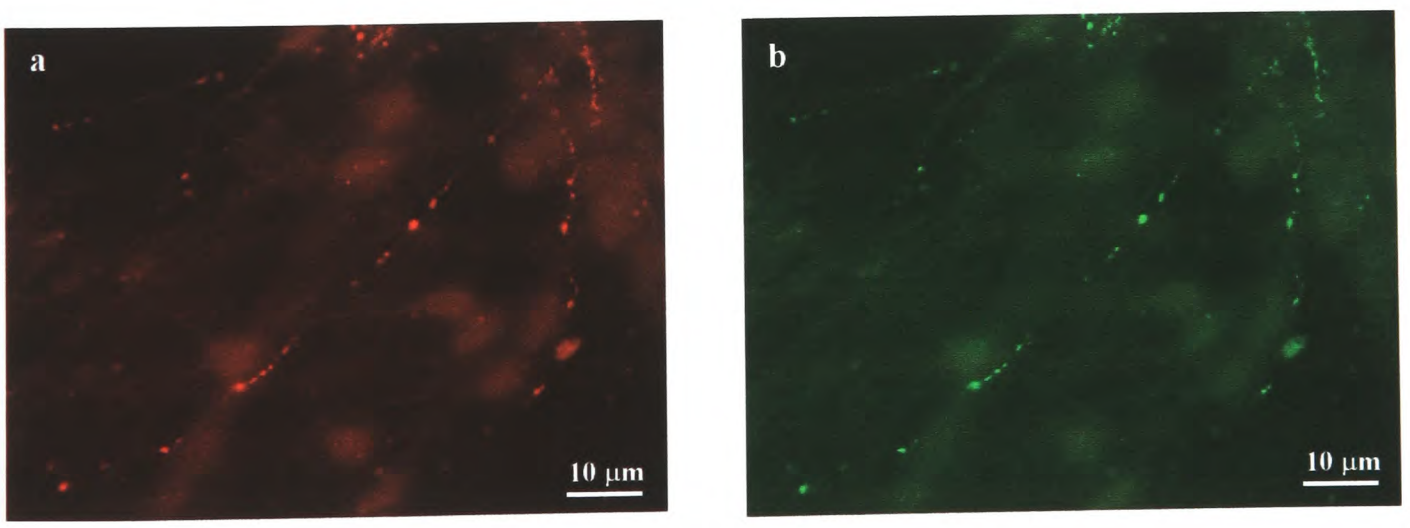

Figure 7.5 C: Time course (i) and electrophysiological traces (ii) for whole-cell calcium current characterisation in a single TH+ve cell at day 7 *in vitro*. (iii) Post-recording identification of cell phenotype: (a) Identification of the biocytin-filled neurites with streptavidin-Texas Red. (b) Co-localisation with FITC anti-TH antibody. *Occasionally, when the pipette was removed from the cell body at the end of the experiment, the cell body was pulled away with it. The cell was then identified by the streptavidin-Texas Red labelling of the neurites left in the culture, as illustrated above.*

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7.3.5 Characterisation of voltage-dependent calcium currents in GAD+ve cells at days 1, 3 and 7 *in vitro*

The contribution of the currents from the P/Q-, L-, N-, and R-type VDCC to I_{max} of GAD+ve cells, expressed as a percentage of I_{max} are given in Table 7.6 and Figure 7.6. The actual values for the VDCC component contribution to I_{max} in pA are given in Table 7.7 and Figure 7.7.

Representative electrophysiological traces and time courses for the characterisation of the constituent calcium channels of GAD+ve cells at each time point are shown in Figures 7.8 A, B and C, with corresponding examples of post-recording triple fluorescent labelling. Concentrations of VDCC antagonists used are as given in Table 7.2

Day *in vitro*	Calcium channel component (% I_{max} ± s.e.m.)			
	P / Q	N	L	R
1	6.1 ± 0.8	32.5 ± 2.4	47.4 ± 3.9	14.0 ± 1.5
3	4.8 ± 0.7	25.2 ± 1.9	52.9 ± 2.0	17.1 ± 2.3
7	4.9 ± 0.8	20.6 ± 1.7	50.5 ± 1.3	24.0 ± 1.8

Table 7.6 Percentage of I_{max} attributable to each VDCC component in GAD+ve cells at days 1, 3 and 7 *in vitro*

Values expressed as % of I_{max} ± s.e.m. For each $n \geq 13$.

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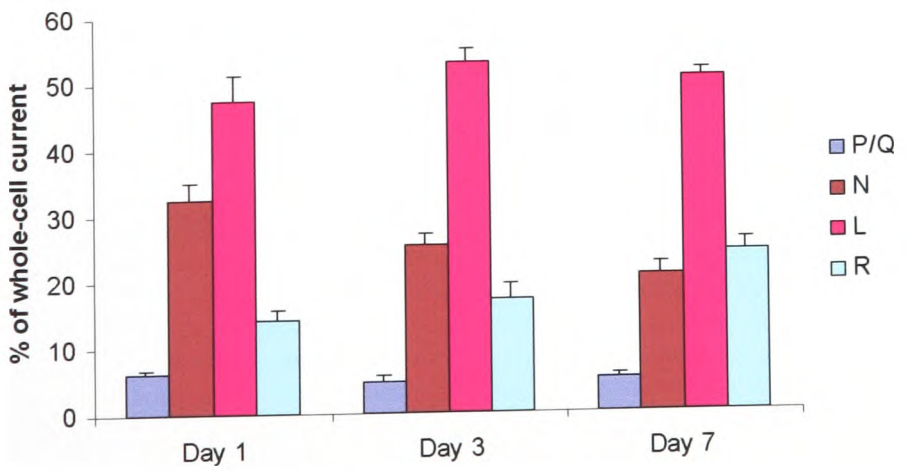

Figure 7.6 Percentage of I_{max} attributable to each VDCC component in GAD+ve cells at days 1, 3 and 7 *in vitro*.

Values expressed as % of I_{max} ± s.e.m. For each $n \geq 13$

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Day *in vitro*	I_{max} (pA)	Calcium channel component (pA)			
		P / Q	N	L	R
1	105.2 ± 10.6	6.4 ± 0.8	34.2 ± 3.9	49.9 ± 7.1	14.7 ± 2.0
3	204.9 ± 18.5	9.8 ± 1.6	51.6 ± 5.5	108.4 ± 9.6	35.0 ± 4.7
7	415.9 ± 38.0	20.4 ± 4.5	85.7 ± 18.6	210 ± 21.2	99.8 ± 14.9

Table 7.7 Actual values for each VDCC component contribution to I_{max} of GAD+ve cells at days 1, 3 and 7 *in vitro*.

Values given are the mean ± s.e.m. For each n ≥ 13

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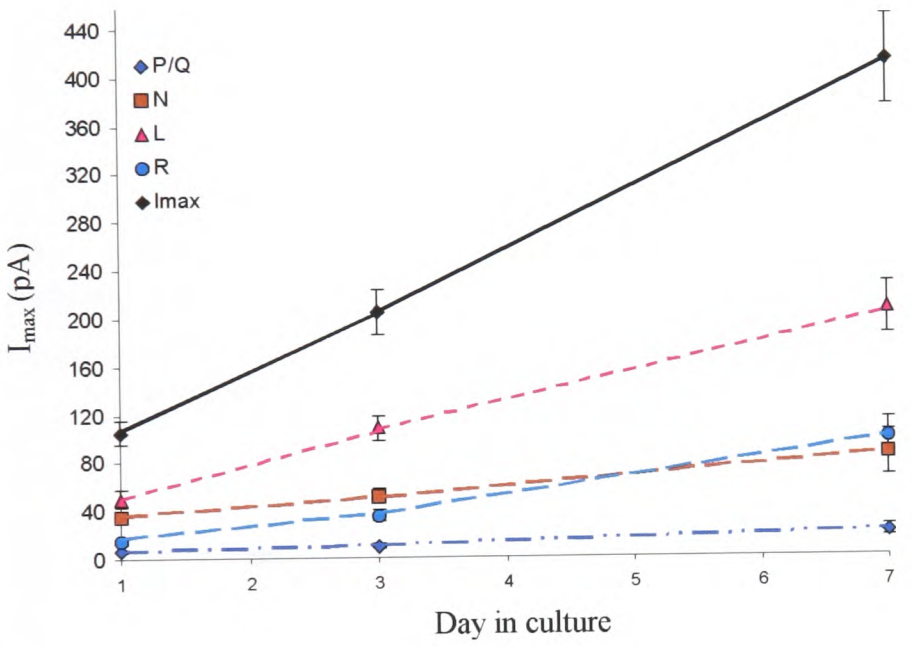

Figure 7.7 Graph to illustrate the change in the actual current value of each VDCC component of the whole-cell calcium current of GAD+ve cells at days 1, 3 and 7 *in vitro*.

Values given are the mean ± s.e.m. For each n ≥ 13

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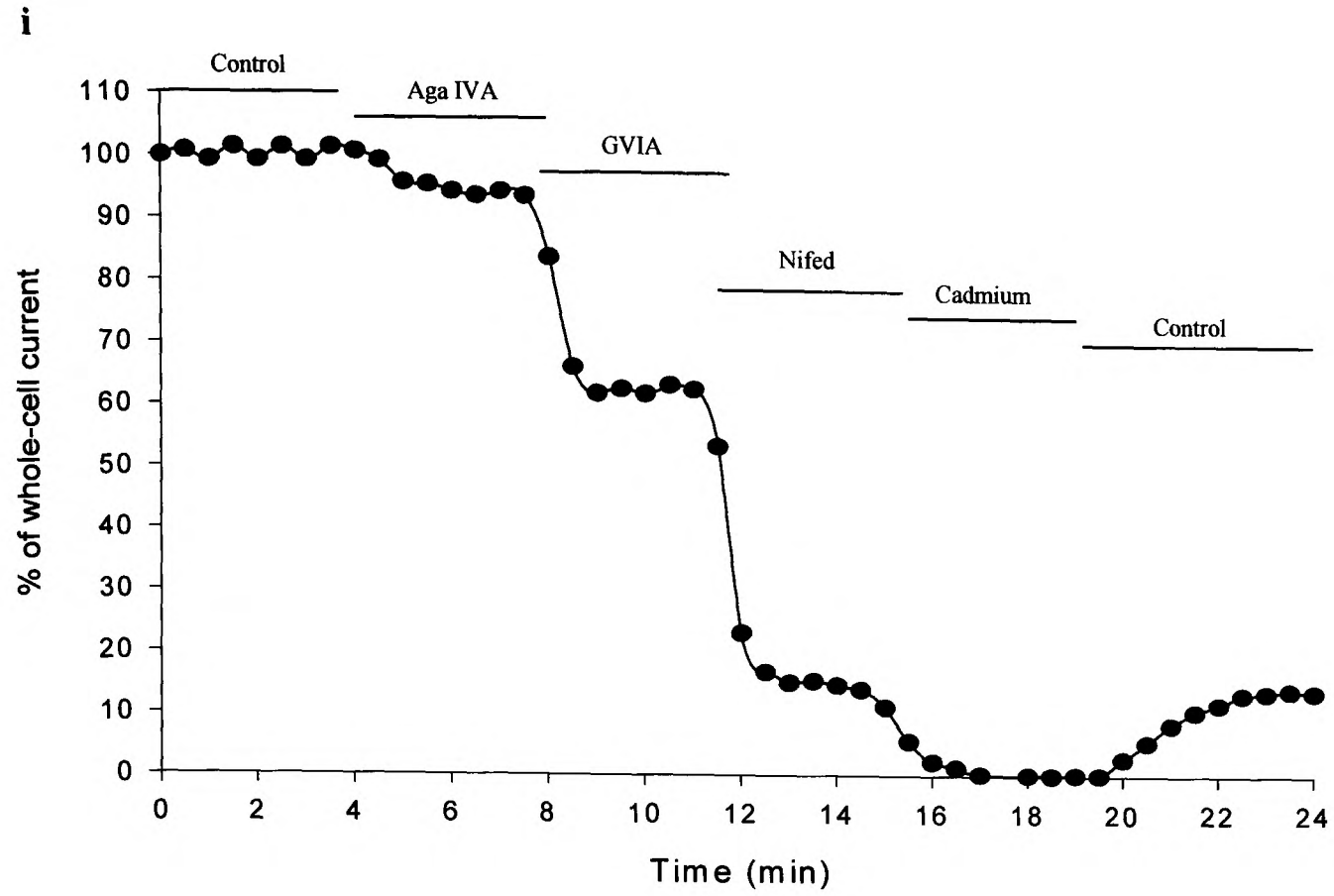

Figure 7.8 A: Time course (i) and electrophysiological traces (ii) for whole-cell calcium current characterisation in a single GAD+ve cell at day 1 *in vitro*. (iii) Post-recording identification of cell phenotype: (a) Identification of the cell recorded from by streptavidin-Texas Red labelling of biocytin. (b) Co-localisation with Cy5 anti-GAD antibody; arrowheads (▶) indicate other GAD+ve cells in culture that were not recorded from.

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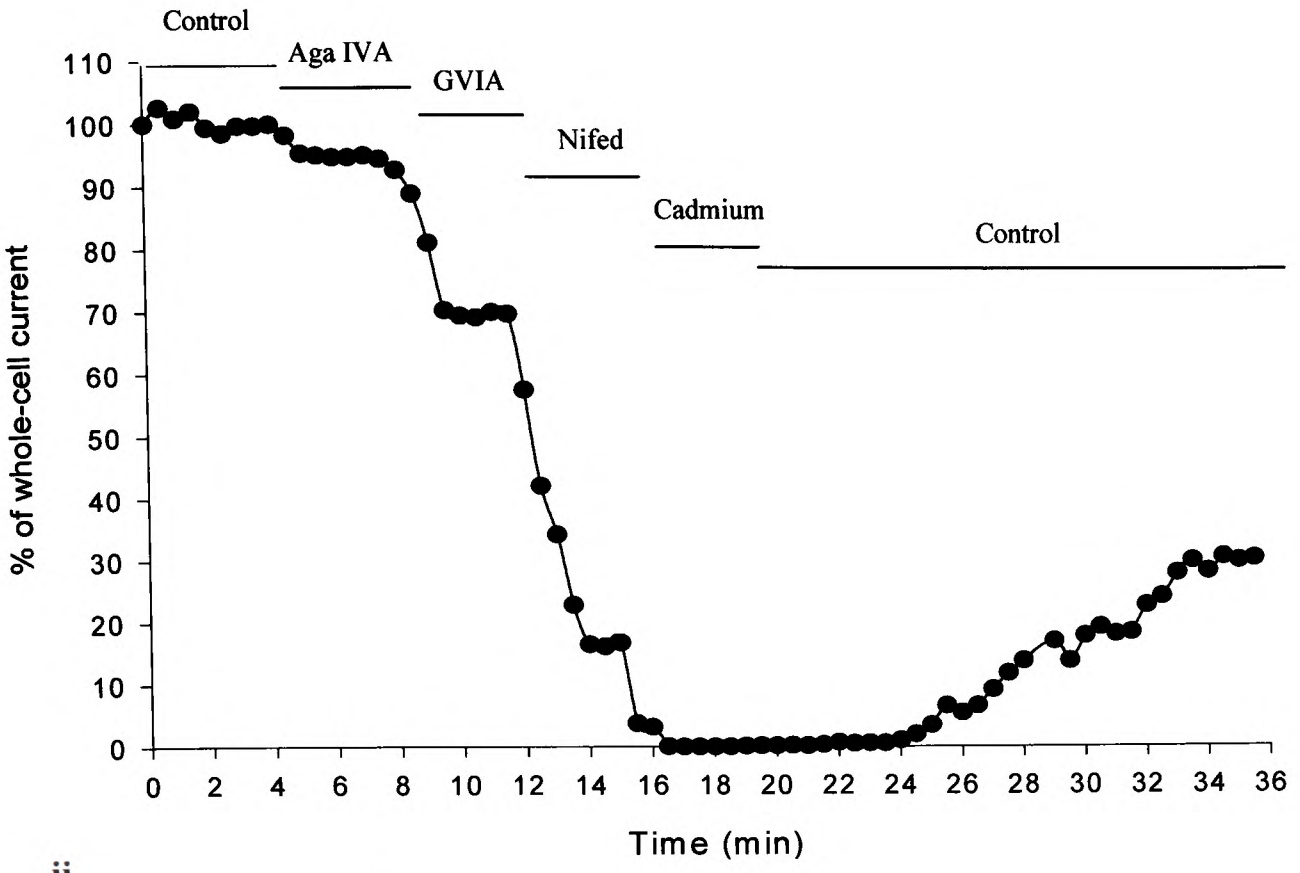

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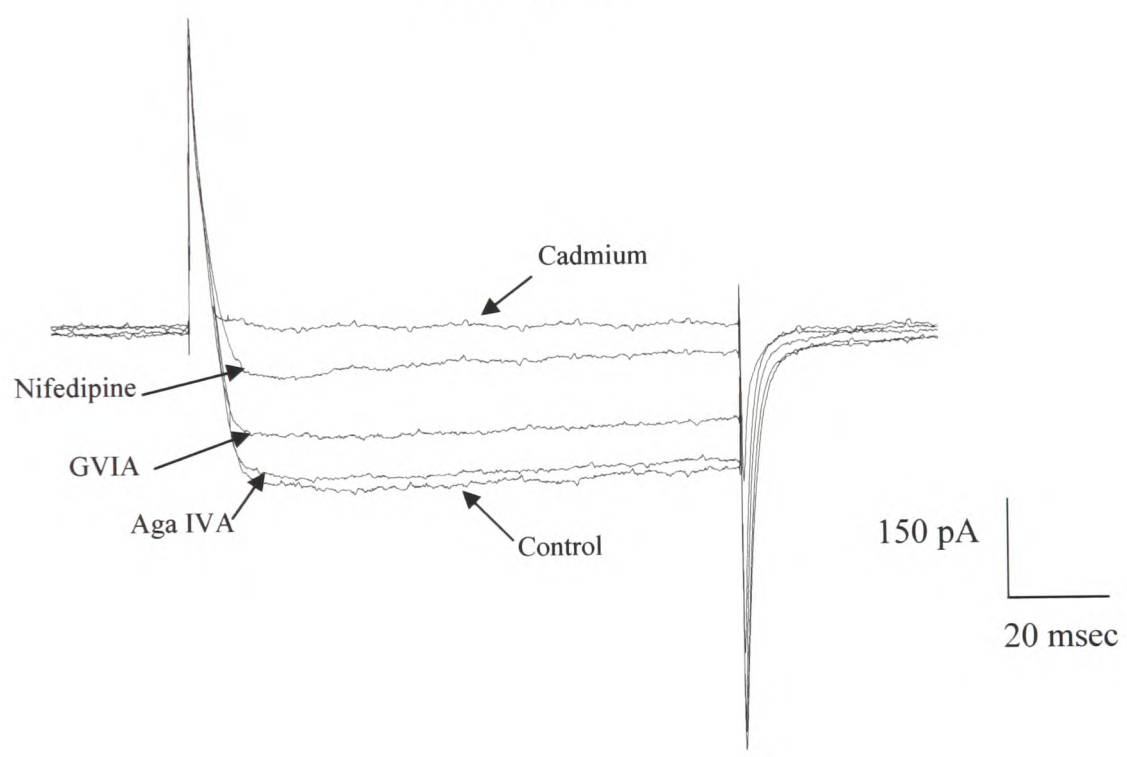

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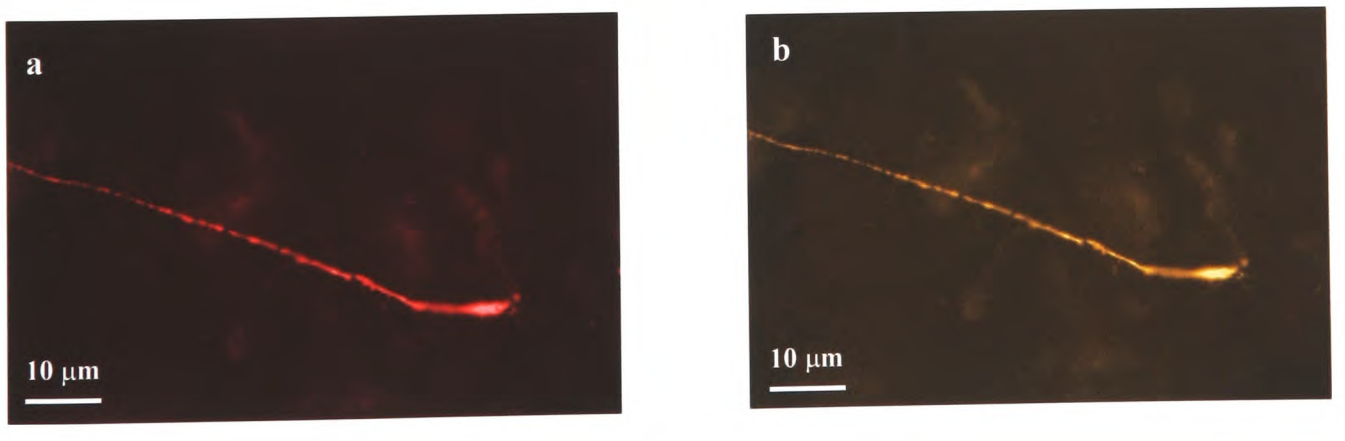

Figure 7.8 B: Time course (i) and electrophysiological traces (ii) for whole-cell calcium current characterisation in a single GAD+ve cell at day 3 *in vitro*. (iii) Post-recording identification of the cell's phenotype: (a) Identification of the cell recorded from by streptavidin-Texas Red labelling of biocytin. (b) Co-localisation with Cy5 antibody against GAD. *On occasion, when the pipette was removed from the cell body at the end of the experiment, the cell body was pulled away with it. The cell was then identified by the streptavidin-Texas Red labelling of the neurites left in the culture, as illustrated above.*

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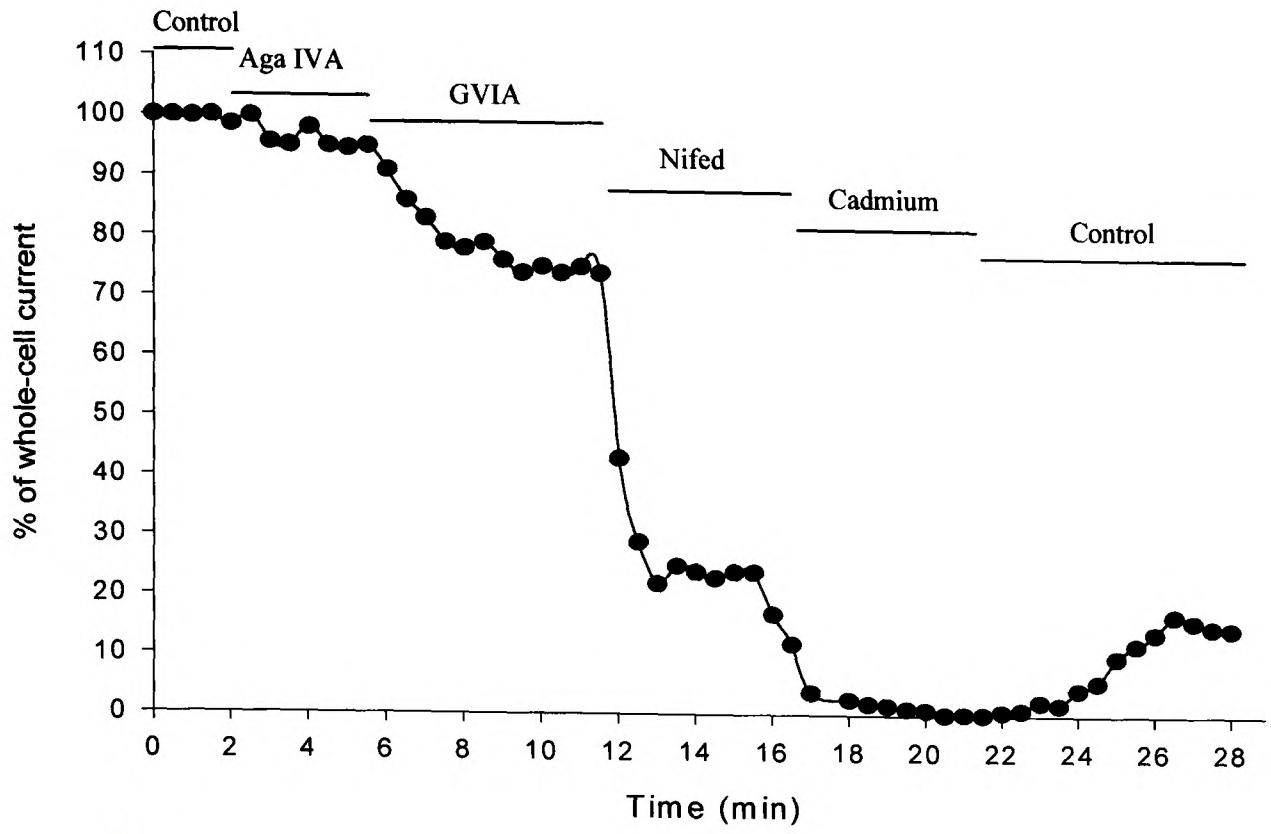

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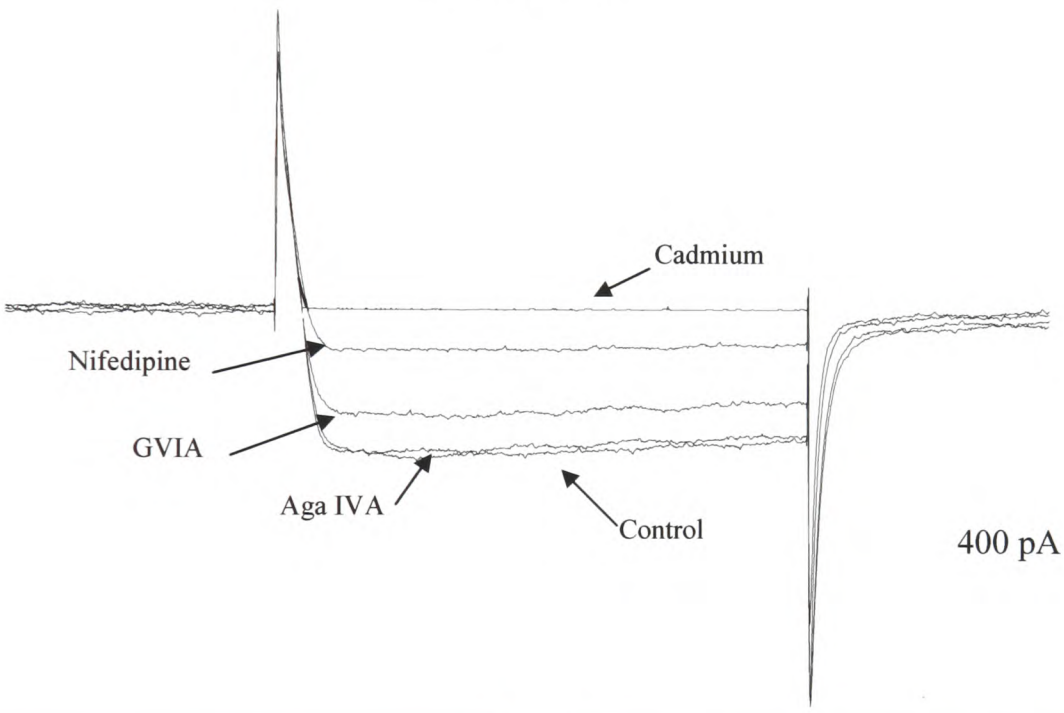

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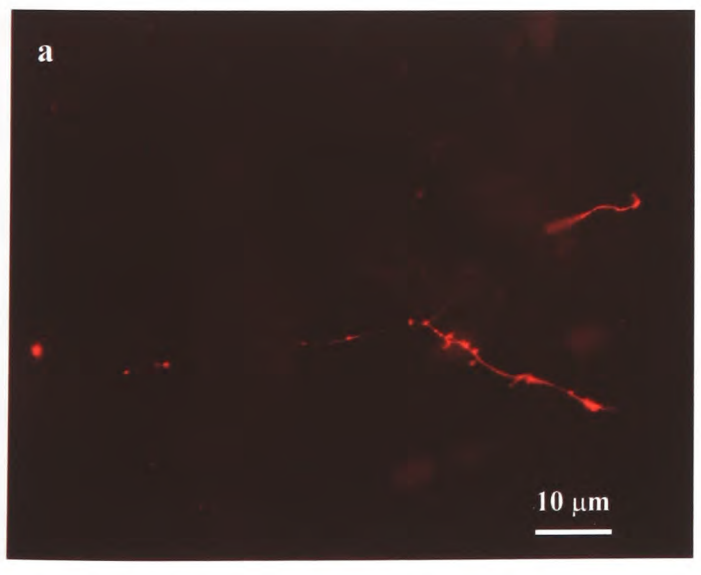

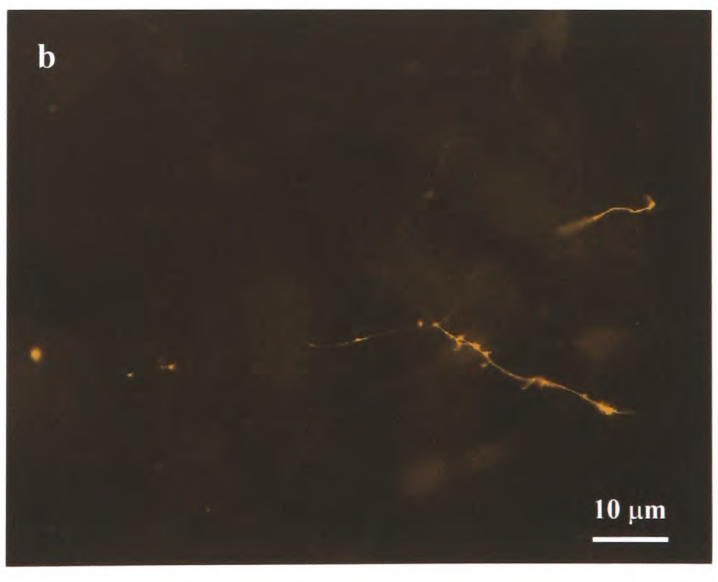

Figure 7.8 C: Time course (i) and electrophysiological traces (ii) for whole-cell calcium current characterisation in a GAD+ve cell at day 7 *in vitro*. (iii) Post-recording identification of cell phenotype: (a) Identification of the biocytin-filled neurites with streptavidin-Texas Red. (b) Co-localisation with CY5 anti-GAD antibody. *On occasion, when the pipette was removed from the cell body at the end of the experiment, the cell body was pulled away with it. The cell was then identified by the streptavidin-Texas Red labelling of the neurites left in the culture, as illustrated above.*

Nifed = nifedipine

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7.4 DISCUSSION

The aim of this chapter was to characterise the expression of the VDCC components of whole-cell calcium currents in embryonic dissociated cells of the ventral mesencephalon at days 1, 3 and 7 *in vitro*. Whilst the primary purpose was to record from dopaminergic cells within the culture, cell phenotype could not be confirmed until after the experiment was terminated, so data from both TH+ve and GAD+ve cells was inevitably obtained and has been presented.

7.4.1 Current-voltage (IV) relationship

For both cell phenotypes maximal inward current was consistently observed upon stimulation from a resting membrane potential of –60 mV to a test potential of 0 mV (Figures 7.1 A and B). The reversal potential for both cell phenotypes was ~ +20 mV. This is far lower than the theoretical equilibrium potential for calcium as defined by the Nernst equation (~ +130 mV; Appendix 5), which represents the point of equilibrium between chemical and electrical driving forces for entry of ions into a cell. There are several possible reasons for the low reversal potentials observed, including the presence of endogenous potassium within the cell and the absence of a calcium chelator in the intracellular solution used. It is also likely that the low reversal potentials were due to outward currents carried by monovalent ions through calcium channels (Reuter and Scholz, 1977; Fenwick *et al.*, 1982; Lee and Tsien, 1982), for example, caesium that was present in the intracellular solution to eliminate potassium currents (Hille, 1994). Nevertheless, the purpose of examining the IV-relationships for TH+ve and GAD+ve cells was to establish the voltage required to produce I_{max} at each time point, to ensure that the stimulation protocol used for subsequent characterisation

experiments elicited the maximal calcium current. As recording conditions were the same on each day, the constancy of the voltage step needed to produce I_{max} is a valid observation.

7.4.2 Relationship between cell capacitance and current size

As illustrated by Figures 7.2 A and B, a linear relationship between cell capacitance and I_{max} was identified in both TH+ve and GAD+ve cells. Within these correlations it is also noticeable that current size and capacitance increased, in general, with time in culture for both phenotypes i.e. cells at day 1 *in vitro* generally had smaller currents and lower capacitance than those at day 3 *in vitro*. Similarly, cells tended to have larger currents and greater capacitance at day 7 *in vitro* than at day 3 *in vitro*. Capacitance can be used as a measure of the surface area of the cell. From the studies carried out in Chapters 3, 4 and 5, and from observation of cells recorded from, it was apparent that the size of cultured cells increased over the 7 day culture period, so it is to be expected that capacitance would increase.

The linear relationship between I_{max} and capacitance seen in both TH+ve and GAD+ve cells is indicative that the increase in total current amplitude is proportional to the increase in cell surface area. As the amplitude of the calcium current is proportional to the conductance of the ion through calcium channels, I_{max} may serve as a measure of the number of calcium channels in the cell being recorded from. Thus, the linear relationship between capacitance and I_{max} seen in the studies presented here may be indicative that the increase in cell surface area is proportional to the increase in the number of calcium channels being expressed in the cell membrane. This would indicate that the density of calcium channels (the number of channels per unit area of membrane) remained constant over the culture period. An

alternative possibility is that the increase in I_{max} was due to a change in the gating properties of VDCC, resulting in an increase in the calcium conductance from a static number of channels.

A caveat concerning the data presented here is that it may not represent the calcium conductance of every VDCC in the cell. It is possible that application of a depolarisation step to the cell soma may not have activated all distal VDCC. It is obvious from the studies presented in Chapters 3, 4 and 5 of this thesis that neurite length of cells in dissociated embryonic VM cultures increased over the 7-day culture period. A study by Takada *et al.* (2001) has demonstrated the presence of VDCC on neurites of VM cells. It is possible that the data presented here may be a representation of somatic VDCC conductance as oppose to the calcium conductance of the soma and all neurites.

7.4.3 Inhibition of voltage-dependent calcium currents by selective antagonists

Dose response experiments were carried out for each VDCC antagonist in order to determine the concentration of each inhibitor that produced complete inhibition of the relevant VDCC subtype. IC_{50} values for application of VDCC antagonists, Aga IVA, GVIA and nifedipine concur with the findings of other studies in neuronal preparations (Mintz *et al.*, 1992; Mintz and Bean, 1993; Randall and Tsien, 1995).

7.4.4 Characterisation of voltage-dependent calcium currents in TH+ve and GAD+ve cells at days 1, 3 and 7 *in vitro*

The results presented here illustrate that TH+ve and GAD+ve cells from the VM of embryonic day 14 rat pups possess a full complement of HVA VDCC by day 1 in culture.

VDCC of dissociated embryonic VM cells have not been previously characterised by electrophysiology. Autoradiographic studies in adult animal brain slices have identified dihydropyridine-binding sites (Gould *et al.*, 1985) and GVIA-binding sites (Takemura *et al.*, 1989) in the substantia nigra, and immunohistochemical studies have localised N and L-type channels to dopamine neurons of the substantia nigra (Takada *et al.*, 2001).

Furthermore, two studies in dispersed postnatal d3-d10 rat VM cells have used specific VDCC inhibitors to electrophysiologically characterise voltage-dependent calcium currents. The results of these studies, by Cardozo and Bean (1995) and Ishibashi *et al.* (1995) revealed similar percentage contributions of current components to the total calcium current; where P/Q = ~ 33 %, N = ~ 24 %, L = ~ 32 % and R = ~ 12 %. These findings vary considerably from the findings of this study, where, most noticeably, in this study the P/Q-type channel contribution to the total calcium current was much lower, both in TH+ve and GAD+ve cells. The variation between the results presented in this chapter and the studies of Cardozo and Bean (1995) and Ishibashi *et al.* (1995) could be attributable to differences in experimental design; for example, variations in dissociation procedure, recording solutions, or stimulation protocol. However, such disparate findings may be a result of the difference in ages of the cells being investigated; the studies of Cardozo and Bean (1995) and Ishibashi *et al.*, (1995) used postnatal VM cells, whereas the studies presented in this chapter used embryonic VM cells. The results of this chapter illustrate that the individual channel contributions vary over time, and whilst this cannot be extrapolated to the *in vivo* situation, it is indicative that VDCC expression is developmentally regulated.

As this study illustrates, (Tables 7.4 and 7.6 and Figures 7.3 and 7.6) the contribution by the N-type channel as a percentage of the maximal current decreases over the 7 day period, and the contribution from the R-type channel increases, for both cell types. Upon comparison of the actual values for current contribution of the components to I_{max}, (Tables 7.5 and 7.7 and Figures 7.4 and 7.7) it becomes apparent that the increases in the currents attributable to the various channel-types over the 7 day time period are not all proportional to the increase in I_{max} over the same time period. Figures 7.4 and 7.7 illustrate that the currents attributable to the P/Q-type and L type channels increase in an approximately proportional manner to the increase in I_{max}. However, the currents attributable to the N-type and R-type channels in both cell types increase disproportionally; in the former case the current amplitude increases less steeply than I_{max} and in the latter case the current amplitude increases more steeply than I_{max}.

The current contributed by a channel type is proportional to the conductance through that type of channel in the cell membrane. It is possible, as discussed above, that the number of each channel type remains constant over the 7 day culture period, but the conductance of each channel type increases, with the increases in conductance of the N and R-type channel being disproportionate to the total increase in channel conductance. Changes in gating patterns of VDCC, wherein alterations are seen in the open–probability, or time of opening of a channel have been shown to occur under patch-clamp conditions in response to application of neurotransmitters (Jones and Elmslie, 1997; Lee and Elmslie, 2000). However, changes in gating patterns of VM cells in development have not been investigated.

Alternatively, the size of the current from a particular VDCC subtype could be considered to be a measure of the number of channels of that type in the cell being recorded from. If this

approach is taken, the total density of calcium channels, as discussed in Section 7.4.2, above, appears to remain constant throughout the culture period. The proportional increases in P/Q and L-type channel currents are indicative that the density of these channel types also remains constant, whilst it could be concluded that the density of N-type channels per cell decreased over the 7 day period and the density of R-type channels increased. Changes in N- and R-type channel density could be accounted for by the formation, or lack of formation, of new channels, the formation of new subunits that alter stoichiometry of channel configuration, or, in the case of the increased density of R-type VDCC, the recruitment of previously silent channels.

Changes in VDCC expression in developing cells have been a subject of some debate, as the correlation between various developmental processes and VDCC expression varies widely between cell types. Arnhold *et al.* (2000) showed that the contribution to the whole-cell current shifted from N- and L-type channels to P/Q- and R-type channels with differentiation in embryonic stem-cell derived neurones. This is in contrast to the work of Hilaire *et al.* (1996) who demonstrated that during ontogenesis in embryonic mouse sensory neurons, the P-type current contribution to the total current decreased markedly and the Q-type contribution increased, whilst the R-type contribution remained constant. Other studies in a variety of cell types have suggested that selective expression of VDCC subtypes in developing tissues plays a key role in processes such as cell migration (Filoux *et al.*, 1994; Komuro and Rakic, 1992) and synaptogenesis (Dourado and Dryer, 1992; Jones *et al.*, 1997; Verderio *et al.*, 1995; Rossi *et al.*, 1994; Basarsky *et al.*, 1994). In these studies, the subunit co-expressed with the developmental process varied, suggesting that the correlation between VDCC expression and developmental process is cell specific. As discussed in Chapter 6,

calcium plays a highly important role in developmental procedures such as cell differentiation, migration and synaptogenesis. These procedures are mediated by the activation of secondary messenger enzymes as a result of calcium entry into the developing cell. As such, initiation of these developmental procedures will require mechanisms for regulating calcium influx, such as VDCC. It is therefore not surprising that VDCC expression correlates to developmental processes. It is possible that the variation in the correlation between channel subtype expression and developmental process seen in different cell phenotypes is dependent upon the conductance of the channel subtype in that particular cell phenotype.

The results presented in this chapter demonstrate that the contribution to the whole-cell current by any VDCC component at any of the days examined is different in TH+ve and GAD+ve cells. However, the findings of this chapter also demonstrate a uniformity in the pattern of expression of VDCC subtypes in TH+ve and GAD+ve cells over the 7-day period, wherein P/Q- and L-type channel contributions to the whole-cell current are constant, the N-type contribution decreases and the R-type contribution increases. The constituent cells of cultures used in the studies presented here are taken from such functionally different structures as the STN and the SNpc. It is therefore interesting that the contribution of individual VDCC subtypes to the total calcium current in GAD+ve and TH+ve cells from within these cultures varies, whilst the pattern of VDCC expression is so similar. As discussed in Chapter 6, the $[Ca]_i$ required for developmental processes can vary between cell types, and as discussed above, the expression of a channel subtype in development may depend upon its contribution to the total calcium current. It could be suggested that midbrain TH+ve and GAD+ve cells have different calcium requirements for certain developmental

processes and therefore individual subtype contribution to the total calcium current may vary. However, N- and R- type channels may be fundamentally involved in these processes in both cell types, hence the pattern of expression of these channels is similar. In support of a specific role for N-type channels, studies have demonstrated that N-type VDCC are involved in mediating neurotransmitter release (Takahashi and Momiyama, 1993; Wheeler *et al.*, 1994; Mochida *et al.*, 1998). The decrease in N-type VDCC conductance in the studies presented here may represent developmental changes in synaptic activity.

The changes in contribution to I_{max} of the various channel subtypes in developing dopaminergic cells over time also presents an intriguing possibility with respect to the physiology of these cells when exposed to growth factors. As discussed in Chapter 1 of this thesis, growth factors are often incorporated into graft tissue used for foetal transplantation as a treatment for PD (Chapter 1, Section 1.2.4.3) to aid survival and outgrowth of TH+ve cells within the graft. However, the literature regarding the effectiveness of growth factors and the optimal time of addition of factors to maximise TH+ve cell survival and outgrowth varies significantly despite, in many cases, close similarity in the methodology of cell preparation.

For example, the study by Clarkson *et al.* (1998), reported that pre-treatment of graft tissue taken from the VM of embryonic day 15 rats with bFGF had no effect on the survival of TH+ve neurons within the graft. In contrast, a methodologically similar study by Mayer *et al.* (1993a) demonstrated that pre-treatment of embryonic rat VM graft tissue with bFGF significantly increased the survival of TH+ve neurons within the graft. In the latter study, the age of the donor foetus was not provided, but a crown-rump measurement of 10-11 mm was given (methodology for Mayer *et al.*, 1993a provided in Mayer *et al.*, 1993b). According to

the review of staging and dissection of rat embryos by Dunnett and Bjorklund (1992), this indicates an earlier gestational age than the foetuses used in the study by Clarkson *et al.* (1998).

As discussed in Chapter 6, evidence exists to support an action of bFGF on VDCCs (Shitaka *et al.*, 1996; Katsuki *et al.*, 1998). The data presented in this chapter illustrates variation in the contribution by channel subtypes to the whole-cell calcium current in TH+ve cells. It is therefore possible that the discrepancies reported in the trophic actions of bFGF on developing TH+ve cells may be due to variation in the correspondence between addition of bFGF, and the presence (or sufficient number) of the VDCC channel subtype through which bFGF has an action.

Further to this, a study by Knusel *et al.* (1990) examined the ChAT activity of embryonic septal cholinergic (SC) neurons following application of NGF or bFGF at various times points after cell plating. The results showed that bFGF only enhanced ChAT activity when given immediately or 24 hours after cell plating, and that NGF only enhanced ChAT activity when give at 3 or 8 days after cell plating. As discussed in Chapters 6, NGF, like bFGF, is known to have an effect upon VDCC (Koike *et al.*, 1989; Rogers and Hendry, 1990; Nikodijevic and Guroff, 1992; Levine *et al.*, 1995; Jia *et al.*, 1999). Whilst the actions of bFGF have been shown to be mediated through the L-type VDCC in the studies by Shitaka *et al.* (1996) and Katsuki *et al.* (1998) both the L- and N-type VDCC have been implicated in the actions of NGF (Levine *et al.*, 1995). Given the variations in VDCC subtype expression in numerous cell types, as discussed above, it is possible that VDCC subtype expression also varies during embryonic SC neuron development. Thus, the time-dependent effects of bFGF

and NGF may depend upon (sufficient) expression of the relevant VDCC subtype. This may explain the lack of effect of NGF upon cultured TH+ve cells from the rostral ventrolateral medulla (Copray *et al.*, 1999) or within mesencephalic grafts transplanted into lesioned rat striatum (Zeng *et al.*, 1996), when a trophic role for NGF on TH+ve cells has been documented by other studies (Date *et al.*, 1990, Date *et al.*, 1994).

In conclusion, this chapter presents data to illustrate that embryonic GAD+ve and TH+ve cells of the VM cultured from ED14 rat pups possess a full complement of HVA VDCC within 24 hours of culturing. Furthermore, the pattern of expression of VDCC subtypes varies over the 7 day culture period, with the contribution to the whole-cell calcium current by the N-type channel decreasing and the contribution to the whole-cell calcium current by the R-type channel increasing for both cell types. The aim of the next chapter is to use whole-cell patch-clamp electrophysiology to investigate the effect of direct application of G_4- and G_1-AChE and BuChE to voltage-dependent calcium currents elicited from embryonic VM cells.

CHAPTER 8

The effect of BuChE, G_4- and G_1-AChE on voltage-dependent calcium currents elicited from dissociated embryonic VM cells

8.1 INTRODUCTION

As discussed in Chapter 6, neurite extension is dependent upon a very particular concentration of calcium within the cell. Variation above or below this set-point results in retardation of outgrowth or even neurite retraction (Kater *et al.*, 1988; Rogers and Hendry 1990; Franklin and Johnson, 1992; Neely and Nicholls, 1995). VDCC compose the primary route of calcium into neuronal cells in response to depolarisation, and numerous studies have shown that alteration of the conductance through VDCC significantly affects neurite extension (Mattson *et al.*, 1988b; Robson and Burgoyne, 1989 Porter *et al.*, 1997; Starikova *et al.*, 1998). Further to this, evidence from several studies has implicated a role for bFGF and NGF in the modulation of calcium channels, which may result in the well-documented stimulatory effects on neurite outgrowth seen upon addition of these factors both *in vitro* and *in vivo* (Koike *et al.*, 1989; Nikodijevic and Guroff, 1992; Levine *et al.*, 1995; Shitaka *et al.*, 1996; Katsuki *et al.*, 1998; Collings *et al.*, 1999; Jia *et al.*, 1999). The mechanism of action of the trophic effects of BuChE and G_4- and G_1-AChE on neurite outgrowth of embryonic TH+ve cells seen in the studies presented in Chapter 3 of this thesis is not certain. The evidence for bFGF and NGF exerting an effect upon VDCC therefore raises the possibility that BuChE and G_4- and G_1-AChE may also be capable of doing so. Having characterised the expression of VDCC in dissociated embryonic VM cells in Chapter 7, the aims of the experiments presented in this chapter are firstly, to examine whether BuChE, G_4- and G_1-AChE and relevant controls have an effect upon voltage-dependent calcium currents of embryonic VM cells using whole-cell patch-clamp electrophysiology. Secondly, preliminary investigations are conducted into the channel subtype involved in mediating any effects of BuChE and G_1- and G_4-AChE on voltage-dependent calcium currents.

8.2 EXPERIMENTAL PROCEDURES

8.2.1 Preparation of dissociated cultures of the VM

Suspensions of ventral mesencephalon cells from E14 rats were prepared as described in Chapter 2, Section 2.2.2 and plated onto astrocyte-covered coverslips. Cultures were used for electrophysiological recordings from day 1 to day 7 *in vitro*.

8.2.2 Electrophysiological recordings

As described in Chapter 2 (Sections 2.2.5.1 – 2.2.5.6) a culture was placed into the recording chamber and perfused with extracellular solution containing 5mM Ba^{2+}. A cell was chosen for recording based upon its morphological characteristics. The aim was to record primarily from dopaminergic cells within the culture. From the biotinylated-labelling of dopaminergic cells in the work of Chapters 3, 4 and 5, it was known that dopaminergic cells tended to have larger soma and were often pyramidal in shape. However, as cell phenotype could not be confirmed until after the experiment was terminated, data from both TH+ve and GAD+ve cells was inevitably obtained and has been analysed.

A pipette was filled with intracellular solution containing 0.2 % biocytin and a gigaseal was formed between the pipette and the chosen cell. Upon whole-cell access the cell was voltage-clamped at a holding potential (Vh) of –60 mV. Series resistance and cell capacitance were determined by analysing instantaneous membrane responses to 5 mV, 30 msec voltage steps. The mean series resistance was 5.1 ± 0.26 MΩ, and partial compensation (60-85 %) was applied.

8.2.3 Post-recording triple labelling

To identify the phenotype of the cell recorded, the biocytin fill was detected using Texas Red-conjugated streptavidin followed by labelling with fluorescent antibodies for TH (FITC fluorophore) and GAD (Cy5 fluorophore) as described in Chapter 2, Sections 2.2.5.7 and 2.2.5.8.

8.2.4 Experimental design

The primary aim of the experiments presented in this chapter was to establish whether BuChE, G_4- and G_1- AChE had an effect upon voltage-dependent calcium currents elicited from dissociated VM cells. To control for the possibility of any action of these cholinesterases being a result of a non-specific protein effect, BSA was applied to a number of cells. In addition to this work, preliminary investigations were also conducted into the channel subtype involved in mediating any effects of cholinesterase application on voltage-dependent calcium currents. Finally, a set of experiments were carried out to ensure that any effects of BuChE, G_4- and G_1- AChE on voltage-dependent calcium currents were not a result of an increase in intracellular calcium, by incorporating 10 mM ethylene glycol-bis(aminoethylether) N,N,N',N'-tetraacetic acid (EGTA), a calcium chelator, into the intracellular solution. For all experiments a single 60 mV, 100 msec depolarisation step was applied every 30 seconds.

For experiments to investigate the effects of cholinesterases upon voltage-dependent calcium currents test substances were applied when the current had stabilised (defined by three depolarisation steps eliciting currents within 2 pA of each other). Test substances and their concentrations are given in Table 8.1. Following test substance application, the cell was

perfused with extracellular solution to test the reversibility of the inhibited current. Cells were recorded from on each day from day 1 to day 7 *in vitro*. Only one test substance was applied per cell and recordings for each test substance were taken from a variety of days *in vitro* and the data was pooled.

Test Substance	Concentration
BuChE	0.01, 0.03, 0.3, 3 U/ml
G_4-AChE	
G_1-AChE	
BSA	1 mg/ml

Table 8.1 Concentrations of test substances applied to whole-cell calcium currents elicited from dissociated cells of the VM

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For preliminary experiments to investigate the channel subtype involved in mediating any effects of cholinesterase on voltage-dependent calcium currents, a selective VDCC antagonist was applied when the current had stabilised. VDCC antagonists used were one of nifedipine (5 μM), GVIA (1 μM), or Aga IVA (200 nM). Following this, when the inhibited current had stabilised, 0.3 U/ml of G_4- or G_1-AChE or 0.3 U/ml of BuChE was applied, again until the current had stabilised. Cells were recorded from on each day from day 1 to day 7 *in vitro*. Only one VDCC antagonist and one test substance were applied per cell and results for each antagonist/test substance were taken from a variety of days *in vitro* and the data was pooled.

For experiments to examine whether any effects of cholinesterases upon voltage-dependent calcium currents were due to an increase in intracellular calcium concentration, 10 mM EGTA was incorporated into the intracellular solution and cells were recorded from as described in Section 8.2.2, above. 3 U/ml of BuChE or G_4- or G_1-AChE was applied when the current had stabilised. Cells were recorded from on each day from day 1 to day 7 *in vitro*.

Only one test substance was applied per cell and recordings for each test substance were taken from a variety of days *in vitro* and the data was pooled.

In all experiments recordings were ended when the current began to deteriorate. Test substances and VDCC antagonists were applied via delivery to the bath in extracellular solution through a three-way tap system. Complete exchange of the bath solution occurred in approximately 1.5 mins.

8.2.5 Source of test substances

As outlined in Chapter 3 (Section 3.2.3), G_4-AChE was purified from a commercial stock of *Electrophorus electricus* AChE (Sigma-Aldrich Company Ltd. Poole, UK, type V-S, 1000 U/ml) using procainamide-ECH sepharose affinity chromatography. G_1-AChE was produced from stably transfected constructs from the mouse pcDNA-3 clone expressed in a HEK cell line kindly provided by Professor Palmer Taylor (Department of Pharmacology, University of California, San Diego, USA) and purified using a meta inhibitor affinity resin. BuChE was purified from a commercial equine stock (Sigma-Aldrich company Ltd., Poole, UK, 500 U/ml) using procainamide ECH sepharose affinity chromatography. Cholinesterase activity of each of the above was assessed by the Ellman assay, followed by protein determination using the Lowry method (Appendix 3). The purity of fragments in each case was assessed using gel filtration with UV detection (SMART^{TM} machine, Amersham Pharmacia Biotech. Ltd., UK). Stock solutions of BuChE (450 U/ml), G_4- (520 U/ml) and G_1-AChE (480 U/ml) were made in 20% glycerol: 80 % PBS, aliquoted and stored at –80 °C. BSA was obtained from Sigma-Aldrich Company Ltd. (Poole, UK) and solutions of BSA (1 mg/ml) were made in extracellular solution immediately prior to use. Cadmium chloride and nifedipine were

obtained from Sigma-Aldrich Chemical Company Ltd. (Poole, UK), Aga IVA was obtained from Calbiochem-Novabiochem Corp., UK., and GVIA from Bachem (UK). Stock solutions of Aga IVA (100 μM), GVIA (100 μM) and cadmium chloride (100 mM) were made in distilled water. A stock solution of nifedipine (100 mM) was made in DMSO. VDCC antagonist stock solutions were aliquoted and frozen at –20 °C. All test substances were diluted in extracellular solution immediately prior to use.

8.3 RESULTS

8.3.1 The effect of BuChE, G_4- and G_1- AChE and BSA on voltage-dependent calcium currents from TH+ve cells

BuChE and G_4- and G_1-AChE inhibited whole-cell calcium currents elicited from TH+ve cells in dissociated cultures of the VM in a concentration dependent manner. Perfusion of the cell with extracellular solution following application of test substance resulted in partial recovery of the inhibited current for each of the test substances. Values for inhibition of I_{max} by the test substance, expressed as a percentage of $I_{max} \pm$ s.e.m., are given in Table 8.2 and illustrated graphically by Figure 8.1 (for each data point $n \geq 6$). Representative electrophysiological traces of the inhibition of I_{max} by BuChE, G_4- and G_1-AChE are shown in Figures 8.2 A, B and C respectively, with a representative time course for application of 3 U/ml of each substance. Values for recovery of the inhibited current following perfusion with extracellular solution, expressed as a percentage of the inhibited current ± s.e.m., are given in Table 8.2. BSA had no effect upon whole-cell calcium currents (n = 5).

Treatment		Inhibition of I_{max} (% ± s.e.m.)	Recovery (% ± s.e.m.)
BuChE	0.01 U/ml	1.75 ± 0.5	94.7 ± 8.6
	0.03 U/ml	3.46 ± 0.6	86.5 ± 4.4
	0.3 U/ml	14.2 ± 1.3	64.0 ± 4.2
	3 U/ml	31.8 ± 4.8	42.6 ± 2.9
G_4-AChE	0.01 U/ml	12.8 ± 1.8	81.1 ± 3.0
	0.03 U/ml	19.4 ± 1.4	51.9 ± 3.4
	0.3 U/ml	31.4 ± 1.6	38.1 ± 2.9
	3 U/ml	43.6 ± 3.0	36.0 ± 4.3
G_1-AChE	0.01 U/ml	10.1 ± 0.9	77.4 ± 1.6
	0.03 U/ml	15.6 ± 1.0	69.5 ± 2.7
	0.3 U/ml	25.3 ± 1.0	44.1 ± 3.2
	3 U/ml	34.5 ± 1.3	33.1 ± 3.2
BSA	1 mg/ml	-	-

Table 8.2 Effect of BuChE, G_4- and G_1-AChE and BSA on whole-cell calcium currents of TH+ve cells

Values for inhibition expressed as % of I_{max} ± s.e.m. Values for recovery expressed as a % of the inhibited current. For each $n \geq 5$

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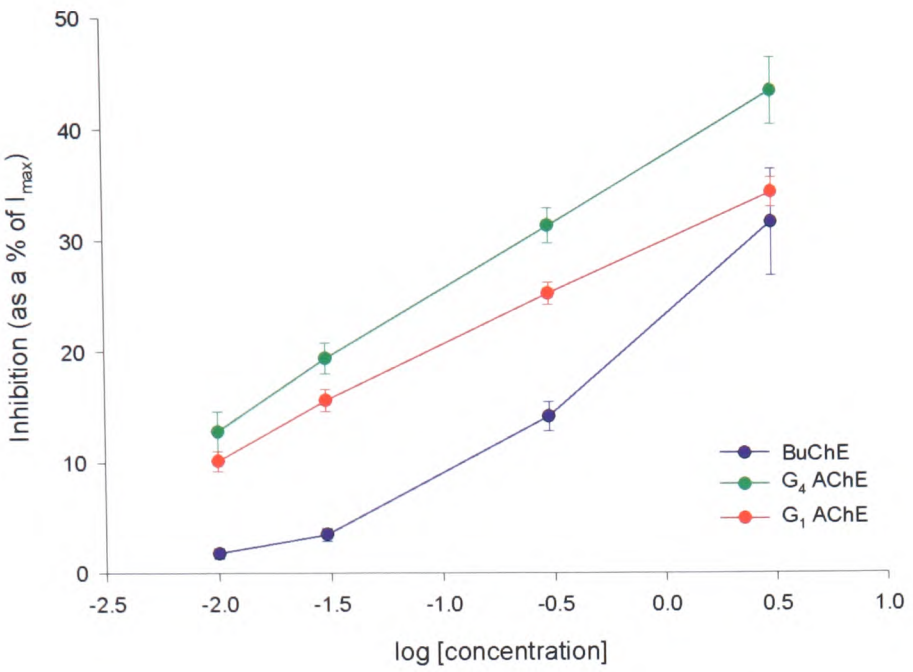

Figure 8.1 Inhibition of whole-cell calcium currents of TH+ve cells by BuChE, G_4- and G_1-AChE.

Values expressed as % of I_{max} ± s.e.m. For each $n \geq 6$

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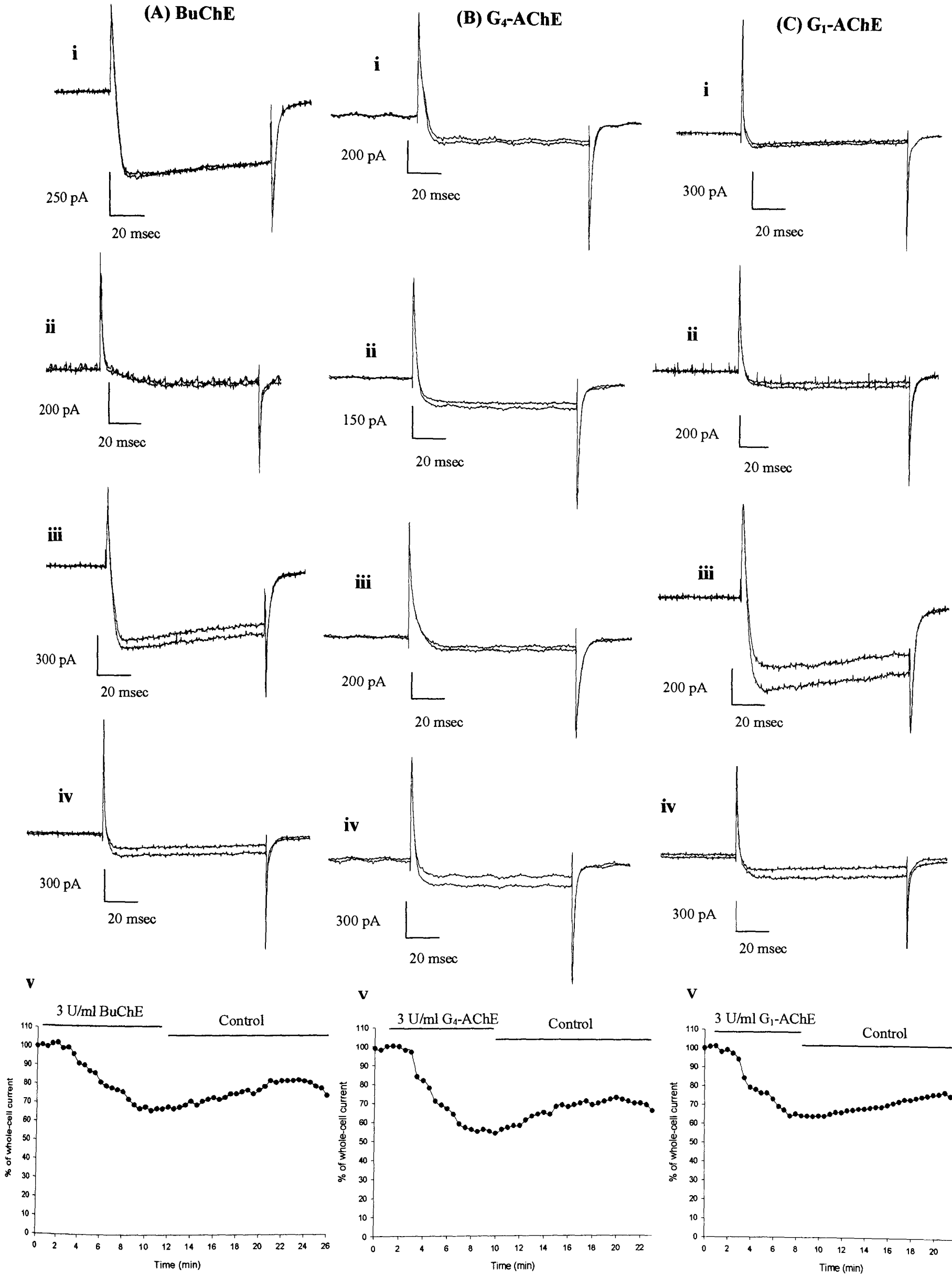

Figure 8.2 Representative traces of the effects of (i) 0.01 U/ml, (ii) 0.03 U/ml (iii) 0.3 U/ml (iv) and 3 U/ml and (v) representative time course of the effect of 3 U/ml (A) BuChE, (B), G_4-AChE (C) and G_1-AChE on whole-cell calcium currents elicited from embryonic TH+ve cells in dissociated cultures

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8.3.2 The effect of BuChE, G_4- and G_1- AChE and BSA on voltage-dependent calcium currents from GAD+ve cells

BuChE and G_4- and G_1-AChE inhibited whole-cell calcium currents elicited from GAD+ve cells in dissociated cultures of the VM in a concentration dependent manner. Perfusion of the cell with extracellular solution following application of test substance resulted in partial recovery of the inhibited current for each of the test substances. Values for inhibition of I_{max} by the test substance, expressed as a percentage of $I_{max} \pm$ s.e.m., are given in Table 8.3 and illustrated graphically by Figure 8.3 (for each data point $n \geq 7$). Representative electrophysiological traces of the inhibition of I_{max} by BuChE, G_4- and G_1-AChE are shown in Figures 8.4 A, B and C respectively with a time course for application of 3 U/ml of each substance. Values for recovery of the inhibited current following perfusion with extracellular solution, expressed as a percentage of the inhibited current ± s.e.m., are given in Table 8.3.

BSA had no effect upon whole-cell calcium currents (n = 6).

Treatment		Inhibition of I_{max} (% ± s.e.m.)	Recovery (% ± s.e.m.)
BuChE	0.01 U/ml	1.3 ± 0.3	88.3 ± 2.3
	0.03 U/ml	3.7 ± 0.6	91.1 ± 2.1
	0.3 U/ml	17.1 ± 2.0	48.1 ± 4.4
	3 U/ml	35.6 ± 2.3	26.1 ± 3.0
G_4-AChE	0.01 U/ml	13.2 ± 1.0	78.6 ± 1.6
	0.03 U/ml	21.8 ± 1.9	42.6 ± 2.2
	0.3 U/ml	36.6 ± 1.1	31.2 ± 2.2
	3 U/ml	48.5 ± 3.8	19.8 ± 5.6
G_1-AChE	0.01 U/ml	10.4 ± 0.7	72.2 ± 2.2
	0.03 U/ml	17.4 ± 1.1	58.5 ± 1.9
	0.3 U/ml	28.2 ± 0.8	30.0 ± 4.2
	3 U/ml	35.2 ± 2.6	24.3 ± 4.0
BSA	1 mg/ml	-	-

Table 8.3 Effect of BuChE, G_4- and G_1-AChE and BSA on whole-cell calcium currents from GAD+ve cells

Values for inhibition expressed as % of I_{max} ± s.e.m. Values for recovery expressed as a % of the inhibited current. For each $n \geq 6$

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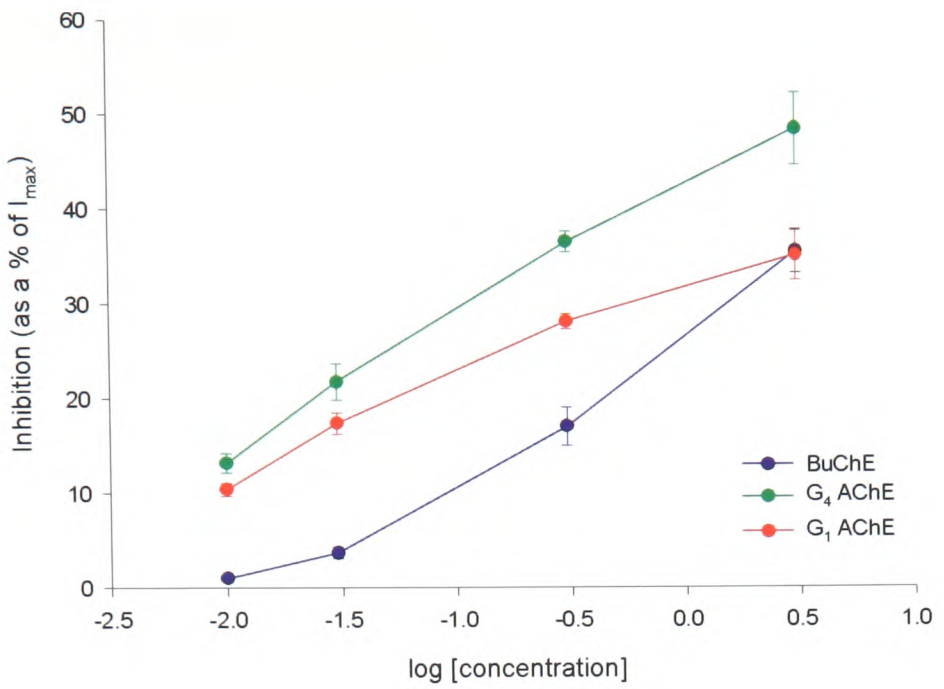

Figure 8.3 Inhibition of whole-cell calcium currents from GAD+ve cells by BuChE, G_4- and G_1- AChE.

Values expressed as % of I_{max} ± s.e.m. For each $n \geq 7$

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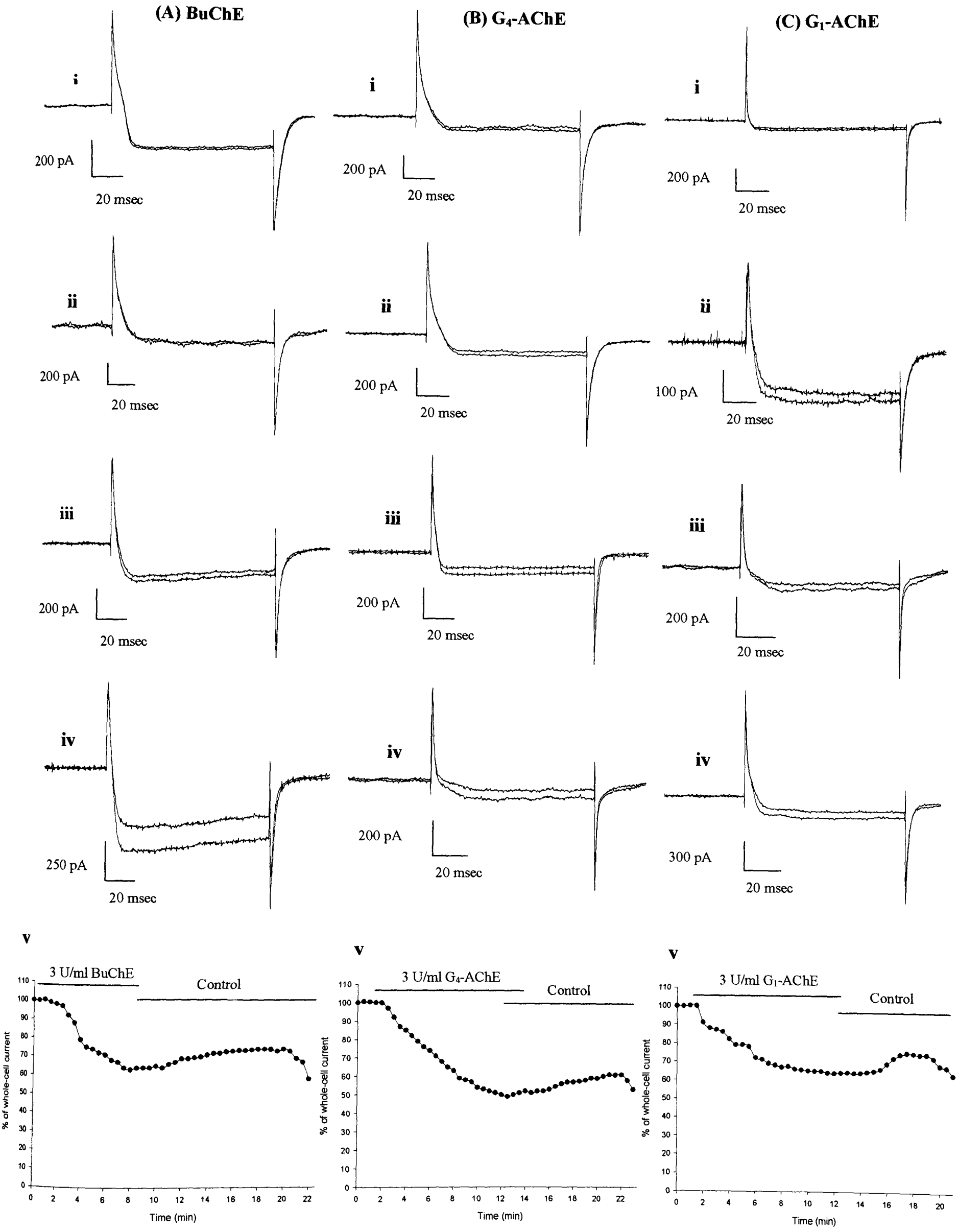

Figure 8.4 Representative traces of the effects of (i) 0.01 U/ml, (ii) 0.03 U/ml (iii) 0.3 U/ml (iv) and 3 U/ml and (v) representative time course of the effect of 3 U/ml (A) BuChE, (B) G_4-AChE (C) and G_1-AChE on whole-cell calcium currents elicited from embryonic GAD+ve cells in dissociated cultures

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8.3.3 Preliminary results for identification of VDCC subtype involved in the inhibition of voltage-dependent calcium currents from TH+ve cells by BuChE or G_4- or G_1-AChE

The results for application of 0.3 U/ml BuChE or G_4- or G_1-AChE alone to TH+ve cells given in this section are taken from Section 8.3.1, above.

In TH+ve cells, application of 0.3 U/ml BuChE or G_4- or G_1-AChE following inhibition of the P/Q-type current with 200 nM Aga IVA did not greatly differ from application of 0.3 U/ml BuChE or G_4- or G_1-AChE alone. Application of 0.3 U/ml BuChE or G_4- or G_1-AChE following inhibition of the N-type current with 1 μM GVIA resulted in a diminished inhibition in comparison to application of 0.3 U/ml BuChE or G_4- or G_1-AChE alone. Likewise, application of 0.3 U/ml BuChE or G_4- or G_1-AChE following inhibition of the L-type current with 5 μM nifedipine resulted in a diminished inhibition in comparison to application of 0.3 U/ml G_4- or G_1-AChE or BuChE alone.

Values for inhibition of I_{max} by 0.3 U/ml BuChE or G_4- or G_1-AChE following application of either Aga IVA (200 nM), GVIA (1 μM) or nifedipine (5 μM), expressed as a mean percentage inhibition of I_{max}, are shown in Table 8.4 and Figure 8.5. Due to the small n values for all data groups, s.e.m. and significance values were not calculated. Values for mean percentage inhibition of $I_{max} \pm$ s.e.m. by 0.3 U/ml BuChE or G_4- or G_1-AChE alone are shown in Table 8.4 and Figure 8.5 for comparison.

VDCC antagonist	0.3 U/ml BuChE	0.3 U/ml G_4-AChE	0.3 U/ml G_1-AChE
	Inhibition of I_{max} (% ± s.e.m.)	Inhibition of I_{max} (% ± s.e.m.)	Inhibition of I_{max} (% ± s.e.m.)
No VDCC antagonist	14.2 ± 1.3	31.4 ± 1.6	25.3 ± 1.0
200 nM Aga IVA	14.4	32.1	26
1 μM GVIA	9.4	24.6	17.7
5 μM Nifedipine	4.5	13.2	12.9

Table 8.4 Effect of 0.3 U/ml BuChE, G_4- and G_1-AChE on whole-cell calcium currents from TH+ve cells, with and without prior addition of VDCC antagonists.

Values for application of 0.3 U/ml BuChE or G_4- or G_1-AChE alone are expressed as mean % inhibition of I_{max} ± s.e.m; $n \geq 6$. Values for application of 0.3 U/ml BuChE or G_4- or G_1-AChE following application of VDCC antagonist are expressed as mean % inhibition of I_{max}. $n = 2$-3

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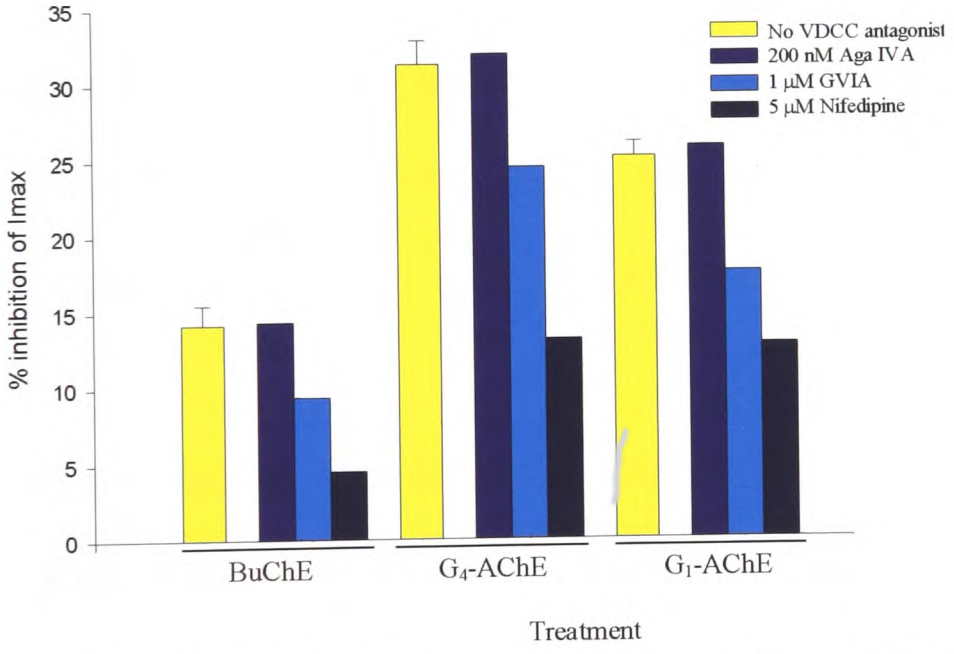

Figure 8.5 Graphical representation of effect of application of 0.3 U/ml BuChE or G_4- or G_1-AChE either alone (yellow; values taken from Section 8.3.1 of this chapter), or following application of a VDCC antagonist (shades of blue) upon TH+ve cells in culture

Values for application of 0.3 U/ml BuChE or G_4- or G_1-AChE alone are expressed as mean % inhibition of I_{max} ± s.e.m; $n \geq 6$. Values for application of 0.3 U/ml BuChE or G_4- or G_1-AChE following application of VDCC antagonist are expressed as mean % inhibition of I_{max}. $n = 2$-3

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8.3.4 Preliminary results for identification of VDCC subtype involved in the inhibition of voltage-dependent calcium currents from GAD+ve cells by BuChE or G_4- or G_1-AChE

The results for application of 0.3 U/ml U/ml BuChE or G_4- or G_1-AChE alone to GAD+ve cells given in this section are taken from Section 8.3.2, above.

In GAD+ve cells, application of 0.3 U/ml BuChE or G_4- or G_1-AChE following inhibition of the P/Q-type current with 200 nM Aga IVA did not greatly differ from application of 0.3 U/ml BuChE or G_4- or G_1-AChE alone. Application of 0.3 U/ml BuChE or G_4- or G_1-AChE following inhibition of the N-type current with 1 μM GVIA resulted in a diminished inhibition in comparison to application of 0.3 U/ml BuChE or G_4- or G_1-AChE alone. Likewise, application of 0.3 U/ml BuChE or G_4- or G_1-AChE following inhibition of the L-type current with 5 μM nifedipine resulted in a diminished inhibition in comparison to application of 0.3 U/ml G_4- or G_1-AChE or BuChE alone.

Values for inhibition of I_{max} by 0.3 U/ml BuChE or G_4- or G_1-AChE following application either Aga IVA (200 nM), GVIA (1 μM) or nifedipine (5 μM), expressed as a mean percentage inhibition of I_{max}, are shown in Table 8.5 and Figure 8.6. Due to the small n values for all data groups, s.e.m. and significance values were not calculated. Values for mean percentage inhibition of $I_{max} \pm$ s.e.m. by 0.3 U/ml BuChE or G_4- or G_1-AChE alone are shown in Table 8.5 and Figure 8.6 for comparison.

VDCC antagonist	0.3 U/ml BuChE	0.3 U/ml G_4-AChE	0.3 U/ml G_1-AChE
	Inhibition of I_{max} (% ± s.e.m.)	Inhibition of I_{max} (% ± s.e.m.)	Inhibition of I_{max} (% ± s.e.m.)
No VDCC antagonist	17.1 ± 2.0	36.6 ± 1.1	28.2 ± 0.8
200 nM Aga IVA	16.8	37.4	26.1
1 μM GVIA	12.5	22.8	22.1
5 μM Nifedipine	7.2	16.2	12.3

Table 8.5 Effect of 0.3 U/ml BuChE, G_4- and G_1-AChE on whole-cell calcium currents from GAD+ve cells, with and without prior addition of VDCC antagonists.

Values for application of 0.3 U/ml BuChE or G4- or G1-AChE alone are expressed as mean % inhibition of I_{max} ± s.e.m; $n \geq 7$. Values for application of 0.3 U/ml BuChE or G_4- or G_1-AChE following application of VDCC antagonist are expressed as mean % inhibition of I_{max}. $n = 1$-3

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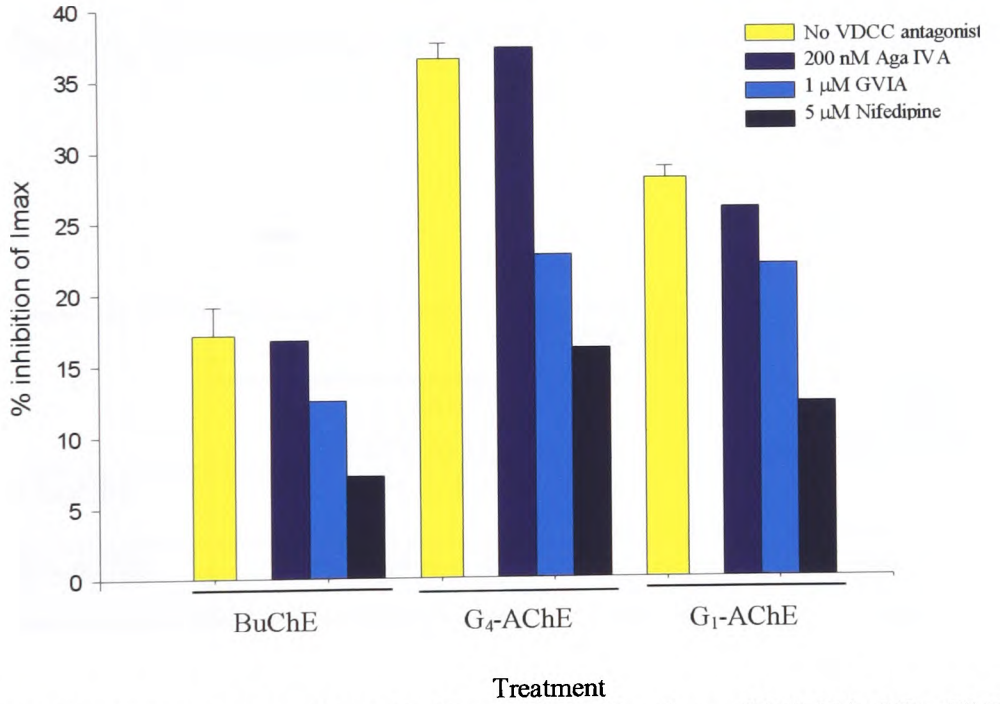

Figure 8.6 Graphical representation of effect of application of 0.3 U/ml BuChE or G_4- or G_1-AChE either alone (yellow; values taken from Section 8.3.2 of this chapter), or following application of a VDCC antagonist (shades of blue) upon GAD+ve cells in culture

Values for application of 0.3 U/ml BuChE or G4- or G1-AChE alone are expressed as mean % inhibition of I_{max} ± s.e.m; $n \geq 7$. Values for application of 0.3 U/ml BuChE or G_4- or G_1-AChE following application of VDCC antagonist are expressed as mean % inhibition of I_{max}. $n = 1$-3

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8.3.5 The effect of BuChE, G_4- and G_1- AChE on voltage-dependent calcium currents following intracellular calcium chelation.

Inhibition of whole-cell calcium currents by 3 U/ml of BuChE or G_4- or G_1-AChE following incorporation of 10 mM EGTA into the intracellular pipette solution to chelate intracellular calcium did not significantly differ from inhibition by 3 U/ml of BuChE or G_4- or G_1-AChE in experiments without intracellular EGTA.

Values for inhibition of I_{max} by 3 U/ml BuChE or G_4- or G_1-AChE with or without EGTA incorporated into the intracellular solution are given in Table 8.6. Values for inhibition by BuChE or G_4- or G_1-AChE without EGTA in Table 8.6 are taken from Section 8.3.1, above. Representative electrophysiological traces of the inhibition of I_{max} by 3 U/ml BuChE or G_4- or G_1-AChE following incorporation of EGTA into the intracellular solution are shown in Figure 8.7

	Inhibition of I_{max} (% ± s.e.m.)	
	- EGTA in intracellular solution	+ EGTA in intracellular solution
3 U/ml BuChE	31.8 ± 4.8	34.2 ± 1.7
3 U/ml G_4-AChE	43.6 ± 3.0	46.2 ± 2.3
3 U/ml G_1-AChE	34.5 ± 1.3	34.8 ± 1.7

Table 8.6 Effect of 3 U/ml BuChE or G_4- or G_1-AChE on whole-cell calcium currents with and without incorporation of calcium chelator EGTA into the intracellular solution.

Values are expressed as mean % inhibition of I_{max} ± s.e.m. Values for application of 3 U/ml BuChE or G_4- or G_1-AChE without EGTA are taken from Section 8.3.1 above. $n \geq 4$.

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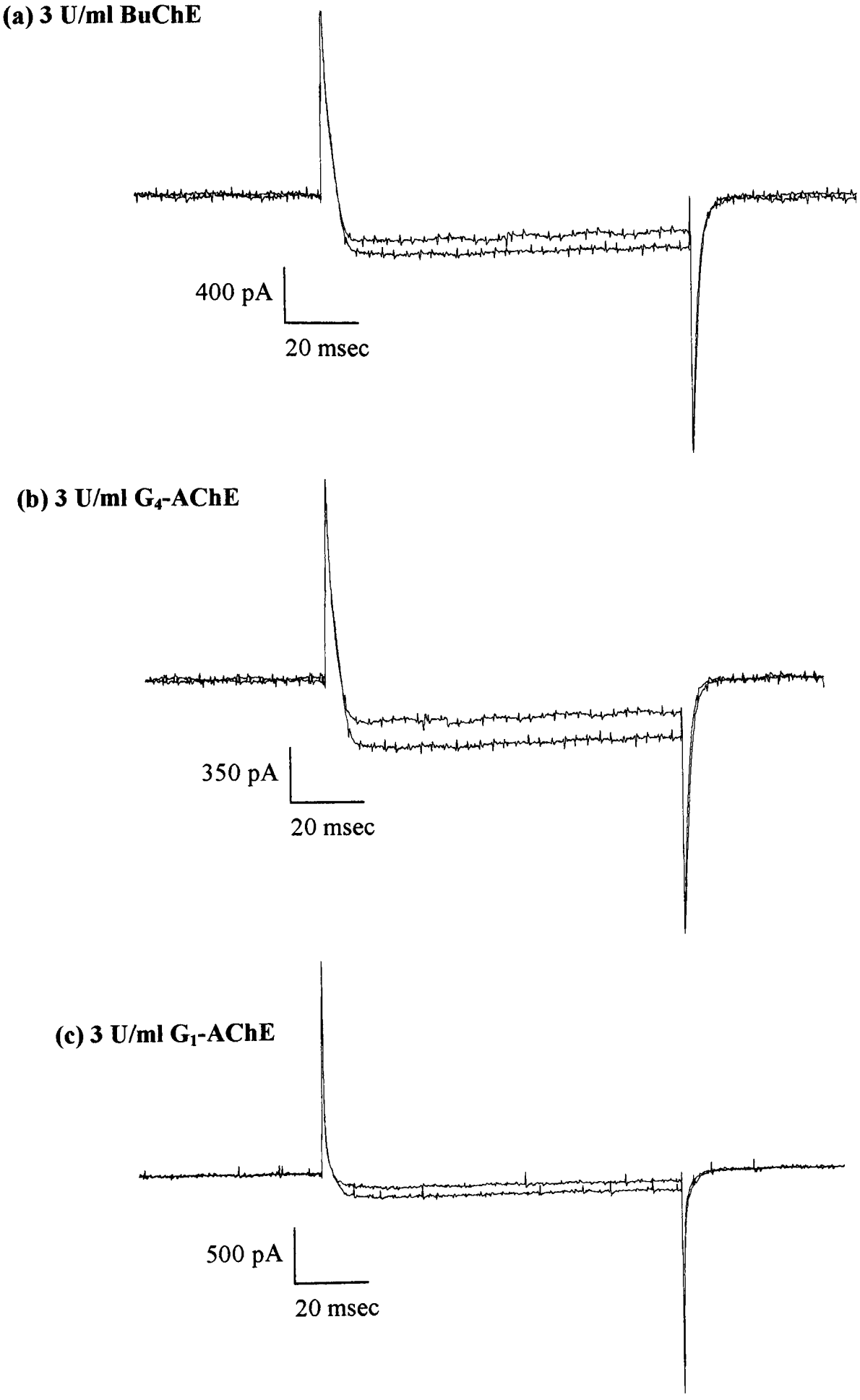

Figure 8.7 Representative traces of the effects of 3 U/ml (a) BuChE, (b) G_4-AChE, and (c) G_1-AChE on whole-cell calcium currents from embryonic dissociated VM cells following incorporation of 10 mM EGTA into the intracellular solution.

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8.4 DISCUSSION

8.4.1 Effect of BuChE and G_4- and G_1-AChE on voltage-dependent calcium currents from embryonic TH+ve and GAD+ve VM cells in culture

The results presented here illustrate that BuChE and G_4- and G_1-AChE inhibit whole-cell calcium currents elicited from both TH+ve and GAD+ve embryonic VM cells in a dose-dependent manner. It is unlikely that the inhibition by these substances results from a non-specific protein effect, as BSA had no effect upon whole-cell calcium currents elicited from either cell type.

The preliminary studies conducted to identify the VDCC subtype involved in the inhibition of whole-cell calcium currents in both GAD+ve and TH+ve cells by BuChE, G_4- and G_1-AChE implicate a role for the N-type and L-type channels, but not the P/Q-type channel. This pilot data is suggestive of an effect of BuChE, G_4- and G_1-AChE either on, or mediated through, the L- and N-type channels. This may be indicative of an interaction of BuChE, G_4- and G_1-AChE with the $\alpha_{1C/D}$ and α_{1B} VDCC subunits, or an indirect effect of these substances being mediated through the $\alpha_{1C/D}$ and α_{1B} subunits of VDCC.

The partial recovery of calcium currents from the inhibition caused by BuChE, G_4- and G_1-AChE may indicate that these substances are binding irreversibly, or causing an irreversible change to VDCC. However, it is possible that the current deteriorated and hence recording was ended before a full recovery from treatment with test substance was achieved. The cell was often perfused by extracellular solution for 10 minutes or more following application of the test substance, without full recovery. This is suggestive of either an irreversible component to the inhibition, or a longer time requirement for reversal of test substance effect.

8.4.1.1 A cholinergic effect of BuChE and G_4- and G_1-AChE on voltage-dependent calcium currents

The possibility that the effects of BuChE and G_4- and G_1-AChE on whole-cell calcium currents are a result of cholinergic activity seems unlikely. Although ventral mesencephalic cultures contain cholinergic neurons (as discussed in Chapter 2, Section 2.1.3.1), an effect on the voltage-dependent calcium current due to the hydrolytic ability of AChE would require ACh to be present within the culture during electrophysiological recordings. The cultures recorded from were continuously perfused with extracellular solution at a rate of 2.6 mls/min. The time taken to obtain a stable whole-cell calcium current following placement of the culture in the recording chamber varied according to the success of obtaining a giga-Ohm seal and whole-cell access. Frequently, attempts were made to patch several cells within the culture before access to the cell that was recorded from was obtained. Under these circumstances the culture was perfused with extracellular solution for up to 30 minutes before a viable clamp was achieved and a test substance applied to the current elicited. Given that cultures comprised a single layer of neuronal cells, it is unlikely that a sufficiently high concentration of ACh remained during perfusion to result in an effect on the voltage-dependent calcium current upon hydrolysis by applied AChE.

The study by Peacock *et al.* (1988) investigated the responses of cultured embryonic mouse ventral mesencephalic cells to direct, or indirect (application to a synaptically connected cell to the one recorded from) application of ACh using intracellular microelectrodes. Direct application of ACh caused depolarisation in 94 % of cells, hyperpolarisation in 4 % of cells, and in 1 % of cells reduced action potential firing was observed. Indirect application of ACh resulted in an increase in post-synaptic potential (PSP) activity in 83 % of cells recorded

from, which was excitatory in 66 % of cells and inhibitory in 17 %. A reduced level of PSP activity occurred in 17 % of cells, which was excitatory in 7 % of cells and inhibitory in 10 % of cells. The variation in the responsiveness of neurons to ACh in this study was suggested to be partly attributable to the heterogeneity of the population in the culture, and therefore the variable expression of AChR in cells recorded from. The studies presented in this chapter also utilised an embryonic VM preparation, and it is possible to consider the results of this chapter in the context of the findings of Peacock *et al.* (1988). As the SNpc receives a cholinergic input from the PPN, it is likely that some of the TH+ve cells recorded from in the experiments presented here were cholinoceptive. Given the consistency of effect of BuChE and G_4- and G_1-AChE on TH+ve cells it is unlikely that hydrolysis of ACh resulted in the inhibition of voltage-dependent calcium currents by BuChE and AChE.

A definitive method of resolving whether the acute inhibitory effects of AChE on voltage-dependent calcium currents are due to a cholinergic effect would be to use AChE that had been modified so that its hydrolytic ability was irreversibly inhibited. Work is being carried out by the Biological and Chemical Defence Establishment at Porton Down, in collaboration with the Greenfield Lab., to irreversibly inhibit the cholinergic ability of G_1-AChE using 1,2,2-trimethyl-propyl-methylphophonofluoridate (soman). Soman, commonly described as a nerve-gas, is a highly toxic organophosphorus compound. It acts by phosphonylating the serine hydroxyl at the active site of AChE, producing irreversible inhibition. Systemic administration of soman results in rapid accumulation of ACh, resulting in peripheral muscarinic manifestations such as excessive smooth muscle contractions, exaggerated nicotinic responses characterised by skeletal muscle fasciculations and weakness, and central effects manifested as seizures. The result of exposure to soman is death by respiratory

depression and cardiovascular collapse (Sterling *et al.*, 1988; Zimmer *et al.*, 1998). Co-incubation of G_1-AChE and soman would produce a G_1-AChE-soman complex which, following dialysis to remove unbound soman, could be used as a test substance in electrophysiology experiments as described in this chapter to establish whether the inhibitory effects of G_1-AChE on calcium currents persist following inhibition of its catalytic ability.

8.4.2 Mechanism of action of BuChE, G_4- and G_1-AChE on voltage-dependent calcium currents

The inhibition of voltage-dependent calcium currents by BuChE, G_4- and G_1-AChE is a novel finding, and the mechanism of action of these substances on VDCC conductance is not clear from the studies presented here. BuChE, G_4- and G_1-AChE could either be modulating the activity of calcium channels directly by interacting with a component of the VDCC to decrease entry of extracellular calcium, or producing some other signal which indirectly affects calcium channels, resulting in a decrease in voltage-dependent calcium conductance.

8.4.2.1 A direct action

It is possible that BuChE, G_4- and G_1-AChE may have had a direct effect upon a VDCC component, or components, resulting in a decrease in calcium conductance. Such inhibition could result from interaction of BuChE, G_4- or G_1-AChE either directly with the α_1- pore-forming subunit, or with a distinct site, which then affects conductance through the α_1-subunit. The hypothetical interaction could be specific, as a result of binding of BuChE or AChE to a particular aspect of the VDCC, or non-specific, resulting, perhaps, in occlusion of the pore-forming channel.

An example of a substance causing direct inhibition of an ionic channel is hexamethonium. This is a nAChR antagonist that inhibits cellular responses to ACh by blocking the cation-selective ionic channels that are controlled by these receptors. Hexamethonium can only block ion channels after they have opened, thus it is referred to as an 'open-channel blocker', hence, increasing the concentration of agonists actually increases the degree of block.

From the data presented in this chapter, and the absence of other studies that have investigated the involvement of VDCC in the actions of cholinesterases, it is not possible to speculate further on a direct mechanism of action for BuChE, G_4- and G_1-AChE on voltage-dependent calcium currents. Completion of the preliminary investigations presented into this chapter of the VDCC subunit(s) involved in the inhibition of voltage-dependent calcium currents by BuChE, G_4- and G_1-AChE would provide further insight into the specificity of the inhibition.

8.4.2.2 An indirect action

Several indirect mechanisms of action for the inhibitory effects of acute application of BuChE, G_4- and G_1-AChE can be postulated. These include the possibility that BuChE, G_4- and G_1-AChE may actually cause an increase in $[Ca]_i$, which then results in inhibition of the voltage-dependent calcium current.

$[Ca]_i$ is known to play a fundamental role in controlling the flow of calcium into the cell through VDCC. Increases in $[Ca]_i$ can result in inhibition of the voltage-dependent calcium conductance by inactivation of VDCC (for review see Hering *et al.*, 2000). This well-recognised phenomenon of calcium-dependent calcium inactivation is illustrated by

comparing a voltage-dependent calcium current under control conditions and following injection of the cell with EGTA, a calcium chelator (Figure 8.8). Inactivation of VDCC can occur directly, through binding of intracellular calcium to a specific EF-hand motif at the C-terminus of the α_{1A} VDCC subunit (De Leon *et al.*, 1995; Bernatchez *et al.*, 1998). Alternatively, inactivation can occur through dephosphorylation of VDCC by a protein kinase (Boland *et al.*, 1991), mediated by calmodulin (Armstrong, 1989; Lee *et al.*, 1999; Peterson *et al.*, 1999; Zühlke *et al.*, 1999).



Figure 8.8 Calcium-dependent inactivation of calcium channels.

An Aplysia central neuron is pre-loaded with caesium to block potassium channels. Under control conditions, depolarisation from –40 mV to +20 mV elicits a voltage-dependent calcium current that appears to inactivate fully in 200 ms. After the cell is injected with EGTA to keep the intracellular free Ca^{2+} buffered at a low level, the inactivation of the calcium current is slower. From Hille, 1994.

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However, the findings of Section 8.3.5 of this chapter, where inhibitory effects of BuChE, G_4- and G_1-AChE remained following addition of 10 mM EGTA to the intracellular solution to chelate intracellular calcium, preclude the possibility that the inhibitory effects of BuChE, G_4- and G_1-AChE caused an increase in $[Ca]_i$, that resulted in a decrease in the voltage-dependent calcium conductance.

A second possible mechanism for indirect calcium channel modulation is through activation of a pertussis-toxin-sensitive (PTX) G-protein, G_o which results in inhibition of VDCC, causing a reduction in amplitude of the voltage-dependent calcium current. This inhibition is not mediated by the conventional cyclic-nucleotide changes associated with G-proteins. Rather, the depression of calcium currents by PTX-sensitive G-proteins is described as 'membrane-delimited'. This is as defined by an experiment which illustrates that bath application of a substance known to cause current inhibition through G_o to almost the entire cell surface is unable to depress the VDCC isolated in an on-cell patch (Figure 8.9). This implies that the signal is not carried any distance through the cytoplasm by a diffusable messenger (for reviews see Hille 1994; Dolphin 1996; Miller, 1998). Instead, it is has been shown that calcium channel inhibition is mediated by the direct binding of G-protein β/γ subunits to one or more sites on the α_1 pore-forming subunit of the VDCC (Herlitze *et al.*, 1996; Ikeda, 1996; Zhang *et al.*, 1996; Qin *et al.*, 1997; Cantí *et al.*, 1999; Simen and Miller, 2000; Zhou *et al.*, 2000).

Several neurotransmitters have been shown to inhibit calcium currents through activation of G_o, including ACh (Higashida *et al.*, 1990), serotonin (Dunlap and Fischbach, 1978), GABA through $GABA_B$ receptors (Dunlap and Fischbach, 1978; Dolphin *et al.*, 1986; Holz *et al.*, 1986; Schiff *et al.*, 2000) and noradrenaline via α-receptors (Holz *et al.*, 1986; Bean, 1989; Lee *et al.*, 2000) (for reviews see Anwyl, 1991; Jones and Elmslie, 1997). The recognised inhibitory effect of ACh on voltage-dependent calcium currents lends support to the suggestion in Section 8.4.1.1 (above) that the effects of BuChE, G_4- and G_1-AChE are not a result of the cholinergic properties of these substances.

It is possible that BuChE, G_4- and G_1-AChE may have caused inhibition of voltage-dependent calcium currents in a similar way to neurotransmitters, as discussed above. However, neurotransmitter-mediated inhibition of voltage-dependent calcium currents is generally characterised by a slowing of activation kinetics (Herlitze *et al.*, 1996; Ikeda, 1996; Qin *et al.*, 1997; Zhou *et al.*, 2000). No change in the activation kinetics of voltage-dependent currents were apparent upon application of BuChE, G_4- and G_1-AChE, as illustrated by the representative traces provided in Figures 8.2 and 8.4. Nevertheless, to test whether the effects of BuChE, G_4- and G_1-AChE seen in experiments presented in this chapter are mediated through a G-protein effect, cultures could be incubated with PTX prior to application of BuChE, G_4- or G_1-AChE during electrophysiological recording of voltage-dependent calcium currents. If the hypothesis is correct, the inhibitory effects of BuChE, G_4- and G_1-AChE on voltage-dependent calcium currents should be blocked.

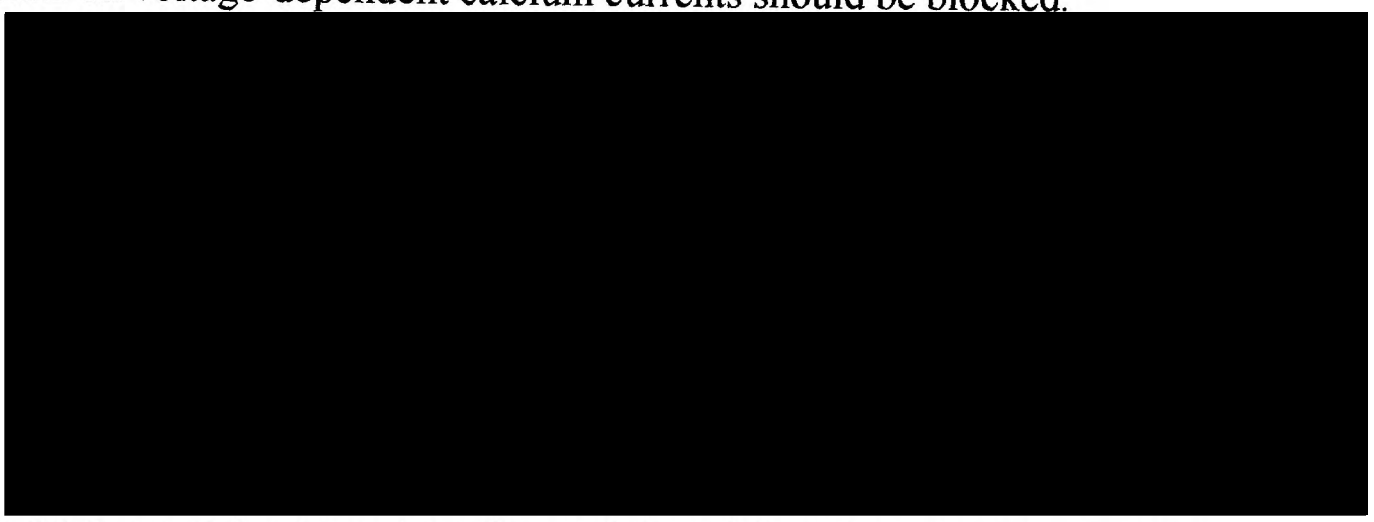

Figure 8.9 Protocol for determining if modulation of the voltage-dependent calcium current is mediated by a diffusable cytoplasmic second messenger or is membrane delimited.

With a messenger-mediated mechanism, agonist in the bath acts on many receptors (R) to make a messenger that diffuses to the channels (black ovals) in the patch and modulates their function (asterisk). On the other hand, in a membrane-delimited mechanism, there is no cytoplasmic messenger, and channels in the patch are not modulated by agonist in the bath. Taken from Hille, 1994.

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A further suggestion of an indirect pathway for the effects of BuChE, G_4- and G_1-AChE on voltage-dependent calcium currents can be proposed following the recent findings of

homology between AChE and neuroligins. As discussed in Chapter 1 (Section 1.3.4.1.3.iii), studies on neuroligin 1, 2 and 3 have demonstrated an extracellular domain homologous to AChE (Ichtchenko *et al.*, 1995 and 1996) and a study by Tsigelny *et al.* (2000), revealed a common EF-hand motif in BuChE, AChE and neuroligins. Neuroligins bind to neurexins, and this interaction is thought to bring about processes that mediate many protein-protein interactions, including cell adhesion, inter-neuronal recognition pathways regulating axon pathfinding, and synaptic vesicle exocytosis (Ichtchenko *et al.*, 1996; Song *et al.*, 1999; Scheiffele *et al.*, 2000). It has been proposed that AChE may replace, or compete with certain types of neuroligins for binding to neurexins, and in doing so, affect intracellular events, with corresponding repercussions on developmental processes such as neurite outgrowth (see Grisaru *et al.*, 1999). Evidence for this theory has been provided by the work of Grifman *et al.* (1998), who demonstrated that overexpression of neuroligin-1 restored both neurite extension and neurexin expression following antisense suppression of AChE in PC12 cells.

The mechanism of action of the neurexin/neuroligin complex is thought to be mediated through interactions of the intracellular regions of neurexins, which associate with other proteins after binding to neuroligin and so mediate the formation of protein complexes which subsequently affect a variety of cellular processes. One such intracellular interaction is the binding of neurexin to calcium-calmodulin-dependent serine protein kinase (CASK). CASK, together with Veli1, -2, and –3, which are *ver*tebrate homologues of *Caenorhabditis Elegans lin*-7 protein and Mint1, a further lin homologue which is a putative vesicle trafficking protein, forms a scaffolding complex (Hata *et al.*, 1996; Butz *et al.*, 1998). There is evidence to suggest that these proteins may then be involved in mediating synaptic vesicle clustering

(Butz *et al.*, 1998) and organelle transportation (Setou *et al.*, 2000). Of relevance to the findings presented in the chapter, however, is the work of Maximov *et al.* (1999), who demonstrate a specific association of the cytosolic carboxy terminus of the N-type VDCC pore-forming α_{1B} subunit with the proteins Mint1 and CASK. This association has been proposed to be of importance to synaptic vesicle exocytosis (Maximov *et al.*, 1999). However, it also provides a feasible pathway by which BuChE, and AChE may modulate VDCC conductance.

The hypothesis that the effects of BuChE and AChE on voltage-dependent calcium currents seen in the experiments presented in this chapter are a result of an interaction with neurexin could be investigated by inhibiting neurexin prior to examining the effects of BuChE or AChE on voltage-dependent calcium currents. Neurexin Iα is known to bind to α-latrotoxin, a component of black widow spider venom (Tzeng *et al.*, 1979; Ushkaryov *et al.*, 1992). However, α-latrotoxin is also known to bind to a G-protein-coupled receptor called latrophilin-1 (or CL1 or CIRL), and is a potent neurotoxin (Krasnoperov *et al.*, 1997; Lelianova *et al.*, 1997; Sugita *et al.*, 1998). The use of α-latrotoxin in an assay that requires specific inhibition of neurexin is thus far from ideal. An alternative suggestion for investigating the involvement of neurexin in the effects of BuChE and AChE on voltage-dependent calcium currents is manipulation of neurexin mRNA levels with antisense neurexin cRNA.

A further consideration in the mechanism of action of BuChE and G_4- and G_1-AChE on voltage-dependent calcium currents is whether the effects are caused by a direct action of test

substances on the cell being recorded from, or indirectly by an effect on neighbouring, synaptically connected cells. Cultures consist of a network of cells (as illustrated by Chapter 2, Figure 2.9 and Chapter 3, Figure 3.8). Perfusion of cultures with test substances (as oppose to direct application of test substance to the single cell being recorded from) results in exposure of all cells in the culture to the test substance. It is therefore possible that alterations in the calcium current of the cell being recorded from are caused indirectly by the effect of the test substance on neighbouring cells. However, if this were the case, the effects of application of test substance on recorded calcium currents would depend upon the number and phenotype of cells projecting on to the cell being recorded from, giving rise to variable data. As demonstrated by the study of Peacock *et al.* (1988), the findings of which are described in Section 8.4.1.1 above, highly variable responses were produced by indirect application of ACh to cells within embryonic dissociated VM cultures. This variation was suggested to be partly attributable to the heterogeneity of the cell population in culture, and therefore the varying responses to ACh of cells projecting onto the cell recorded from. The consistency of the inhibitory effect seen upon application of BuChE, G_4- and G_1-AChE to both TH+ve and GAD+ve cells in this study suggests against the possibility that these effects were caused indirectly by an effect on a neighbouring cell.

8.4.3 A neurotrophic action of BuChE and G_4- and G_1-AChE mediated through VDCC

As discussed in Chapter 6, several studies have demonstrated an effect of two well-known growth factors on VDCC. Jia *et al.* (1999) and Nikodijevic and Guroff, (1992) demonstrated that NGF increased $[Ca]_i$ by acting upon VDCC of PC12 cells and SCG cells respectively. Studies by Shitaka *et al.* (1996) and Katsuki *et al.* (1998) demonstrated similar effects of

application of bFGF to cultured foetal hippocampal cells. Both bFGF and NGF have been shown to improve cell survival and increase neurite outgrowth in cell culture systems. The experiments presented in Chapter 3 of this thesis demonstrated trophic effects of BuChE and G_4- and G_1-AChE upon neurite outgrowth of TH+ve cells in embryonic cultures. The inhibitory effects of BuChE and G_4- and G_1-AChE on whole-cell calcium currents elicited from TH+ve cells are therefore interesting.

Neurite outgrowth is very sensitive to levels of intracellular calcium, with optimal growth cone motility occurring at a highly specific concentration, the set-point, as discussed in Chapter 6. It would appear from the numerous studies conducted to investigate the effect of altering $[Ca]_i$ in developing cells, that the set-point is specific to the cell type (Connor, 1986; Mattson and Kater, 1987; Mattson *et al.*, 1988a and b; Holliday and Spitzer, 1992; Komuro and Rakic, 1996). Studies that illustrate an effect of NGF and bFGF on VDCC, have postulated that the trophic effects of these factors are a result of modulation of $[Ca]_i$ due to alterations in calcium conductance through VDCC by these factors. It is therefore possible that the inhibitory effects of BuChE and G_4- and G_1-AChE on voltage-dependent calcium currents of developing TH+ve cells are at least partly responsible for their trophic actions on neurite outgrowth.

The inhibitory effects of BuChE, G_4- and G_1-AChE on whole-cell calcium currents elicited from GAD+ve cells as well as TH+ve cells demonstrate that the effects of these substances on voltage-dependent calcium currents are not cell specific. As the aim of this thesis was to provide further information about the potential of AChE to act as a trophic factor for embryonic dopaminergic cells, the experiments described in Chapters 3-5 did not examine

the effects of test substances upon the cultured GAD+ve cell population. However, a recent study within the Greenfield Lab. has shown that G_1-AChE enhances neurite outgrowth of GABA-ergic cells in organotypic cultures of postnatal hippocampus (Dr. Toni Day, personal communication). Given the findings of this chapter, perhaps it is not unreasonable to postulate that this trophism may also be mediated by the effects of G_1-AChE on GAD+ve cell voltage-dependent calcium currents.

A weakness of the data presented in this chapter is that values for the effect of each test substance were obtained and pooled from cells recorded from on different days in culture. Having presented data to illustrate that the density of certain VDCC subtypes changes over the 7 day culture period in Chapter 7, data in this chapter would have ideally constituted a dose response series for each test substance at days 1, 3 and 7 *in vitro*. Unfortunately, due to the limited amount of BuChE, G_4- and G_1-AChE available, this was not possible. The pooling of data from several days may have repercussions for interpretation of the data obtained. If the inhibitory effect of BuChE and G_1- and G_4-AChE on calcium currents are a result of a specific interaction with a VDCC subtype or subtypes, then application of the same concentration of these substances at different days could produce disparate results due to a change in the density, or conductance of the relevant VDCC subtype(s). Pooling of data from different time points would mask such discrepancies. A dose response series for each test substance at each time point, taken with the data that is already known about the change in channel density, may also have provided further information about the channel subtypes involved in mediating the effects of BuChE and G_4- and G_1-AChE.

In conclusion, the results presented in this chapter demonstrate an inhibitory action of acute application of BuChE and G_4- and G_1-AChE on whole-cell calcium currents elicited from dissociated embryonic TH+ve and GAD+ve cells in culture. It is postulated that these actions may be at least partly responsible for the trophic effects of BuChE and G_4- and G_1-AChE on neurite outgrowth of TH+ve cells, as illustrated by the results presented in Chapter 3 of this thesis. However, demonstration of an acute, partially reversible action of BuChE and G_4- and G_1-AChE on voltage-dependent calcium currents is not evidence that chronic incubation (for 24, 72 or 168 hours) of cultured cells with these substances causes changes in $[Ca]_i$ that result in an increase in neurite outgrowth. Therefore, the aim of the next chapter is to establish whether chronic incubation of embryonic VM cultures with known VDCC antagonists Aga IVA, GVIA and nifedipine affects neurite outgrowth of TH+ve cells. The immunocytochemical and quantification methodologies used in Chapters 3-5 are utilised to quantify any effects on cell survival and neurite outgrowth.

CHAPTER 9

The effect of VDCC antagonists on survival and outgrowth of embryonic dopaminergic cells in dissociated cultures of the VM

9.1 INTRODUCTION

The data presented in Chapter 8 of this thesis demonstrates that BuChE and G_4- and G_1-AChE inhibit whole-cell calcium currents elicited from dissociated embryonic TH+ve and GAD+ve cells of the VM. As discussed in Chapter 8, it is feasible, given the literature citing the stimulatory effects of NGF and bFGF upon VDCC, that the trophic effects of BuChE and AChE upon dopaminergic cells as demonstrated in Chapter 3, are at least partly attributable to their effect upon voltage-dependent calcium currents. If this is the case then it can be postulated that other antagonists of VDCC should also prove trophic. Thus, the aim of this chapter is to examine the effects of the VDCC antagonists used in Chapter 7, Aga IVA, GVIA and nifedipine, upon cell survival and neurite outgrowth of TH+ve cells using the immunocytochemical, and cell number and neurite length quantification techniques used in Chapters 3, 4 and 5.

9.2 EXPERIMENTAL PROCEDURES

9.2.1 Dissociated cultures of the VM: preparation and staining

Suspensions of ventral mesencephalon cells from E14 rats were prepared as described in Chapter 2 (Section 2.2.2). Cultures were chronically treated for 1, 3 or 7 days with media supplemented with the VDCC antagonists Aga IVA, GVIA, nifedipine or cadmium. Untreated cultures served as controls. Every 48 hours 150 µl of media was removed and replaced with 150 µl of fresh media containing, where appropriate the test substance. At days 1, 3 and 7 *in vitro* cultures were fixed and processed for TH immunoreactivity as described in Chapter 2 (Section 2.2.3).

9.2.2 Experimental design

Cultures were chronically treated for 1, 3 or 7 days with media supplemented with Aga IVA, GVIA, nifedipine or cadmium at the concentrations given in Table 9.1. Experiments were repeated a minimum of three times using foetuses from different mothers. Each treatment group contained a minimum of 4 chamber wells, giving a total of at least 12 cultures for each treatment group ($n \geq 12$).

Test Substance	Concentration
Aga IVA	200 nM
GVIA	1 µM
Nifedipine	5 µM
Cadmium	20 µM

Table 9.1 Concentrations of test substances used to inhibit VDCC components

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9.2.3 Collection of data and statistical analysis

9.2.3.1 Cell numbers

As detailed in Chapters 3, 4 and 5, total cell numbers per culture well were counted for each treatment at each time point. For all experiments involving treatment of cultures with test substances, data was normalised to the untreated control.

9.2.3.2 Neurite length

Camera Lucida drawings were made of every TH+ve cell in the chamber-slide well for each treatment group at each time point. The length of each cell's longest neurite was assessed using a sports route planner and results for each treatment group were normalised to the untreated control.

For both parameters, all data was collected in a blind fashion to prevent sampling bias. For both parameters values for each data group were expressed as mean ± s.e.m. Statistical significance was assessed using either analysis of variance (ANOVA) for normally distributed data followed by a Dunnett's multiple comparison t-test, or the Kruskal-Wallis non-parametric test for non-normally distributed data.

9.2.4 Source of test chemicals

Cadmium chloride and nifedipine were obtained from Sigma-Aldrich Chemical Company Ltd. (Poole, UK), Aga IVA was obtained from Calbiochem-Novabiochem Corp., (UK), and GVIA from Bachem (UK). Stock solutions of Aga IVA (100 μM), GVIA (100 μM) and cadmium chloride (100 mM) were made in distilled water. A stock solution of nifedipine

(100 mM) was made in DMSO. Stock solutions were aliquoted and frozen at –20 °C. All test substances were diluted in culture medium immediately prior to use.

9.3 RESULTS

9.3.1 The effect of VDCC antagonists on dopaminergic neurons in dissociated embryonic cultures of the VM

The data obtained for the effects of treatment with VDCC antagonists on cell survival and neurite outgrowth of dopaminergic cells at days 1, 3, and 7 *in vitro* is shown in Table 9.2.

Treatment	Number of TH+ve cells (normalised to untreated control)			Neurite length (normalised to untreated control)		
	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7
Untreated control	1.0 ± 0.05	1.0 ± 0.06	1.0 ± 0.09	1.0 ± 0.07	1.0 ± 0.08	1.0 ± 0.13
200 nM Aga IVA	0.83 ±0.05	0.99 ± 0.08	1.03 ± 0.14	1.44 ± 0.08**	1.46 ± 0.10**	0.82 ± 0.05
1 μM GVIA	0.90 ± 0.06	1.02 ± 0.07	1.16 ± 0.09	1.71 ± 0.12***	1.69 ± 0.13***	0.83 ± 0.06
5 μM Nifedipine	0.88 ± 0.06	1.0 ±0.08	0.97 ± 0.09	1.78 ±0.14***	1.66 ± 0.13***	0.56 ± 0.05*
20 μM Cadmium	0.18 ± 0.12***	-	-	0.08 ±0.008***	-	-

* denotes $P < 0.05$
** denotes $P < 0.01$
*** denotes $P < 0.001$

Table 9.2 The effect of VDCC antagonists on TH+ve neurons in dissociated embryonic cultures of the VM

Values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. Statistical significance was assessed by the Kruskal-Wallis non-parametric test. $n \geq 12$. Values for cell numbers and neurite outgrowth at days 3 and 7 for treatment with cadmium are missing, as examination of cultures at these time points revealed a complete absence of cells.

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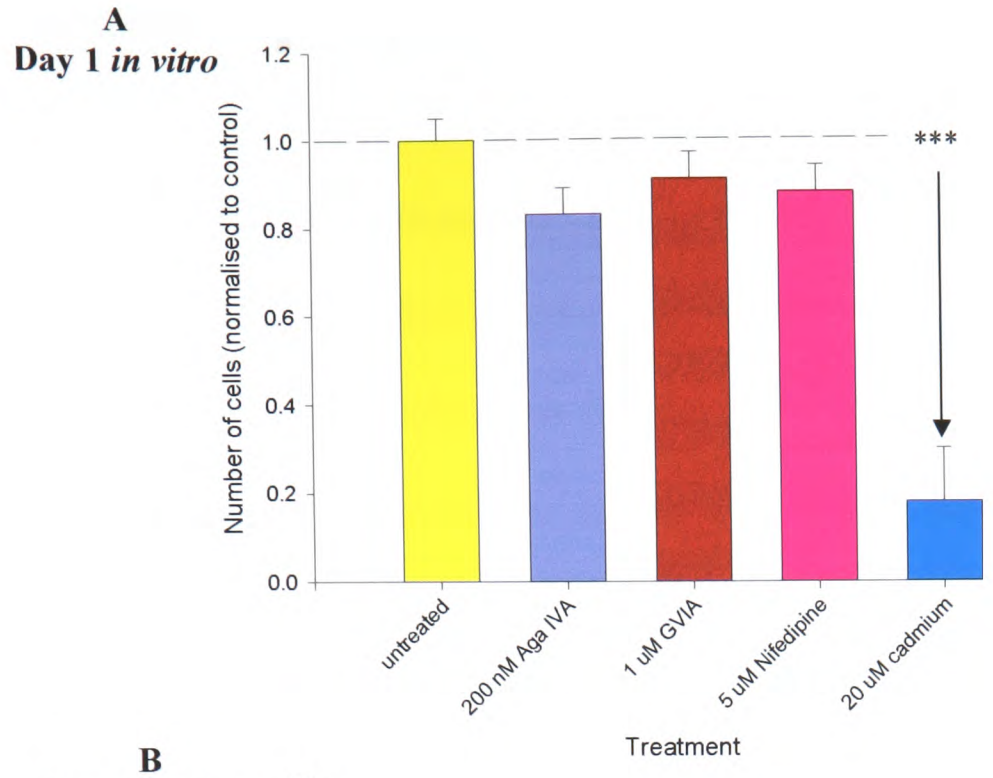
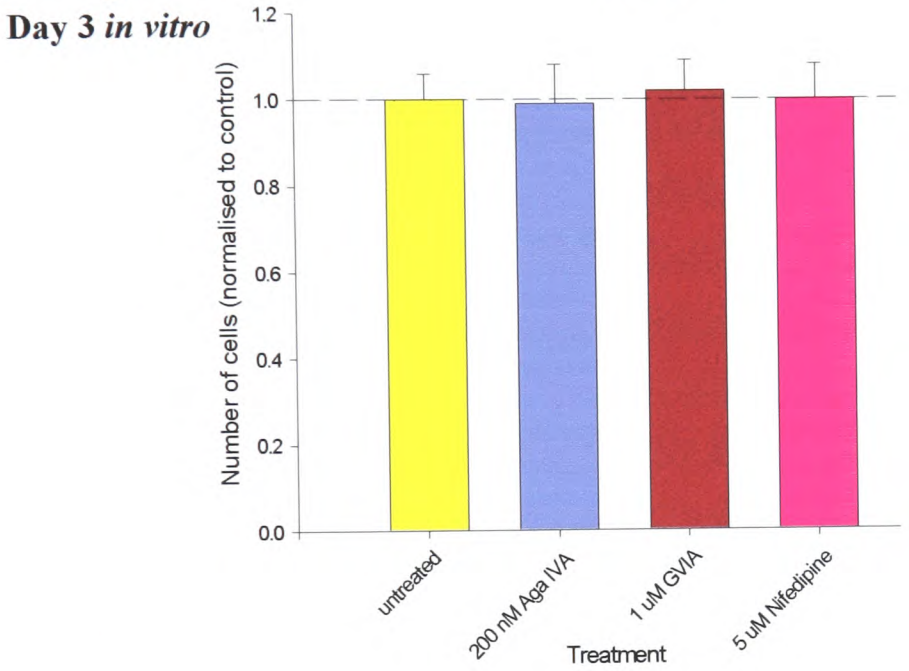
9.3.1.1 The effect of VDCC antagonists on TH+ve cell numbers

As illustrated by Figures 9.1 A, B and C, addition of VDCC antagonists Aga IVA, GVIA and nifedipine had no effect upon total TH+ve cell number per culture from the untreated control at any of the time points. However, addition of cadmium significantly decreased the number of TH+ve cells in comparison to the control at day 1 *in vitro* ($P < 0.001$). Examination of cultures incubated with cadmium at day 3 and day 7 *in vitro* (72 and 168 hours of exposure to cadmium respectively) revealed a complete absence of cells at both time points.





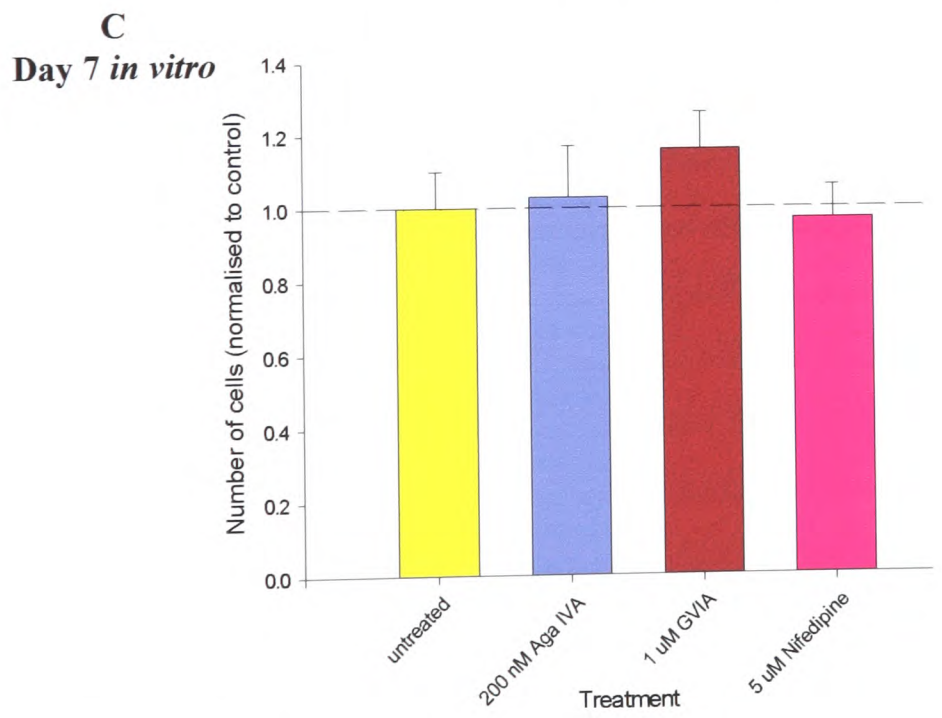


Figure 9.1 Effects of VDCC antagonists on TH+ve cell numbers

*Addition of 200 nM Aga IVA, 1 μM GVIA, or 5 μM Nifedipine had no effect upon total TH+ve cell number per culture from the untreated control at any of the time points. Addition of 20 μM cadmium significantly decreased the number of TH+ve cells in comparison to the control at day 1 in vitro (*** denotes $P < 0.001$; Kruskal-Wallis non-parametric test). Examination of cultures incubated with cadmium at day 3 and day 7 in vitro (72 and 168 hours of exposure to cadmium respectively) revealed a complete absence of cells at both time points. Values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. $n \geq 12$.*

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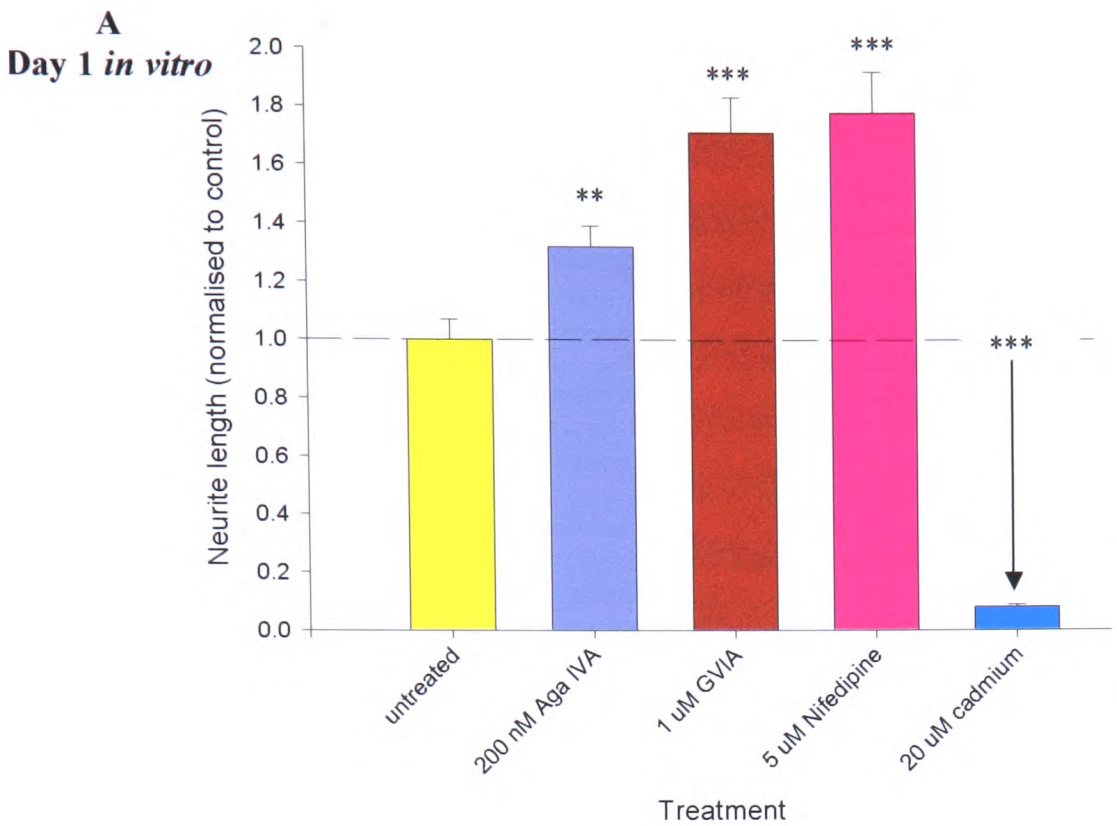
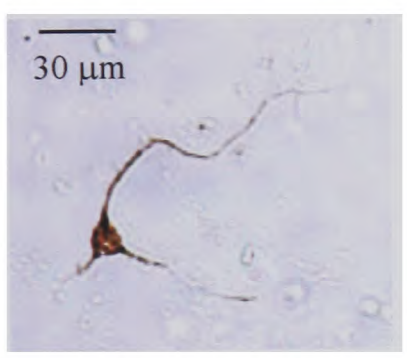
9.3.1.2 The effect of VDCC antagonists on TH+ve neurite outgrowth

At day 1 *in vitro* (24 hour exposure to test substance; Figure 9.2 A) addition of 200 nM Aga IVA, 1 μM GVIA and 5 μM nifedipine had significantly increased neurite outgrowth in comparison to the untreated control (for Aga IVA $P < 0.01$, for GVIA and nifedipine $P < 0.001$; Kruskal-Wallis non-parametric test). Addition of 20 μM cadmium had significantly decreased neurite outgrowth in comparison to the untreated control ($P < 0.001$; Kruskal-Wallis non-parametric test). At day 3 *in vitro* (72 hour exposure to test substance; Figure 9.2B), addition of 200 nM Aga IVA, 1 μM GVIA and 5 μM nifedipine had also significantly increased neurite outgrowth in comparison to the untreated control (for Aga IVA $P < 0.01$, for GVIA and nifedipine $P < 0.001$; Kruskal-Wallis non-parametric test). At days 3 and 7 *in vitro*, neurite length measurement was not possible in cultures incubated with cadmium, due to the complete absence of cells in these cultures. By day 7 *in vitro* (168 hour exposure to test substance; Figure 9.2C), neurite outgrowth had been significant decreased in comparison to the untreated control by incubation of cultures with 5 μM nifedipine. No effect of Aga IVA or GVIA was apparent. Photographic representations of effects upon neurite outgrowth of the various treatments are shown alongside relevant figures.





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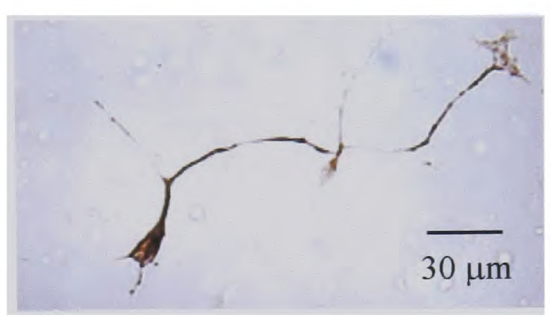
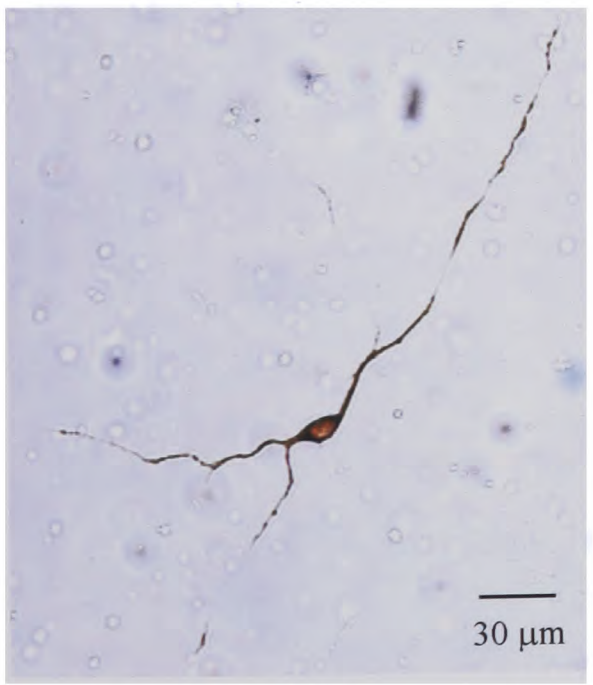


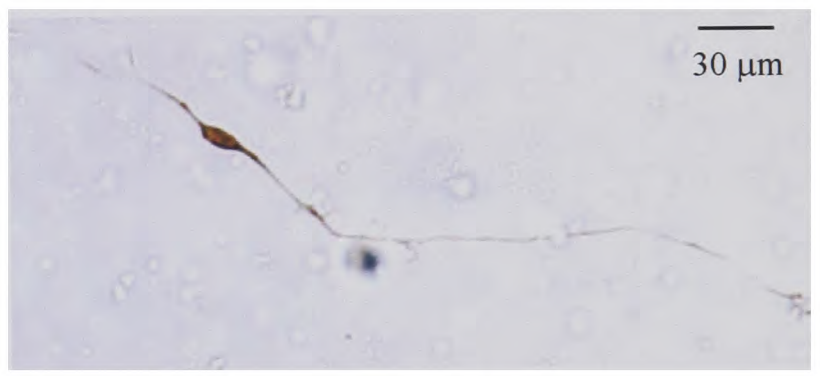
200 nM Aga IVA



20 µM Cadmium



1 µM GVIA



5 µM Nifedipine

Figure 9.2A The effect of VDCC antagonists on neurite outgrowth of TH+ve cells. Day 1 *in vitro* (24 hour exposure to test substances)

*Addition of 200 nM Aga IVA, 1 µM GVIA, or 5 µM nifedipine increased neurite outgrowth in comparison to the untreated control. Addition of 20 µM cadmium significantly decreased neurite outgrowth in comparison to the untreated control. (** denotes $P < 0.01$ and *** denotes $P < 0.001$; Kruskal-Wallis non-parametric test). Graphical values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. $n \geq 12$.*

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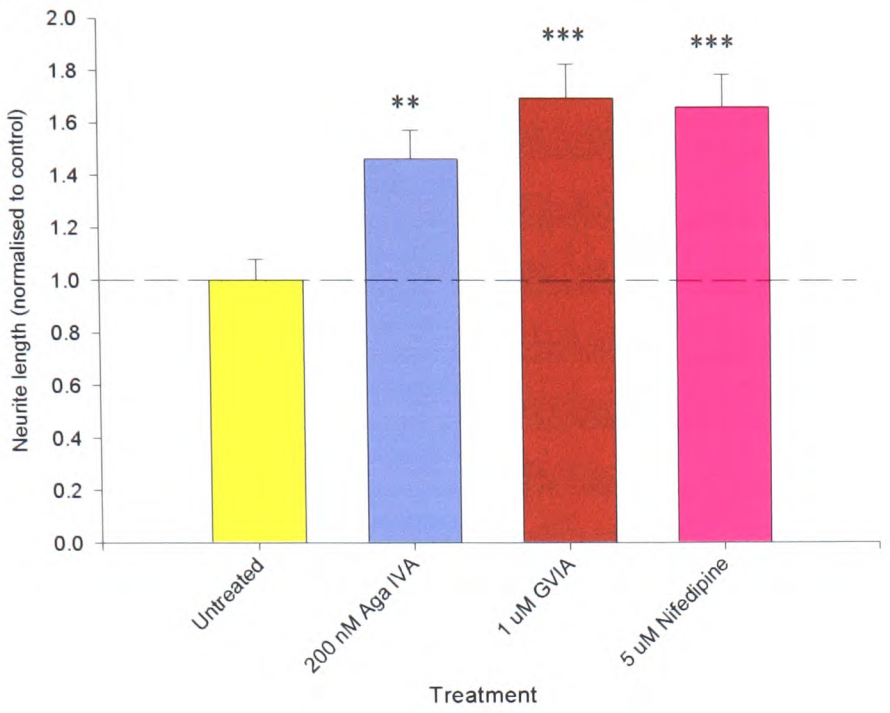
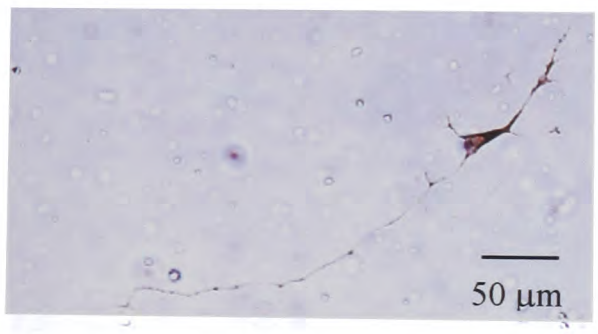
B
Day 3 *in vitro*





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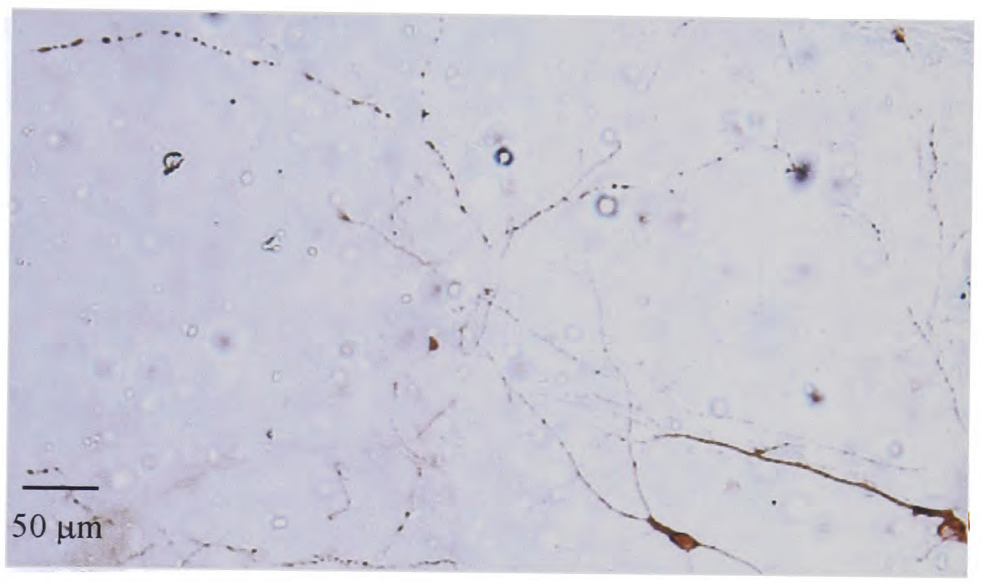
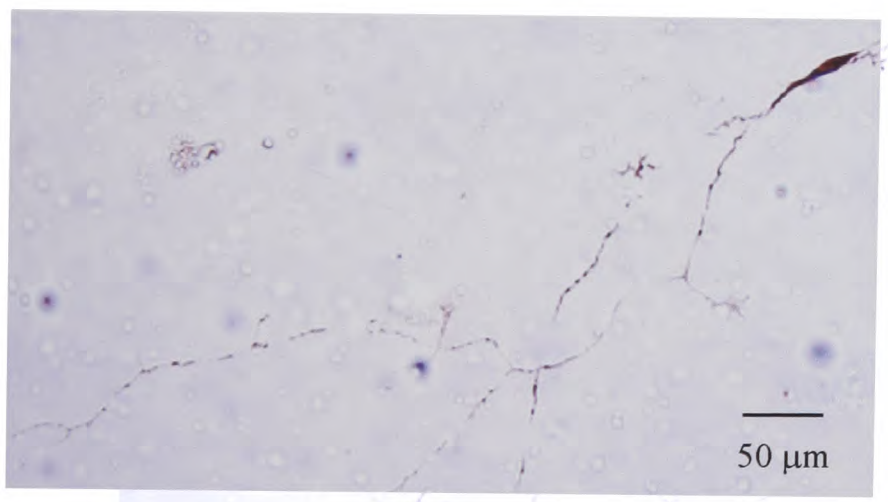
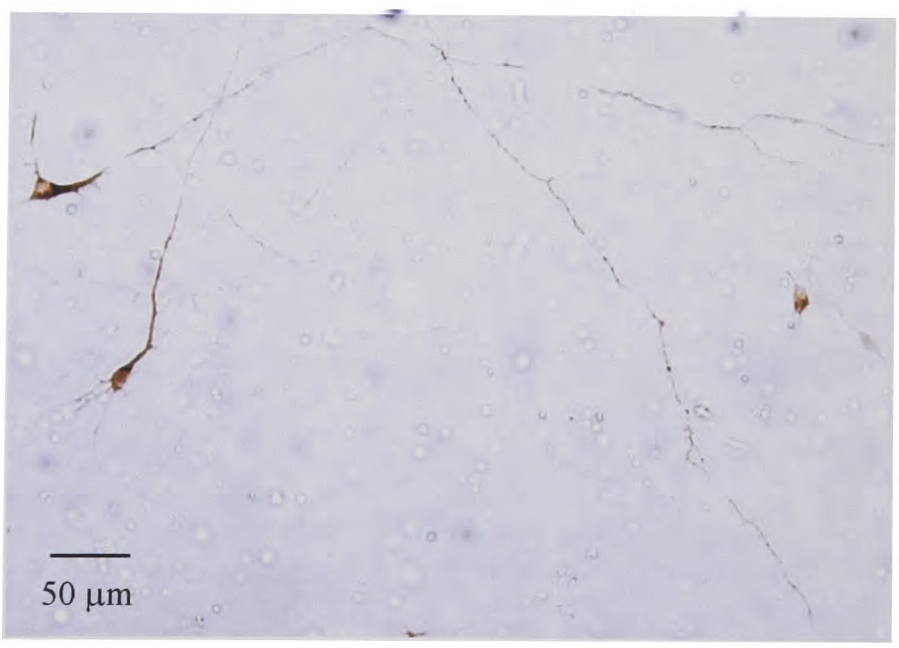


200 nM Aga IVA



1 µM GVIA



5 µM Nifedipine

Figure 9.2B The effect of VDCC antagonists on neurite outgrowth of TH+ve cells. Day 3 *in vitro* (72 hour exposure to test substances)

*Addition of 200 nM Aga IVA, 1 µM GVIA, or 5 µM nifedipine increased neurite outgrowth in comparison to the untreated control (** denotes $P < 0.01$ and *** denotes $P < 0.001$; Kruskal-Wallis non-parametric test). Neurite length measurement was not possible in cultures incubated with cadmium, due to the complete absence of cells in these cultures. Graphical values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. $n \geq 12$.*

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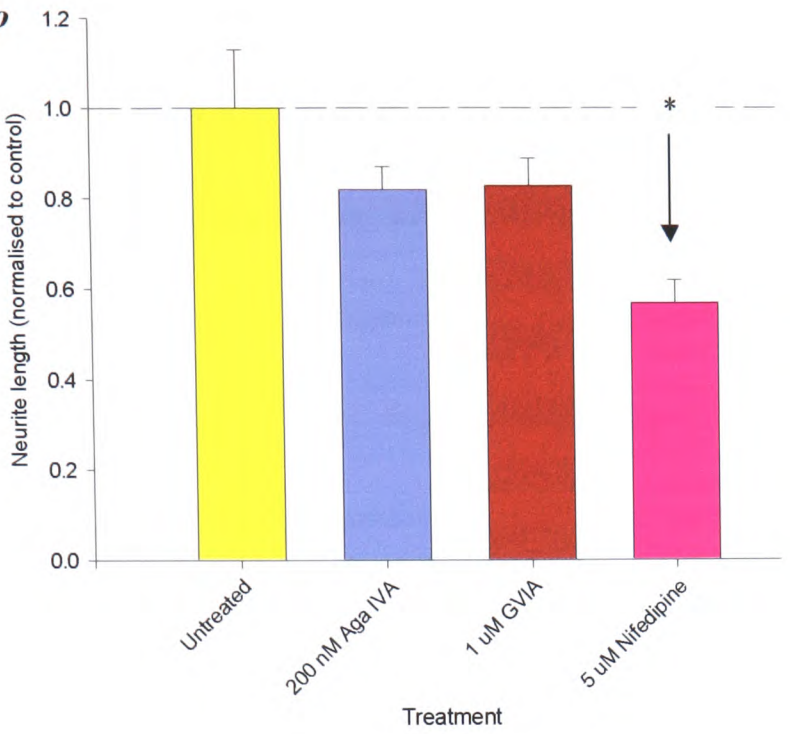
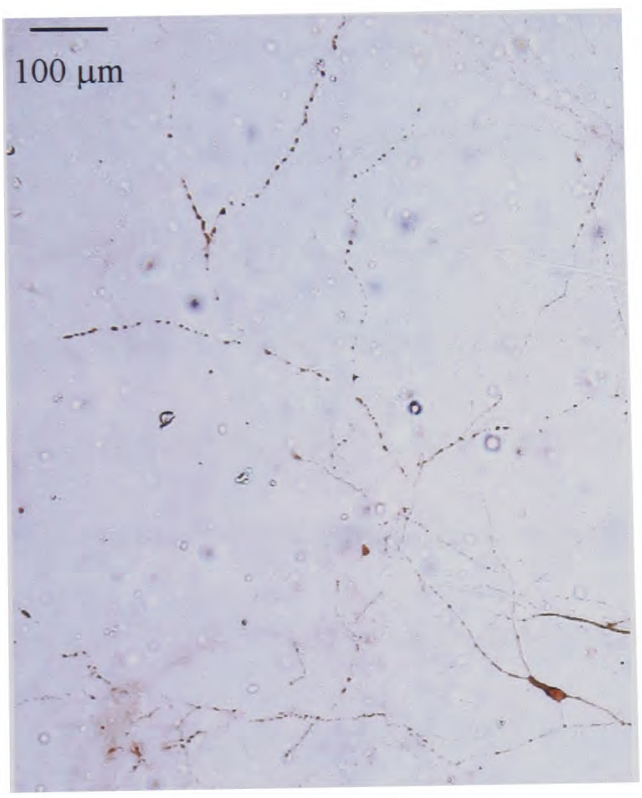
C
Day 7 *in vitro*





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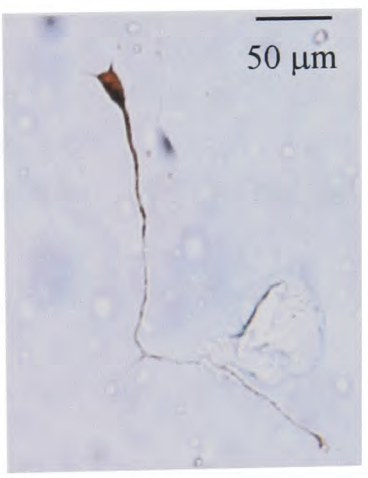

5 µM Nifedipine

Figure 9.2C The effect of VDCC antagonists on neurite outgrowth of TH+ve cells. Day 7 *in vitro* (168 hour exposure to test substances)

Addition of 5 µM nifedipine decreased neurite outgrowth in comparison to the untreated control (denotes $P < 0.05$; Kruskal-Wallis non-parametric test). Neurite length measurement was not possible in cultures incubated with cadmium, due to the complete absence of cells in these cultures. Graphical values shown are mean ± s.e.m. and have been normalised to the untreated control for each time point. $n \geq 12$*

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9.4 DISCUSSION

9.4.1 Effect of VDCC antagonists on TH+ve cell numbers

This chapter demonstrates that incubation of dissociated embryonic VM cultures with 20 μM cadmium for 24 hours results in a significant decrease in TH+ve cells in comparison to the untreated control. By 72 hours of exposure to cadmium, cultures were devoid of TH+ve cells and it was also apparent that there was a complete absence of all cells. These results illustrate the importance of calcium to the developing cell. As discussed in Chapter 6, entry of calcium into the developing cell activates a number of secondary messenger enzymes that influence many fundamental cellular processes. VDCC constitute the primary route of entry of calcium into the cell in response to depolarisation, and complete inhibition of VDCC by cadmium will therefore severely deprive the developing cell of calcium. The consequence of calcium deprivation will be a loss of calcium-dependent developmental processes, including those processes fundamental to cell-substrate attachment and cell survival. The importance of calcium to cell survival is illustrated by several studies. Koike *et al.* (1989) investigated the effects of chronic VDCC agonist and antagonist application on cultures of embryonic SCG that had been NGF deprived, but treated with 40 mM K^+, which confers protection against cell death. It was found that removal of extracellular calcium, pre-loading of cells with a calcium chelator, or addition of nifedipine and nimodipine blocked the survival-promoting effect of high concentrations of K^+. Furthermore, VDCC agonists augmented the protective effects of 40 mM K^+. A similar study by Collins *et al.* (1991) used isolated embryonic chick ciliary neurons loaded with calcium-sensitive fluorescent dye to illustrate that 40 mM K^+ raised $[Ca]_i$ and improved cell survival.

Addition of selective VDCC antagonists Aga IVA, GVIA and nifedipine to culture medium had no effect upon TH+ve cell numbers at any of the time points examined. It could be hypothesised that inhibition of individual VDCC subtypes did not reduce calcium levels sufficiently to detrimentally affect cell survival. The percentage contribution of each channel type to the whole-cell calcium current at days 1, 3 and 7 *in vitro* in TH+ve cells is known from the experiments presented in Chapter 7 (Table 7.3). The greatest contribution made by any single VDCC component to the whole-cell calcium current at each time point examined is ~ 40 %. It could thus be concluded that reduction of the whole-cell current by this amount at any time point examined is not detrimental to cell survival.

Given the stimulatory effect of Aga IVA, GVIA and nifedipine on TH+ve cell neurite outgrowth, it is interesting that these substances had no stimulatory effect upon survival of TH+ve cells at any time point examined. As detailed in Chapter 3, it was also the case that addition of BuChE, G_4- and G_1-AChE to culture medium of embryonic VM cells increased TH+ve neurite outgrowth, but had no effect upon cell numbers. It is probable, as discussed in Chapter 3, that death of TH+ve cells in culture is a result of both naturally occurring cell death and death due to traumatisation during the culture procedure. It was hypothesised that the lack of effect of BuChE, G_4- and G_1-AChE upon TH+ve cell numbers may be due to the delay of two hours in addition of these factors to cultures (Chapter 3 Section 3.4.2.2). Upon addition, the mechanisms causing cell death whether a result of natural occurrence or due to traumatisation, may have been sufficiently advanced to prevent reversal by the trophic factor. This may also be the case with addition of VDCC antagonists, as the experimental procedure was the same as for the BuChE and G_4- and G_1- AChE experiments.

9.4.2 Effect of VDCC antagonists on TH+ve neurite outgrowth

Addition of 20 μM cadmium to culture medium of dissociated embryonic VM cells resulted in a significant decrease in neurite outgrowth of TH+ve cells in comparison to the untreated control at day 1 *in vitro*. Neurite length measurement was not possible in cultures incubated with cadmium at days 3 and 7 *in vitro*, due to the complete absence of cells in these cultures. Addition of Aga IVA, GVIA and nifedipine to culture medium for 1 – 3 days to inhibit the P/Q-, N-, and L-type VDCC respectively resulted in an increase in neurite length of TH+ve cells in comparison to the untreated control. However, after incubation with these antagonists for 7 days no increases in neurite length were detected in comparison to the untreated control, and incubation with 5 μM nifedipine had significantly decreased neurite outgrowth.

As discussed in Chapter 6 (Section 6.2), entry of calcium into the growth cone of the developing cell activates CaM-dependent secondary messenger enzymes, such as protein kinases, protein phosphatases and adenylate cyclases. One of the consequences of activation of such enzymes is modulation of the rate of assembly and disassembly of microtubules and actin filaments in the growth cone, resulting in alterations in neurite extension and retraction (Bamburg and Chapman, 1986; Lankford and Letourneau, 1989, Mattson and Kater, 1987). The variation in the effects of increasing or decreasing calcium concentration on neurite outgrowth have demonstrated that the permissibility of calcium concentrations for maximal neurite outgrowth is narrow, and specific to cell type (Connor, 1986; Collins and Lile, 1989; Collins *et al.* 1991; Lankford and Letourneau, 1989; Matttson and Kater, 1987; Mattson *et al.*, 1988a; Holliday and Spitzer, 1992).

It would appear from the findings presented here that the concentration of intracellular calcium required for maximal neurite outgrowth of TH+ve cells at days 1 and 3 *in vitro* in this culture system is lower than the physiological $[Ca]_i$ in these cells. Hence, addition of VDCC antagonists that inhibit calcium conductance through selective channel subtypes increases neurite outgrowth at these time points. The greater effect of addition of GVIA and nifedipine to culture medium, in comparison to Aga IVA, is likely to be a reflection of the calcium conductance through each channel subtype. It is known from the results presented in Chapter 7 that the percentage contribution to the whole-cell current made by the N- and L-type channels is significantly higher than the percentage contribution from the P/Q-type channel in TH+ve cells at day 1 and 3 *in vitro*. Thus, inhibition of the N- and L-type channels at these time points will inhibit a larger percentage of the whole-cell calcium current than inhibition of the P/Q-type current. As addition of GVIA and nifedipine enhanced outgrowth more than addition of Aga IVA, it can be postulated that VDCC inhibition by GVIA and nifedipine brings $[Ca]_i$ closer to the narrow range for optimal neurite outgrowth than inhibition by Aga IVA.

By day 7 *in vitro* (168 hour exposure of cells to test substances), no significant effect upon TH+ve neurite outgrowth was seen with incubation of cultures with P/Q- and N-type VDCC antagonists Aga IVA and GVIA. However, the L-type antagonist, nifedipine had significantly reduced neurite outgrowth in comparison to the untreated control. From the results presented in Chapter 7 it is known that in TH+ve cells at day 7 *in vitro* the mean percentage contribution to the whole-cell current made by the L-type channel is higher than the mean percentage contribution from the P/Q- and N-type channels. It is possible that by day 7 *in vitro* the $[Ca]_i$ of TH+ve cells is similar to the set-point, meaning that neurite

outgrowth is occurring at near maximal rate. Reduction of the whole-cell calcium current by addition of Aga IVA or GVIA may not, at this time point, dissociate $[Ca]_i$ from the range required for optimal neurite outgrowth. The increased inhibition of the whole-cell calcium current in TH+ve cells at day 7 *in vitro* by nifedipine in comparison to Aga IVA and GVIA may, however, sufficiently deprive cells of calcium to result in disruption of the processes required for neurite outgrowth. Alternatively, the detrimental effects of incubation with nifedipine may implicate a specific role for the L-type channel in dopaminergic neurite outgrowth.

It has been assumed so far in this discussion that chronic incubation of cultures with a VDCC antagonist results in a decrease in $[Ca]_i$ due to inhibition of a component of the whole-cell calcium current by the antagonist. It is possible, however, that VDCC antagonist binding results in an increase in $[Ca]_i$, through up-regulation of the particular subtype involved, or of other VDCC subtypes, in compensation for the loss of calcium conductance through the inhibited channel. No studies have examined the effect of chronic incubation of cultured neurons with VDCC antagonists on VDCC density. However, Porter *et al.*, (1997) observed an increase in survival of cultured hippocampal neurons upon chronic incubation with nimodipine, and suggested that this may be due to an increase of the density VDCC. Furthermore, chronic administration of DHPs is used in models of both ageing and diabetes. A control in a diabetes study by Kostyuk *et al.* (2000) demonstrated that peroral treatment of normal mice with nimodipine for 3 weeks resulted in a small increase in peak amplitude of intracellular calcium in dorsal root ganglion cells following membrane depolarisation. Further to this, a study by Yamada *et al.* (1996) demonstrated that chronic (3 week) oral administration of nimodipine and nicardipine to senescence-accelerated prone mice resulted

in an increase in calcium binding sites within the cerebral cortex and hippocampus, which was thought to reflect an up-regulation of L-type VDCC receptors.

The studies by Kostyuk *et al.* (2000) and Yamada *et al.* (1996) suggest that up-regulation of VDCC can occur *in vivo.* Whilst it is not accurate to draw direct parallels between *in vitro* and *in vivo* situations, it is possible that similar up-regulation of VDCC may have occurred in the culture system utilised here, due to chronic exposure of cells to VDCC antagonists. Postulations concerning trophic effects upon neurite outgrowth being a result of the *lowering* of calcium levels by VDCC antagonists would then be the antithesis of what, in fact, may occur. However, the schemes suggested to account for the variation of effect of the different VDCC antagonists are still applicable if the inverse effect of VDCC antagonists on calcium levels, i.e. *raising* of calcium levels due to up-regulation, are applied.

In conclusion, the results presented in this chapter demonstrate the effects on the survival and neurite outgrowth of TH+ve cells in embryonic dissociated cultures of the VM following chronic treatment with VDCC antagonists Aga IVA, GVIA, nifedipine and cadmium. Addition of cadmium resulted in a rapid loss of all cells from cultures. Addition of Aga IVA, GVIA, nifedipine had no effect upon cell survival, and increased neurite outgrowth at days 1 and 3 *in vitro.* At day 7 *in vitro* no effect upon neurite outgrowth was seen in cultures incubated with Aga IVA or GVIA, and in cultures treated with nifedpine, a decrease in neurite outgrowth was seen. These experiments were conducted following the findings of Chapters 3 and 8, which demonstrated trophic effects of BuChE, G_4- and G_1-AChE upon TH+ve neurite outgrowth, and effects of BuChE, G_4- and G_1-AChE on voltage-dependent calcium currents respectively. As discussed in Chapter 6, various studies have demonstrated

the effects of well-known growth factors NGF and bFGF upon VDCC. In view of these studies, it was hypothesised that the actions of BuChE, G_4- and G_1-AChE on TH+ve cell voltage-dependent calcium currents might be, at least partly responsible for the trophic effects of these substances on TH+ve neurite outgrowth. Accordingly, it was postulated that other antagonists of VDCC would also prove trophic to TH+ve cells. The findings of this chapter, that antagonists of VDCC subtypes have a trophic effect on TH+ve neurite outgrowth, are consonant with the possibility that the trophic effects of BuChE, G_4- and G_1-AChE on neurite outgrowth are mediated through their inhibitory action upon VDCC.

The next chapter provides a discussion of the findings obtained throughout this thesis, and puts them in the context of foetal transplantation as a treatment for Parkinson's disease. In addition, several lines of future work are discussed.

CHAPTER 10

Discussion, conclusions and future work

10.1 INTRODUCTION

The aim of this thesis was to investigate the putative non-cholinergic actions of AChE on developing dopaminergic neurons in a preparation analogous to that used in the animal model of foetal transplantation as a treatment for PD. More specifically, experiments presented within this thesis concentrated firstly on examining the effect of exogenous application of AChE and BuChE to cultured foetal dopamine cells. Secondly, establishing a non-cholinergic basis of action for AChE and BuChE within the preparation and finally, examining a novel mechanism of action of AChE and BuChE on cultured embryonic VM cells.

This chapter will provide a summary and discussion of the experimental findings of this thesis and draw conclusions on the implications and relevance of the work. Moreover, suggestions of possible goals for future experiments will be made.

10.2 A neurotrophic effect of BuChE and G_4- and G_1-AChE on embryonic dopaminergic cells in dissociated VM cultures

Exogenous application of BuChE and G_4- and G_1-AChE to dissociated cultures of embryonic VM consistently produced a neurotrophic effect upon neurite outgrowth of dopaminergic cells, as illustrated in Chapter 3 of this thesis. The trophicity of AChE is widely considered to be evidence for a non-classical action of the glycoprotein. However, the stimulatory effects of both BuChE and AChE in the studies presented in Chapter 3 meant that a cholinergic mechanism of action could not be discounted. Thus it was demonstrated in Chapter 4 that inhibition of the active site of endogenous BuChE and AChE with echothiophate had no detrimental effect on neurite outgrowth, suggesting that the effects of

BuChE and AChE on neurite outgrowth were not due to their hydrolytic ability. It was also demonstrated in Chapter 4 that the active site and PAS inhibitor of AChE, BW284c51, markedly reduced dopaminergic cell survival and neurite outgrowth, which, taken with the lack of effect of active site inhibitor echothiophate, implicated a role for the PAS of AChE in these processes. However, the involvement of the PAS in neurite outgrowth is hard to reconcile with the trophic effects of BuChE on neurite outgrowth, when BuChE lacks a PAS. Furthermore, it has been proposed by Postuma *et al.* (1999) that the toxicity caused by BW284c51 may be non-specific. As a specific inhibitor of the PAS is not available, an attempt was made in Chapter 5 to address the question of the toxicity of BW284c51 by an alternative means; by investigating whether toxic effects were due to elevated ACh levels as a result of inhibition of AChE by BW284c51. The results of Chapter 5 demonstrated an alleviation of chronic effects of lower concentrations of BW284c51 and acute effects of higher concentrations of BW284c51 by co-incubation of cultures with a mixture of nAChR antagonists, but no effect on chronic effects of higher concentrations of BW284c51. This suggested that the detrimental effects of BW284c51 on neurite outgrowth and cell survival were partly attributable to elevated ACh levels but did not exclude an interaction of BW284c51 with a distinct site on the AChE molecule, potentially the PAS. It would be satisfying to fully identify the role of the PAS in the trophic effects of AChE. Whilst a specific inhibitor of the PAS is not currently available, work is currently being carried out by the Greenfield group, as discussed in Chapter 5, to modify fasciculin so that it will bind to the PAS without obstructing the catalytic gorge (S. Emmett, Personal Communication). Alternatively, site-directed mutagenesis could be employed to alter the PAS of AChE.

The findings of Chapters 4 and 5 are of relevance to treatments used for Alzheimer's disease (AD). This neurodegenerative disease is characterised by severe memory impairment and pathologically, by loss of cholinergic nuclei in the basal forebrain region and their terminals in the hippocampus (Decker, 1987; Giacobini, 1990). The deficiency in cholinergic markers in the brains of AD patients, has led to the use of compounds that inhibit AChE in order to restore ACh concentrations (Giacobini, 1998). It is recognised that long-term administration of anticholinesterasics, such as 9-amino-1,2,3,4-tetrahydroaminoacridine hydrochloride (tacrine) to AD patients can give rise to parkinsonian events such as tremor and akinesia (Cousins *et al.*, 1997; for review see Masand-Prakash, 2000). Tacrine is an inhibitor of the PAS that also occludes the active site of AChE, thus essentially performing the same function as BW284c51. As shown in Chapters 4 and 5, inhibition of AChE by BW284c51 is toxic to midbrain dopaminergic cells. It is possible that the parkinsonian events observed upon administration of anticholinesterasics such as tacrine to AD patients are due to the detrimental effects of such AChE inhibitors on dopaminergic cells of the nigrostriatal pathway.

10.3 Characterisation of VDCC expression in developing VM cells

Chapters 7, 8, and 9 returned to the question of the trophic effects of both BuChE and AChE on embryonic dopaminergic cells. As discussed above, it is hard to see how the trophic effects of AChE on neurite outgrowth may be mediated solely through the PAS when BuChE, which was shown to enhance neurite outgrowth under identical culture conditions in Chapter 3, has no PAS. Thus, a novel mechanism was proposed; BuChE and AChE may increase neurite outgrowth by modulating $[Ca]_i$ through an action on VDCC.

Firstly, however, the expression of the various VDCC components of the whole-cell calcium current was investigated in Chapter 7. It was found that both embryonic GAD+ve and TH+ve cells displayed a full compliment of VDCC by day 1 *in vitro* and that the contribution of components to the whole-cell calcium current varied over time, with the R-type contribution increasing and the N-type current decreasing in both cell types. This is an interesting and novel finding for developing midbrain dopaminergic neurons. As discussed in Chapter 6, calcium plays a fundamental role in neuronal development, and the variation in VDCC component conductance demonstrates the importance of regulation of calcium influx into the developing cell. As discussed in Chapter 7, such variation may also have implications for the use of growth factors to enhance survival and outgrowth of TH+ve neurons within cultures or grafts for transplantation. If, as suggested by several studies, the actions of growth factors such as bFGF and NGF are mediated by VDCC (Koike *et al.*, 1989; Rogers and Hendry, 1990; Nikodijevic and Guroff, 1992; Levine *et al.*, 1995; Shitaka *et al.*, 1996; Jia *et al.*, 1999; Katsuki *et al.*, 1998), it is possible that the variation seen in the effectiveness of these substances on neurite outgrowth may be a result of variations in expression of the VDCC subtype through which the growth factor effect is mediated. The characteristic pattern of VDCC expression in TH+ve and GAD+ve cells will also be helpful for identification of particular cell types within heterogenous embryonic dissociated VM cultures in future studies.

10.4 An effect of BuChE and G_4- and G_1-AChE on voltage-dependent calcium currents

Experiments described in Chapter 8 demonstrated that BuChE and G_4- and G_1-AChE inhibit voltage-dependent calcium currents in a dose-dependent manner. These are highly

significant findings when considered in conjunction with the trophic effects of VDCC antagonists Aga IVA, GVIA and nifedipine on dopaminergic neurite outgrowth as illustrated in Chapter 9. The concentration of intracellular calcium is vital to cellular processes that govern developmental events such as neurite outgrowth, with a particular, cell-specific $[Ca]_i$ required for maximal neurite outgrowth, the set-point. It is feasible that by inhibiting VDCC, BuChE and G_4- and G_1-AChE moderate $[Ca]_i$ in developing cells, thus bringing $[Ca]_i$ closer to the set-point required for maximal neurite outgrowth. The obvious repercussion of this would be enhanced neurite outgrowth as seen upon addition of BuChE and G_4- and G_1-AChE to cultures in Chapter 3.

Furthermore, the difference in the response of dopaminergic cells to BuChE and G_4- and G_1-AChE seen in Chapter 3 may be a repercussion of the sensitivity of neurite outgrowth processes to $[Ca]_i$. Dopaminergic cells displayed a variation in the time of response to BuChE / G_1-AChE (trophic by day 1 *in vitro*) and to G_4-AChE (trophic by day 3 *in vitro*). As discussed in Chapter 3, this may be representative of a parallel with the *in vivo* situation, whereby cells have a narrow window of response to a trophic factor. *In vivo*, it is known that in development, BuChE and G_1-AChE are expressed before G_4-AChE (Rieger and Vigny, 1976; Layer *et al.*, 1987; Layer, 1988, Willbold and Layer, 1992; Arendt *et al.*, 1992; Inestrosa *et al.*, 1994). From the electrophysiology data presented in the latter chapters of this thesis, a further postulation could be made. It is possible that the changes in VDCC component expression seen in developing GAD+ve and TH+ve cells in Chapter 7 correspond to changes in the concentration of calcium required for outgrowth. Thus, the responsiveness of cells to a trophic factor may vary according to the effect the factor has on calcium conductance. At all concentrations tested, G_4-AChE inhibited a greater percentage of the

whole-cell calcium current than BuChE or G_1-AChE, and it is possible that this may have played a part in determining the timing of the effect of these substances. The postulation that the effect of BuChE and G_4- and G_1-AChE depend upon their action on VDCC, and consequently the presence (or sufficient number) of the VDCC channel subtype through which they act, may account for the lack of effect of BuChE on postnatal midbrain dopaminergic neurons in the study by Holmes *et al.* (1995).

There are several points concerning these experiments that require discussion, however. The absence, for example, of trophic effects seen upon addition of 0.3 U/ml of BuChE and G_4- and G_1-AChE in Chapter 3. Upon direct application to voltage-dependent calcium currents by electrophysiology in Chapter 8, 0.3 U/ml BuChE and G_4- and G_1-AChE caused a minimum of 14.2 % inhibition in either GAD+ve or TH+ve cells. It is known from the studies presented in Chapter 7 that the contribution to the whole-cell current by P/Q-type VDCC in either cell type, on any day examined, is between 4.8 % and 11.3 %, yet inhibition of P/Q-type VDCC by Aga IVA produced an increase in neurite outgrowth. If it is hypothesised that the trophic effects of BuChE and G_4- and G_1-AChE are due to inhibition of the voltage-dependent calcium current bringing the $[Ca]_i$ closer to the set-point, and that higher concentrations of BuChE and G_4- and G_1-AChE are required in order to produce sufficient inhibition to alter $[Ca]_i$, then the trophic effects of Aga IVA are confounding. As discussed in Chapter 8, from the studies presented in this thesis it is not possible to deduce the mechanism by which BuChE and G_4- and G_1-AChE may be acting on VDCC. Whilst a direct, specific interaction of BuChE and G_4- and G_1-AChE with a component or components of the VDCC is possible, it would seem more likely, given the high concentrations required

to affect neurite outgrowth in comparison to selective inhibitors of VDCC, that the interaction is less specific, perhaps due to an occlusion of a VDCC component.

Further to this, all factors found to be trophic to dopaminergic neurite outgrowth in the culture system used here (BuChE, G_4- and G_1-AChE, Aga IVA, GVIA and nifedipine) lost effect by day 7 in culture. It is possible that the presence of these substances at later stages in culture did not dissociate $[Ca]_i$ from the range required for optimal neurite outgrowth. Alternatively however, chronic incubation with VDCC inhibitors may have resulted in cellular changes, for example desensitisation of receptors, which meant that their continued presence did not sufficiently effect $[Ca]_i$, and consequently had no effect on neurite outgrowth.

A further consideration is that although acute application of BuChE, G_1- or G_4-AChE, Aga IVA, GVIA or nifedipine inhibited voltage-dependent calcium currents of developing VM cells as examined by electrophysiology, chronic incubation of cultures with these substances may ultimately increase $[Ca]_i$. As shown by the experiments in Chapter 8 that chelated intracellular calcium with EGTA, the inhibitory actions of BuChE and G_1- and G_4-AChE are not mediated by an increase in $[Ca]_i$. However, it is possible that chronic inhibition of VDCC by BuChE and G_1- and G_4-AChE, as is the case with other VDCC antagonists (see Chapter 9, Section 9.4.2), may cause upregulation of a VDCC component or components, with the eventual repercussion of an increase in $[Ca]_i$. Concurrent with the idea that chronic AChE treatment may enhance $[Ca]_i$ are the studies by Hawkins and Greenfield (1992a, 1992b, and 1995), which revealed long term behavioural effects of AChE on the nigrostriatal pathway, that are consistent with an increase in activity of the nigrostriatal neurons.

However, the question remains, that if trophic effects of the substances examined in this thesis are mediated through alterations in VDCC conductance that bring the $[Ca]_i$ closer to the set-point required for maximal neurite outgrowth, why do developing cells maintain an $[Ca]_i$ that is sub-optimal for such a fundamental process? Perhaps the answer to this lies in the neurotrophic hypothesis and its relation to electrical excitability and naturally occurring cell death (Chapter 6). 'Pruning' of the extensive preliminary network of cells and neurites is thought to ensure that the size of the projection matches the target. The neurotrophic hypothesis dictates that only cells receiving adequate amounts of neurotrophic factor produced by the target will survive. This means that survival of a cell depends upon the rate of extension of its neurite towards the target. The rate of extension may be governed by the electrical activity that the cell receives, as it is known that deafferentation or pharmacological blockade of synaptic transmission between developing neurons can cause neurite inhibition and cell death (Harris, 1981; Ramakers and Boer, 1990; Sherrard and Bower, 1998). Electrical activity causes changes in $[Ca]_i$ (Collins *et al.*, 1991, Hack *et al.*, 1993; Franklin *et al.*, 1995; Koshimura *et al.*, 1999). Thus, if the $[Ca]_i$ of developing cells was maintained at the set-point, the effects of electrical activity could be disastrous. Calcium levels would be moderated away from the set-point resulting in retardation of neurite outgrowth with the eventual repercussion of cell death through a lack of neurotrophic factor, or even cell death directly.

Finally, a conclusion as to the physiology of the cell cultures utilised in this thesis can be drawn from the studies of Chapter 9, which illustrated a trophic effect of well-known VDCC inhibitors on neurite outgrowth. In order for VDCC antagonists to have an effect on a cellular process such as neurite outgrowth, VDCC have to be not only present in cells in the

culture, but also active. Therefore it can be concluded that VDCC in cells in dissociated embryonic cultures of the VM are operational, and active. This supports the evidence of other studies that cells within dissociated cultures form a functional system, with communication between component cells (Ort *et al.*, 1988; Mount *et al.*, 1989; Shimoda *et al.*, 1992).

10.5 Implications of findings

The proposal that cholinesterases may have non-cholinergic/non-classical developmental functions has gained widespread acceptance. The findings of this thesis, that BuChE and G_4- and G_1-AChE have a growth promoting action on embryonic dissociated dopaminergic cells, independently of catalytic activity, add further support to the theory of non-cholinergic, morphogenic actions of cholinesterases.

The fact that non-cholinergic stimulatory actions of AChE were seen on dopaminergic neurons of the SN is of particular interest since this area of the brain displays high AChE activity, but low ChAT activity (Henderson and Greenfield, 1987). It is therefore tempting to speculate that a function of AChE within the SN could be to act as a neurotrophic factor for midbrain dopaminergic neurons. Furthermore, the fact that AChE is secreted from cells within the SNpc (Greenfield *et al.*, 1983) suggests that it could act as an autocrine growth factor. The loss of AChE as a consequence of cell death within the SN in PD may therefore enhance the disease pathology due to removal of an important growth factor.

The demonstration of trophic effects of BuChE and G_4- and G_1-AChE on embryonic dissociated dopaminergic cells by experiments in this thesis have implications for foetal

transplantation, where addition of a trophic factor to the cell suspension is known to improve graft survival (Mayer *et al.*, 1993a; Zawada *et al.*, 1998; Törnqvist *et al.*, 2000). However, although the trophic actions of BuChE and G_4- and G_1-AChE may be non-cholinergic, incorporation of BuChE and G_4- and G_1-AChE into a grafted cell preparation would also result in hydrolysis of endogenous ACh. This has the potential to disrupt cholinergic systems within the site of transplantation, the striatum. The elucidation of a mechanism of action of BuChE and G_4- and G_1-AChE on VDCC is therefore interesting. Manipulation of $[Ca]_i$ of developing dopaminergic cells has the potential to improve neurite outgrowth; a parameter that is essential for graft survival in foetal transplantation as a treatment for PD. However, as discussed in Chapter 6, perturbation of $[Ca]_i$ either side of the set-point in developing cells, can be detrimental to cell survival and neurite outgrowth. Thus, a method would be required wherein calcium levels could be manipulated in order to improve the functionality of grafts without causing cell death, and also, again, without repercussions on endogenous tissue at the site of transplantation.

10.6 Future work

Several lines of future work would aid the interpretation of the findings of this thesis and further understanding of potential pharmacological roles for BuChE and AChE in pathological conditions such as PD. Importantly, identification of the site(s) on the cholinesterase molecules that interacts with VDCC and identification of the mechanism(s) by which BuChE and G_4- and G_1-AChE inhibit voltage-dependent calcium currents. Also, as discussed above, identification of the effect that chronic incubation of cultures with BuChE or G_1- or G_4-AChE, Aga IVA, GVIA or nifedipine have upon $[Ca]_i$. The answers to these

questions may then provide possibilities for manipulation of cellular calcium content within grafted cell suspensions without affecting surrounding tissues.

10.7 Concluding remarks

Experiments presented in this thesis have used embryonic dissociated cultures of the VM to investigate the effect, and mechanism of action of G_4- and G_1-AChE, and the related enzyme, BuChE, on the survival and outgrowth of developing dopaminergic neurons. Results have demonstrated a trophic effect of BuChE and G_4- and G_1-AChE on embryonic dopaminergic cells that is not dependent on hydrolytic activity. Furthermore, a novel action for BuChE and G_4- and G_1-AChE on voltage-dependent calcium channels has been documented. Given the importance of calcium regulation in development, and the known expression of cholinesterases in embryogenesis, the demonstration of a neurotrophic action of BuChE and G_4- and G_1-AChE on midbrain embryonic dopaminergic neurons, mediated through VDCC, substantiates the role of cholinesterases in development of the SN. Consequently, by increasing our knowledge of such developmental processes, a minor step has been taken toward understanding the physiology, and perhaps the pathology, of this vulnerable brain region.

APPENDIX 1

Poly-D-lysine (PDL) coating of coverslips, sterile chamberslides and 75cm^2 sterile flasks

Wash coverslips in 100 % alcohol. Allow to dry. Place each coverslip in a 22mm sterile petridish.

Poly-d-lysine (PDL) was reconstituted in water (50 μg / ml).

150 μl per well was added to each chamber of the chamberslides and to each coverslip and 5 mls to each flask.

Incubate all at 37 °C in a humidified atmosphere comprising 95% air: 5% CO_2 for 2 hours

PDL was removed and all PDL-coated ware was washed twice for 15 mins with ddH_2O before being left to dry at room temperature.

All PDL-coated ware was stored a 4°C until used.

Media for astrocytes and dissociated VM cultures

Dulbecco's modified eagle medium (DMEM, 4500mg/l glucose, Life Technologies Ltd., Paisley, UK) containing 2mM glutamine supplemented with 10 % foetal calf serum and 1:100 penicillin-streptomycin.

Tris buffered saline (pH 7.6, 0.05 M)

416 ml Tris base (0.2M)

1250 ml water

5.9 ml HCl

14.6 g NaCl

Ellman assay reagent

A: 0.1 M potassium dihydrogen orthophosphate buffer (pH 7)

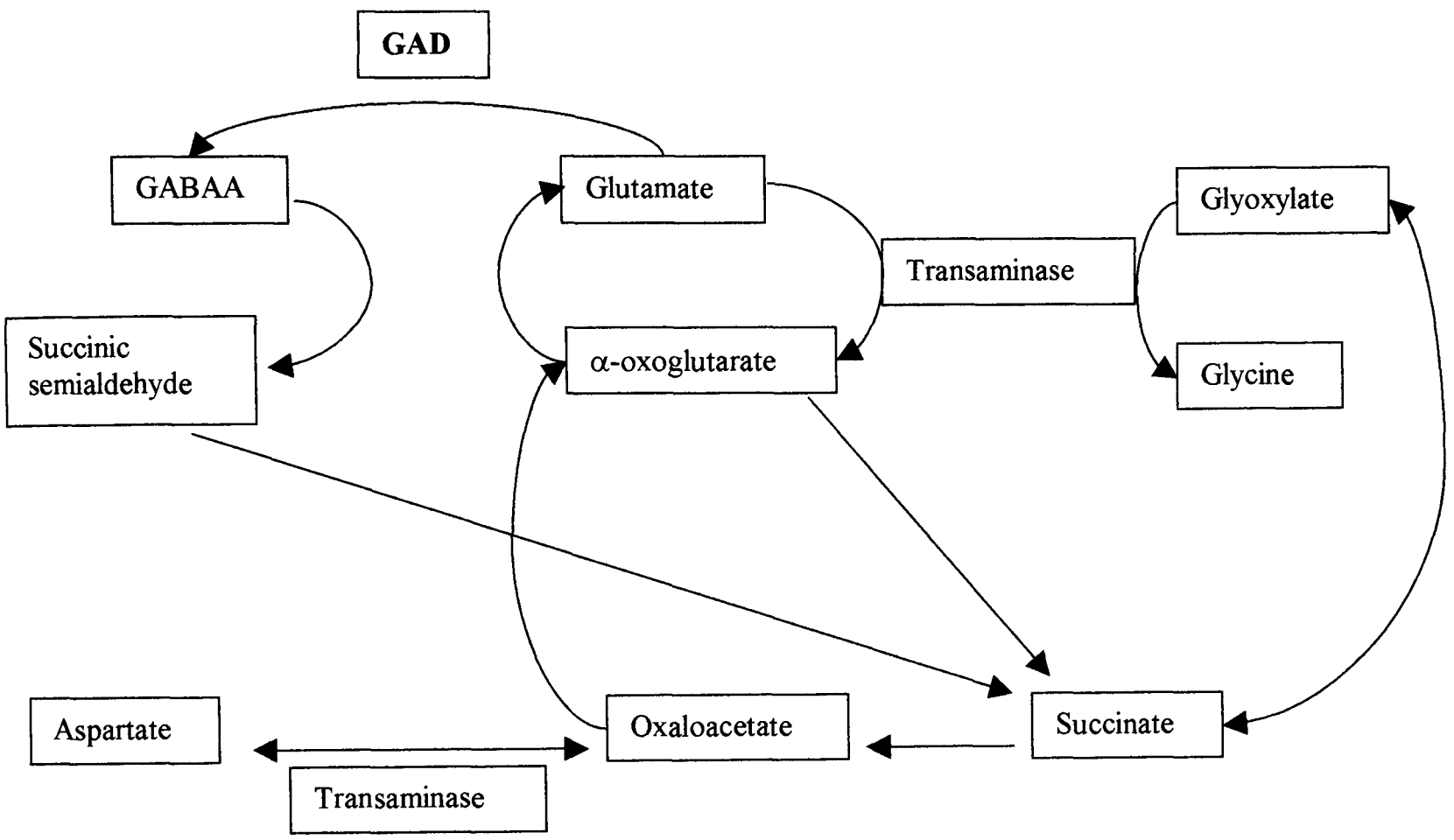
B: Dissolve 31.7 mg DTNB and 12 mg sodium hydrogen carbonate in 10 ml of A

C: Dissolve 28.9 mg acetylthiocholine iodide in 10 ml of water

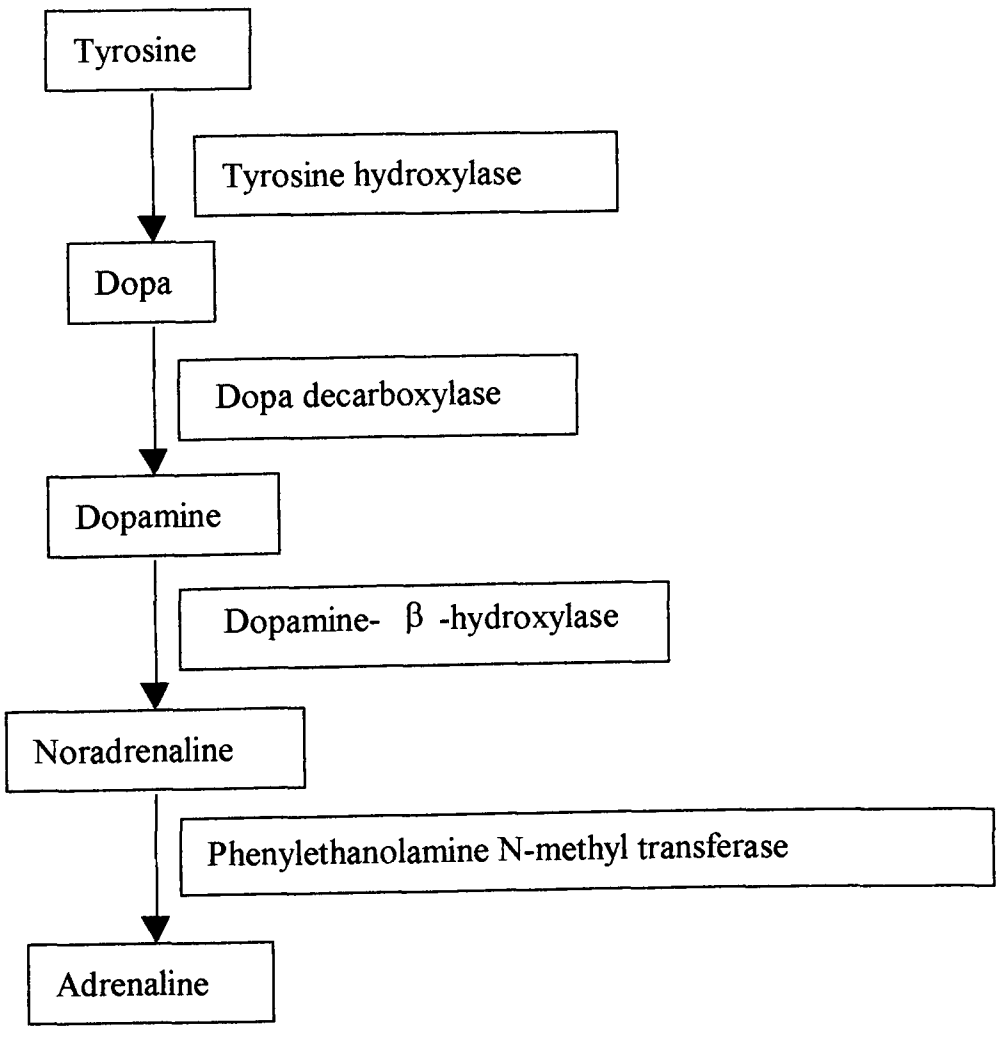
Mix A: B: C in ratio 5: 1: 1

APPENDIX 2

γ amino butyric acid (GABA) synthesis



Dopamine (DA) synthesis



APPENDIX 3

AChE purification

Sepharose affinity chromatography

Preparation of the procainamide ECH sepharose affinity resin

Wash 25 ml ECH sepharose (Pharmacia) with 2 litres (~ 80 ml/ml beads) of 0.5 M sodium chloride (NaCl) in a scintered glass funnel *in vacuo*. Followed by 2 litres of water (deionised).

Re-suspend beads in 50 ml of water (deionised).

Dissolve 3 g of the ligand, procainamide hydrochloride in 2.5 ml of 1 M NaCl and adjust to pH 4.0.

Add 1.75 ml of this mixture to the sepharose and mix.

Dissolve 1 g of the EDC coupler (1-ethyl-3-(3-dimethylethylaminopropyl) carbodiimide hydrochloride) in 1 ml of water (deionised) and adjust to pH 4.0. Add to sepharose mixture and stir for 1 hour at room temperature (monitor pH, as reaction is acid catalysed (condensation) and the pH will drop during this hour).

Transfer to a falcon tube and mix by inversion overnight at room temperature to allow for the completion of the coupling reaction.

Wash the sepharose alternately with high pH saline (0.1 M Tris / hydrochloride with 0.5 M NaCl, pH 8.3) and low pH saline (0.1 M sodium acetate with 0.5 M NaCl, pH 4.0).

The resin can be stored with 0.2 % sodium azide at 4 °C or used immediately.

Running the column

Wash sepharose with PBS and pour into column

Wash the column with PBS to allow the sepharose to settle.

The AChE was prepared in PBS and loaded onto the column at 12 ml / hour at 4 °C

The column was left to recycle at 400 µl / minute overnight at 4 °C.

Following the recycling period the column was washed with 10 x the column volume with [A 0.0] (20 mM di-sodium hydrogen orthophosphate (Na_2HPO_4) + 5 mM EDTA (pH 7.2)) followed by washes with 10 times column volume with [A 0.4] ([A 0.0] + 400 mM NaCl) at 15 mg / ml.

Elute with 400 mM tetraethylammonium (TEA) and 10 mM decamethonium in [A 0.0].

To remove active fragments from TEA and decamethonium samples can be subjected to gel filtration on sephadex G25 (PD10).

The acrylamide gel is poured in to the chamber with 2.3 µl TEMED and mix using a pipette.

Overlay with 0.1 % isobutamol and allow to polymerise for 30 minutes.

Remove isobutyl alcohol and rinse with Tris chloride (pH 8.8).

Load and run protein and molecular weight markers.

Pour the stacking gel (5 %, Table A3.1) directly onto the resolving gel (above).

Insert a dry, clean teflon comb and fill plate immediately with stacking gel.

Allow to polymerise for 30 minutes.

After polymerisation remove the comb and wash with water.

Mount gel and add Tris-glycine buffer to reservoirs.

Prepare samples and markers

Heat samples to 100 °C for 3 minutes in 1x SDS gel-loading buffer:

50 mM Tris. Chloride (pH 6.8) in 100 ml water

100 mM Dithiothreitol

0.1 % Bromophenol blue

0.1% SDS/10 % Glycerol

Solution components	2 ml	5 ml	10 ml
H_2O	1.4	3.4	6.8
30 % acrylamide mix	0.33	0.83	1.7
1.0 M Tris (pH 6.8)	2.5	3.8	5.0
10 % SDS	0.1	0.15	0.2
10 % ammonium persulphate	0.1	0.15	0.2
TEMED	0.004	0.006	0.008

Table A3.1 Stacking gel (5%)

Lowry method for the quantification of proteins

The lowry method quantitates the colour from the reaction of Folin-Ciocalteu phenol reagent with the tyrosyl residues of an unknown protein, and compares this colour value to those derived from a standard protein, usually BSA.

Duplicates of BSA (10, 20, 30 and 40 μl) are added to eppendorf tubes and volumes brought up to 200 μl with water (Blanks = 200 μl of water only).

Add 20 μl of 0.15 % sodium deoxycholate to each tube and vortex. Leave for 10 minutes at room temperature.

Add 20 μl of 72 % (w/v) trichloroacetic acid to each tube and vortex. Microcentrifuge for 15 minutes at 3000 g at room temperature.

Dissolve the protein pellets in 200 μl of water and 200 μl of copper tartrate/carbonate solution (50 ml of a 0.2 % solution of copper sulphate (w/v) to 0.2 % potassium tartrate. Stir vigorously adding 50 ml of 0.2 % sodium carbonate) and leave for 10 minutes at room temperature.

Add 100 μl of 20 % Folin-Ciocalteu reagent and vortex. Leave at room temperature for 30 minutes. Measure at A_{750}.

Meta inhibitor synthesis for the purification of G_1 rAChE (a gift from Prof. P. Taylor)

(A method used by Prof. P. Taylor's group)

Dissolve phenylenediamine in 45 ml of acetic anhydride and add seven drops of sulphuric acid. Heat and stir.

Increase the heat until the reaction starts to boil for about 30 minutes. Transfer to a cold three neck round bottom flask and add 184 ml of water (slowly). Stir at 55 °C for 20 minutes.

Add 1380 ml of sodium hydroxide and leave overnight. Remove the crystals that should have formed and dissolve them in 40 ml of acetone and 3.3 ml of methyl iodide. Begin heating until the reaction is boiling (30 minutes).

Filter and rinse with 40 ml of cold acetone and add 2.2 ml of methyl iodide to the filtrate. Allow the reaction to boil for 1 hour.

Filter and rinse with cold acetone.

Dissolve the powder in 35 ml of ethanol and add 35 ml of hydrochloric acid. Heat and stir

Allow the mixture to boil for 2 hours.

Cool at room temperature and add 75 ml of acetone.

Boil the mixture and stir with a wooden stirrer.

Filter the crystals and wash in cold acetone.

Store the compound in the dark *in vacuo.*

APPENDIX 4A

Chemical reagents

Sigma-Aldrich Company Ltd. Poole, UK

(www.sigma-aldrich.com)

Acetylcholinesterase (E.C. 3.1.1.7)

Acetylthiocholine iodide

Barium chloride

Biocytin

Bovine serum albumin

Butyrylcholinesterase (E.C. 3.1.1.8)

1:5-bis(4-allydimethylammoniumphenyl)-pentan-3-one-dibromide (BW284c51)

Cesium chloride

Cesium hydroxide

Creatinine phosphokinase

3,3'-diaminobenzidine

DNAse

5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB)

Ethylenediaminetetraacetic acid (EDTA)

(1-ethyl-3-(3-dimethylethylaminopropyl) carbodiimide hydrochloride) (EDC)

Folin-ciolcalteus phenol reagent

Goat serum

HEPES

Methyllcaconitine citrate salt

Mecamylamine hydrochloride

MgATP

Nifedipine

Phosphate buffered saline (PBS) tablets

Penicilin/streptomycin solution

Poly-D-lysine

Procainamide

Tetraethylammonium chloride (TEACl)

Tetraethylammonium hydroxide (TEAOH)

N, N, N', N'-Tetramethylethylenediamine (TEMED)

Tetraisopropyl pyrophosphoramide (Iso-ompa)

Triphosphocreatinine

Trizma base (Tris(hydroxymethyl)aminomethane

Life Technologies Ltd. Paisley UK

(www.lifetech.com)

Dulbeccos minimal essential media (DMEM)

Foetal calf serum

Hanks balanced salt solution

Research Biochemicals International, USA

Dihydro-β-erythroidine

Bachem Ltd, UK

(www.bachem.com)

ω-conotoxin GVIA

Novocastra Labs Ltd, Newcastle upon Tyne, UK

(www.novocastra.co.uk)

ABC elite kit

Biotinylated goat anti-mouse secondary antibody

VECTASHIELD™

Calbiochem-Novabiochem (UK) Ltd

(www.cnuk.co.uk)

ω-Agatoxin VIA

Chemicon International Ltd, Harrow, UK

(www.chemicon.com)

Anti-GAD (rabbit) antibody

Anti-TH (mouse) antibody

Amersham Pharmacia Biotech. Ltd., UK

(www.apbiotech.com)

Stretavidin-Texas Red

Jackson Immunoresearch Laboratories Inc., USA

(www.jacksonimmuno.com)

Donkey serum

CY5-donkey anti-rabbit antibody

FITC-donkey anti-mouse antibody

Pharmacia Biotech, Herts, UK

(www.jp.apbiotech.com)

ECH Sepharose 4B affinity resin

Promega Corporation, USA

Basic fibroblast growth factor (bFGF)

BDH/Merck Chemicals Ltd, Warwickshire, UK

(www.merck-ltd.co.uk)

All other reagents

GIFTS

Meta inhibitor resin was a kind gift from Professor P. Taylor (UCSD, USA). Echothiophate iodide was a kind gift from Wyeth Europa, Ltd.(Berks, UK; www.wyeth.com)

APPENDIX 4B

Equipment

BDH/Merck Chemicals Ltd, Warwickshire

(www.merck-ltd.co.uk)

Sterilising oven ULM400

All standard laboratory equipment

Life Technologies Ltd. Paisley UK

(www.lifetech.com)

All disposable, sterile plasticware

Heraeus Equipment Ltd., Essex, UK

(www.iscpubs.com)

Laminair HBB 2448S

Lab-Lime Instruments, USA

Radiant warm wall CO_2 incubator

Raymond A. Lamb, London, UK

(www.ralamb.net)

Glass coverslips

Dissecting instruments

Sartorius AG, 37070, Gottingen, Germany

(www.sartorius.com)

Sterile filters (pore size 0.2 μm)

The Mickle Lab, Engineering Co. Ltd., Surrey

McIlwain mechanical tissue chopper

Leica Microsystems (UK) Ltd, Milton Keynes

(www.leica-microsystems.com)

Diaplan microscope (68202)
Camera Lucida attachment

Medical Air Technology Ltd., Manchester. UK
BioMAT-2 class II microbiological safety cabinet

Molecular Devices Ltd., Sussex UK
(www.moleculardevices.com)
UV Max microplate reader

Amersham Pharmacia Biotech (UK) Ltd., Bucks, UK
(www.apbiotech.com)
SMART system

SANYO Gallenkamp PLC, Leicester, UK
(www.sanyogallenkamp.com)
CO_2 incubator (MCO-17A1C)

Shimanzo Europa GmbH
UV (160A) Visible recording spectrophotometer

Carl Zeiss Ltd. Herts. UK
(www.zeiss.com)
Axioscop-2 fixed-stage light microscope

Narishige Inc., UK
(www.narishige.com)
MX-1 Micromanipulator

Harvard Apparatus Ltd., Kent, UK
(www.harvardapparatus.com)
Glass microelectrodes

Sutter Instruments, USA

(www.sutter.com)

P-87 Flaming / Brown microelectrode puller

Trough filaments

World Precision Instruments, USA

Microfil needles

Axon Instruments, USA

(www.axon.com)

Axopatch 200A whole-cell patch-clamp amplifier

Cambridge Electronics, Cambridge, UK

(www.ced.co.uk)

CED 1401

Gould Inc. UK

(www.gould.com)

Digital recording oscilloscope

APPENDIX 5

The Nernst equation

$$E = \frac{RT}{ZF} \ln \frac{[Ca]_o}{[Ca]_i}$$

Where: E is the equilibrium potential for the given ion species in Volts

R is the gas constant = 8.314 J K^{-1} mol^{-1}

T is the temperature in Kelvin

Z is the valency of the ion species

F is the Faraday constant; 9.6485 x 10^4 C mol^{-1}

[Ca] is the concentration of calcium ions, i for intracellular, o for extracellular. These may be expressed in any unit provided it is the same unit for both concentration values.

Using a value of 293 K for temperature, and the value of $[Ca]_o$ used in the experiments presented in this thesis of 5 mM, and an estimated $[Ca]_i$ of 10^{-7} M, E_{Ca} = +137 mV.

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