Oxford University

The role of catechol-*O*-methyltransferase (COMT) in hippocampal function

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Abstract

Catechol-*O*-methyltransferase (COMT) metabolises catechol-containing compounds, including dopamine. The aim of this thesis was to investigate whether COMT is involved in hippocampal function. This thesis also explored the role of functional polymorphisms within the COMT gene in the pathogenesis of schizophrenia and schizophrenia-related phenotypes.

First, as part of a study investigating the role of COMT in schizophrenia, human hippocampal COMT mRNA levels were shown to be neither altered in schizophrenia or bipolar disease, nor affected by COMT genotype. Hence, functional COMT polymorphisms do not appear to operate by altering gross COMT mRNA expression. Importantly, this study showed that COMT is expressed in the human hippocampus.

Second, the role of COMT in hippocampal neurochemistry was explored by studying the effect of pharmacological COMT inhibition on catecholamines and metabolites in rat hippocampal homogenates, and extracellularly, using microdialysis. Both demonstrated that COMT modulates hippocampal dopamine metabolism. Thus, hippocampal COMT is of functional significance with respect to dopamine.

Third, the effect of COMT inhibition on hippocampus-dependent behaviour was investigated. The results suggested a memory-enhancing effect of pharmacological COMT inhibition on hippocampus-dependent associative and non-associative forms of short-term memory in rats. In contrast, acute COMT inhibition appeared to have no effect on behavioural correlates of ventral hippocampal function i.e. anxiety-like behaviour.

In summary, the expression of COMT mRNA in the human hippocampus, as well as the effect of COMT inhibition on rat hippocampal neurochemistry and hippocampus-dependent behaviour provide evidence for a functional role of COMT in the hippocampus. Moreover, changes in COMT activity alter hippocampal dopamine metabolism, which could be a potential mechanism for the role of COMT in hippocampus-dependent short-term memory.

This thesis contains approximately 45’000 words.

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Table of Contents

Chapter 1: Introduction

1 Catecholamines 3

1.1 Biosynthesis and Release of Catecholamines 4

1.2 Inactivation of catecholamine neurotransmission 4

1.2.1 Inactivation of neurotransmission by catecholamine transporters 5
1.2.2 Enzymatic inactivation of catecholamine neurotransmission 5

1.3 Dopamine 8

1.3.1 Dopamine receptors 8
1.3.2 Evidence for a dopamine system in the hippocampus 11
1.3.3 Modulatory role of dopamine at hippocampal synapses 13
1.3.4 Dopamine in cognition 15
1.3.5 Dopamine in emotion and reward 17
1.3.6 Dopamine in the pathogenesis of schizophrenia 18

1.4 Noradrenaline 22

1.4.1 Adrenergic receptors 22
1.4.2 Noradrenergic projections in the CNS 22
1.4.3 Neuromodulatory role of noradrenaline at hippocampal synapses 23
1.4.4 Role of noradrenaline in cognition and emotion 24

1.5 Relationship between hippocampal dopamine and noradrenaline signalling 25

2 Catechol-*O*-methyltransferase (COMT) 27

2.1 COMT gene structure 27

2.2 Regulation of COMT gene expression 29

2.3 Genetic regulation of COMT activity 29

2.4 COMT protein structure 30

2.5 COMT expression 31

2.5.1 Cellular localisation of COMT in the CNS 32
2.5.2 Sub-cellular localisation of COMT 33

2.6 Psychopathophysiology and COMT 34

2.6.1 COMT may be associated with schizophrenia 34
2.7 COMT is associated with cognitive and emotional phenotypes 36
2.7.1 COMT Val^{158}Met and cognition 36
2.7.2 Tolcapone and cognition 39
2.7.3 COMT Val^{158}Met and emotion 40
2.7.4 COMT Val^{158}Met and evolution 42
3 The Hippocampus 44
3.1 Structure of the hippocampus 44
3.2 Role of the hippocampus 45
3.3 The hippocampus and COMT 46
4 Thesis rationale and aims 47

Chapter 2: COMT expression in the hippocampus

1 Introduction 50
1.1 Structural and functional abnormalities in schizophrenia implicate the hippocampus 50
1.2 Studying hippocampal COMT expression in bipolar disorder 51
1.3 Validation of methodology 52
1.3.1 Use of qRT-PCR 52
1.3.2 Genotyping COMT SNPs 56
1.4 Aims and hypotheses 57
2 Methods 58
2.1 Subjects 58
2.2 RNA extraction and reverse transcription 59
2.3 qRT-PCR 59
2.4 Genotyping 60
2.5 Data analysis 61
3 Results 62
3.1 COMT expression did not differ between diagnostic groups 62
3.2 COMT expression was unaffected by COMT genotype 63
3.3 COMT expression was unaffected by demographic variables 66

4 Discussion **68**

4.1 COMT expression was unaffected by diagnosis and COMT genotype 68

4.2 COMT expression was unaffected by demographic variables 69

4.3 Summary of Chapter 2 70

Chapter 3: Effect of COMT inhibition on tissue catecholamine and metabolite levels in rat brain regions

1 Introduction **72**

1.1 Sexually dimorphic effects of COMT 72

1.2 Moving from humans to rats 74

1.3 Using a tolcapone to manipulate COMT activity 75

1.4 High performance liquid chromatography coupled with electrochemical detection (HPLC-EC) 76

1.5 Aims and hypotheses 78

2 Methods **79**

2.1 Animals 79

2.2 Tissue collection and sample preparation 79

2.3 Drugs 79

2.4 HPLC-EC analysis 80

2.5 Data analysis 80

3 Results **83**

3.1 Tissue levels of dopamine and noradrenaline across the rat brain 83

3.2 Effect of tolcapone on regional brain levels of dopamine and noradrenaline 85

3.3 Tissue levels of DOPAC and HVA across the rat brain 87

3.4 Effect of tolcapone on regional brain levels of DOPAC and HVA 89

3.5 Estimates of dopamine turnover across the rat brain 90

3.6 Hippocampal subregion-specific effects 91

3.7 Sex-specific effects 93

4 Discussion **95**

4.1 COMT plays a role in regulating dopamine metabolism in the hippocampus 95

4.2 Tolcapone increases dopamine, but not noradrenaline levels, in the hippocampus 96

4.3 Dorso-ventral differences in hippocampal neurochemistry 97

4.4 Sex-specific effects of tolcapone are limited to non-hippocampal brain regions 98

4.5 Summary of Chapter 3 99

Chapter 4: Impact of COMT inhibition on extracellular levels of catecholamines in the hippocampus

1 Introduction **102**

1.1 *In vivo* microdialysis 102

1.2 Dissociations along the septotemporal axis of the hippocampus 103

1.3 Aims and hypotheses 104

2 Methods **106**

2.1 Animals 106

2.2 Surgery 106

2.3 Awake microdialysis 107

2.4 HPLC-EC 110

2.5 Drugs 111

2.6 Data analysis 111

3 Results **114**

3.1 Dopamine and noradrenaline were detected in hippocampus perfusates 114

3.2 Effect of tolcapone on basal and evoked dopamine and noradrenaline 115

3.3 DOPAC and HVA were detected in hippocampal perfusates 117

3.4 Effect of tolcapone on basal DOPAC and HVA levels 118

4 Discussion **122**

4.1 Caveats of the microdialysis methodology 122

4.2 Tolcapone may alter hippocampal catecholamine levels 124
4.3 Tolcapone influences DOPAC and HVA levels 125
4.4 Dorso-ventral dissociations? 126
4.5 Summary of Chapter 3 and Chapter 4 127

Chapter 5: Investigation of the effects of COMT inhibition on aspects of anxiety

1 Introduction 129
1.1 Role of the hippocampus in emotion 129
1.2 Validation of the experimental approach 133
1.2.1 Assessing emotionality in rodents 133
1.2.2 Choice of tasks 133
1.3 Aims and hypotheses 135
2 Methods 136
2.1 Animals 136
2.2 Drugs 137
2.3 Successive alleys task 137
2.3.1 Apparatus 137
2.3.2 Behavioural testing 138
2.4 Hyponeophagia test 139
2.4.1 First hyponeophagia test 139
2.4.2 Second hyponeophagia test 140
2.5 Data analysis 141
3 Results 142
3.1 Tolcapone did not influence behaviour during the successive alleys task 142
3.2 Tolcapone does not influence the results of the hyponeophagia tests 144
4 Discussion 146
4.1 Summary of Chapter 5 147

Chapter 6: Investigation of the effects of COMT inhibition on hippocampus-dependent cognition

1 Introduction 149

1.1 The hippocampus and memory 149

1.2 Methods for studying hippocampus-dependent cognition in rodents 150

1.3 Validation of methodology 152

1.3.1 Delayed rewarded alternation task 152
1.3.2 Spatial novelty preference task 153

1.4 Aims and hypotheses 153

2 Methods 155

2.1 Animals 155

2.2 Delayed Rewarded Alternation Task 156

2.2.1 Apparatus 156
2.2.2 Habituation 157
2.2.3 Training 157
2.2.4 Behavioural testing 158

2.3 Spatial novelty preference task 158

2.3.1 Apparatus 158
2.3.2 Behavioural testing 159

2.4 Data analysis 160

3 Results 162

3.1 Delayed rewarded alternation task 162

3.1.1 Performance during pre-training 162
3.1.2 Performance during behavioural testing 162
3.1.3 Proactive interference 165

3.2 Spatial Novelty Preference Task 168

3.2.1 Effect of tolcapone on spatial novelty preference 168
3.2.2 Effect of tolcapone on the number of times rats enter each arm of the Y-maze 169

4 Discussion 171

4.1 Delayed rewarded alternation 171

4.1.1 Task-specific caveats of the rewarded alternation experiment 172

4.2 Spatial novelty preference task 173
4.3 Effects of injection stress 174
4.4 Summary of Chapter 6 177

Chapter 7: General discussion

1 Summary of main findings 179
1.1 Hippocampal COMT mRNA expression is not altered in schizophrenia or bipolar disorder, nor affected by COMT genotype 180
1.2 COMT inhibition alters hippocampal dopamine metabolism 181
1.3 COMT activity affects some aspects of rat behaviour 181
2 Broader perspectives 184
2.1 Cellular and subcellular localisation of COMT 184
2.2 Sexually dimorphic effects of COMT 185
2.3 Dorso-ventral dissociations in the hippocampus 188
2.4 What molecular mechanisms mediate the effects of COMT 189
2.5 Relative involvement of COMT in hippocampal versus non-hippocampal function 192
2.6 Tolcapone as a cognitive enhancer 192
3 Final conclusions 194

Appendices

Appendix I COMT expression data: ANOVAs 197
Appendix II COMT expression data: t-tests 198
Appendix III Tissue extract study data 199
Appendix IV Optimisation of the microdialysis protocol 201
Appendix V Microdialysis data 203
Appendix VI Delayed Rewarded Alternation Data 204

References 205

List of Figures

Figure 1 Biosynthesis of catecholamines 7
Figure 2 A schematic illustration of the major routes of catecholamine metabolism 7
Figure 3 Dopamine signalling via the adenylate cyclase cascade 10
Figure 4 Schematic diagram of the pattern of dopamine (D2) receptor expression and dopamine innervations in the rat and human hippocampus and parahippocampal cortices 12
Figure 5 The inverted U-shaped relationship between DA and PFC function 16
Figure 6 The hippocampal-VTA loop 18
Figure 7 Schematic illustration of the interactions between the mesolimbic and mesocortical dopamine (DA) systems 20
Figure 8 Monoaminergic projections to the hippocampus are sparse 23
Figure 9 Methyl transfer from SAM to catechol in the COMT-catalysed reaction 28
Figure 10 Structure of the COMT gene 28
Figure 11 Association of COMT genotype with performance on the Wisconsin Card Sorting Task (WCST) 37
Figure 12 Simplified illustration of the tri-synaptic circuit of the hippocampus 45
Figure 13 Illustration of a PCR cycle 53
Figure 14 Representative amplification plot, standard curve and dissociation curve from the COMT qRT-PCR experiment 55
Figure 15 Illustration of COMT SNPs with frequencies >0.15 in the coding region of COMT 57
Figure 16 Normalised COMT mRNA expression in control subjects (CON; n=35) and patients with schizophrenia (SZ; n=34) or bipolar disorder (BPD; n=32) 62
Figure 17 Normalised COMT mRNA expression in control subjects (CON; n=35) and patients with schizophrenia (SZ; n=33) or bipolar disorder (BPD; n=32), by Val^{158}Met genotype 63
Figure 18 Normalised COMT mRNA expression in control subjects (CON; n=35, 34 for rs6269) and patients with schizophrenia (SZ; n=34) or bipolar disorder (BPD; n=32, 31 for rs6269), by (a) A-287G, (b) rs4818, (c) rs6269 and (d) rs4633 genotype 65
Figure 19 Normalised COMT mRNA expression in control subjects (CON; n=15) and patients with schizophrenia (SZ; n=9) or bipolar disorder (BPD; n=10), according to their haplotype homozygosity .. 65
Figure 20 Normalised COMT mRNA expression in control subjects (CON; n=35) and patients with schizophrenia (SZ; n=34) or bipolar disorder (BPD; n=32) by sex 66
Figure 21 Structure and function of tolcapone 75
Figure 22 Diagram illustrating the components of a HPLC-EC system and the principles of HPLC using dopamine (DA) as an example compound 77

Figure 23 Example of a HPLC chromatogram corresponding to a standard calibration solution 82
Figure 24 Example of a HPLC-EC chromatogram..... 82
Figure 25 (a) Dopamine and (b) Noradrenaline content in rat brain tissue extracts 84
Figure 26 (a) Dopamine and (b) Noradrenaline content in dorsal (dHC) and ventral (vHC) hippocampal extracts 86
Figure 27 (a) DOPAC and (b) HVA content in rat brain tissue extracts 88
Figure 28 DOPAC/HVA ratio in rat brain tissue extracts 92
Figure 29 (a) DOPAC content and (b) DOPAC/HVA in the dorsal and ventral hippocampus 92
Figure 30 DOPAC/HVA ratio in female (n=11) and male (n=12) rat brain tissue extracts 94
Figure 31 Diagrams (a, c) and example brain sections (b, d) illustrating the location of the MAB6.14.3 microdialysis probes (red line; probe shown in Figure 30e) and guide cannulae (red rectangle) in the ventral (a, b) and dorsal (c,d) hippocampus 108
Figure 32 Illustration of equipment used for an awake, freely moving animal 108
Figure 33 Example of a HPLC chromatogram corresponding to a standard calibration solution 111
Figure 34 Example of two HPLC chromatograms indicating consistency in peak positions and the detection of both NA and DA in hippocampal dialysates 114
Figure 35 (a) Dopamine and (b) Noradrenaline content as a percentage relative to baseline in hippocampal dialysates from rats treated with either tolcapone or vehicle..... 116
Figure 36 (a) DOPAC and (b) HVA content as a percentage relative to baseline in dorsal (dHC) and ventral (vHC) hippocampal dialysates from rats treated with either tolcapone or vehicle..... 120
Figure 37 DOPAC/HVA ratio in dorsal and ventral hippocampal dialysates from rats treated with either vehicle or tolcapone..... 121
Figure 38 The concept of defensive direction 130
Figure 39 A schematic diagram of the septo-hippocampal involvement in conflict resolution 132
Figure 40 Successive alleys apparatus 138
Figure 41 Apparatus for the first hyponeophagia test 140
Figure 42 Apparatus for the second hyponeophagia test..... 140
Figure 43 Effect of tolcapone on (a) total time spent in, (b) number of entries into, and (c) latency to cross into, the four successive alleys 1-4..... 143
Figure 44 Effect of tolcapone on latency to (a) first contact and (b) eat novel food rewards on the first hyponeophagia task 145
Figure 45 Effect of tolcapone on latency to eat novel food during the second hyponeophagia task..... 145

Figure 46 Diagram of delayed rewarded alternation task design and apparatus 156
Figure 47 Diagram of the spatial novelty preference task design and apparatus 159
Figure 48 Effect of tolcapone and vehicle treatment on delayed rewarded alternation 164
Figure 49 Performance on the delayed rewarded alternation task by block of trials 166
Figure 50 Performance on the delayed rewarded alternation task by previous delay duration 167
Figure 51 Effect of tolcapone and vehicle treatment on spatial novelty preference 169
Figure 52 Three attempts at explaining the effects of injection stress and tolcapone administration on cognitive performance using the "U-shaped curve" presented in Figure 5 176

List of Tables

Table 1 Summary of the four principal dopaminergic pathways in the brain 8
Table 2 Primary literature on COMT in the hippocampus 46
Table 3 List of commonly studied COMT SNPs 57
Table 4 Demographic details of SMC brain series 58
Table 5 Primers used for qRT-PCR experiments 60
Table 6 Dopamine (DA) and noradrenaline (NA) content in tissue extracts from vehicle-treated rats 84
Table 7 DOPAC and HVA content, as well as DOPAC/HVA ratios in striatal, cortical, hippocampal and cerebellar tissue extracts from vehicle-treated rats 88
Table 8 DOPAC/DA ratios in striatal, cortical, hippocampal and cerebellar tissue extracts from vehicle-treated rats 90
Table 9 Dopamine and noradrenaline data in ventral and dorsal hippocampal dialysates prior to either vehicle or tolcapone treatment 115
Table 10 DOPAC, HVA and DOPAC/HVA data in ventral and dorsal hippocampal dialysates prior to either vehicle or tolcapone treatment 118
Table 11 Summary of published papers reporting dopamine levels in hippocampal dialysates 124
Table 12 Summary of the main findings of this thesis 179

Abbreviations and Acronyms

3-MT, 3-Methoxytyramine
3-OMD, 3-*O*-methyldopa
5HIAA, 5-Hydroxyindole-3-acetic acid
ab, Angular bundle
aCSF, Artificial cerebrospinal fluid
AD Aldehyde dehydrogenase
ADH, Alcohol dehydrogenase
al, Alveus
ANOVA, Analysis of variance
AR, Aldehyde reductase
BPD, Subjects with bipolar disorder
CA1, Cornu Ammon 1 subregion of the hippocampus
CA3, Cornu ammonis region 1 and 3 of the hippocampus
cAMP, Cyclic AMP
Cer, Cerebellum
CNS, Central nervous system
COMT, Catechol-*O*-methyltransferase (including **MB-COMT**, membrane-bound COMT; **S-COMT**, soluble COMT)
CON, Control subjects
$D_{1/5}$R, D1-like dopamine receptors
DA, Dopamine
DAT, Dopamine transporter
DDC, L-aromatic amino acid decarboxylase
DG, Dentate gyrus
DHPG (or DOPEG), 3,4-Dihydroxyphenylglycol
dlPFC, Dorsolateral prefrontal cortex
DOMA (or DHMA), 3,4-Dihydroxymandelic acid
DOPAC, 3,4-Dihydroxyphenylacetic acid
DOPAL, 3,4-Dihydroxyphenylacetaldehyde
DOPEGAL, 3,4-Dihydroxyphenylglycolaldehyde
DOPET, 3,4-Dihydroxyphenylethanol
DβH, Dopamine β-hydroxylase
EC, Entorhinal cortex
ERE, Oestrogen response element
fi, Fimbria
fMRI, Gunctional magnetic resonance imaging
GAPDH, Glyceraldehyde-3-phosphate dehydrogenase
gcl, Granule cell layer
HC, Hippocampus (including **vHC**, ventral hippocampus; **dHC**, dorsal hippocampus)
HC-VTA loop, Hippocampal-ventral tegmental area loop
hf, Hippocampal fissure
HMPG, 4-Hydroxy-methoxyphenylglycol
HPLC-ED, High performance liquid chromatography coupled with electrochemical detection
HV, homovanillin
HVA, Homovanillic acid
K.O., Genetic knock-out
L-DOPA, 3,4-Dihydroxyphenylalanine
LTP, Long-term plasticity
MAO, Monoamine oxidase
MAPK, Mitogen-activated protein kinase
MHPA, 3,4-Methoxy-4-hydroxyphenylacetaldehyde
MHPG (or MOPEG or HMPG), 3-Methoxy-4-hydroxyphenylglycol
ml, Molecular layer
MN, Metanephrine
mPFC, Medial prefrontal cortex
NA, Noradrenaline
NAT, Noradrenaline transporter
NM, Normetanephrine
OCD, Obsessive compulsive disorder
OCT3, Organic cation transporter 3
Para, Parasubiculum
pcl, Pyramidal cell layer
PFC, Prefrontal cortex
PFC, Prefrontal cortex
PKA, Protein kinase A
pl, Polymorphic cell layer
PMAT, Plasma membrane monoamine transporter
PMI, Post-mortem interval
PNMT, Phenylethanolamine-N-methyltransferase
PP1, Protein phosphatise 1
PPTg, Pedunculopontine tegmental nucleus
Pre, Presubiculum
qRT-PCR/qPCR, Quantitative real-time polymerase chain reaction
RIN, RNA integrity number
RNA, Ribonucleic acid
SAH, S-adenosyl-L-homocysteine
SAM, S-adenosyl-L-methionine
SEM, Standard error of the mean
sl, Stratum lucidum
sl-m, Stratum lacunosum-moleculare
SNP, Single nucleotide polymorphism
STP, Short-term plasticity
so, Stratum oriens
sr, Stratum radiatum
Str, Striatum
SZ, Subjects with schizophrenia
TBP, Tata-box binding protein
TFRC, Transferrin receptor
TH, Tyrosine hydroxylase
V Pallidum; Ventral Pallidum
VCFS, Velocardiofacial syndrome
VMA, Vanillylmandelic acid
VMAT, Vesicular monoamine transporter
VTA, Ventral tegmental area
WCST, Wisconsin card sorting task

Oxford University

Chapter 1

Introduction

Linda Maria Laatikainen

DPhil thesis on the role of COMT in hippocampal function

Catechol-*O*-methyltransferase (COMT) metabolises catechol-containing compounds, including dopamine and noradrenaline. This thesis investigates whether COMT has a functional role in the hippocampus at a molecular, neurochemical and behavioural level.

This introduction begins with an overview of the catecholamine neurotransmitters dopamine and noradrenaline. The second subchapter introduces the COMT gene and proteins, including a discussion of the physiological and pathophysiological roles of the COMT enzyme. The third subchapter introduces the hippocampus, and briefly describes why the hippocampus is a promising brain region to describe the association of COMT with both cognitive and emotional phenotypes. The final subchapter summarises the aims and hypotheses of this thesis.

1 Catecholamines

Catecholamines are organic compounds that contain an amine group and a catechol ring (benzene ring with two adjacent hydroxyl substitutions; see structure of dopamine in Figure 1). In the periphery, catecholamines have extensive roles in the nervous and hormonal systems (Goldstein et al 2003; Goldstein et al 1995; Missale et al 1998). In the central nervous system (CNS), catecholamines help control attention, arousal, emotion, locomotion, cognition, body temperature and neuroendocrine secretion (Arnsten & Li 2005; Beninger 1983; Carlsson 1987; Cooper et al 1965; Glavin 1985; Greengard 2001; Kempf et al 1976; also see subchapter 1). Dysfunction of catecholamine systems is a contributing factor to disorders such as Parkinson's disease, Huntingdon's disease, drug addiction and schizophrenia (Greengard 2001; Jankovic 1988; Koob 1992; also see subchapter 1 below). Hence, catecholamine synthesis, release, uptake and metabolism are carefully controlled in a coordinated fashion (Fillenz 1990; Iversen 1971; Trendelenburg 1990) – as discussed below.

1.1 Biosynthesis and Release of Catecholamines

Dopamine (DA) and noradrenaline (also called norepinephrine, NA) are synthesised from L-tyrosine by successive enzymatic steps initiated by tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis (see Figure 1).

Catecholamine neurotransmitters are stored in pre-synaptic vesicles (through the action of vesicular monoamine transporters, VMATs; Hoffman et al 1998) until they are released by Ca^{2+}-dependent exocytosis, Ca^{2+}-independent dendritic release or the reversal of catecholamine transporters. Both DA and NA neurons have autoreceptors, which regulate catecholamine release, catecholamine synthesis and neuronal firing rates through auto-inhibitory feedback (Cedarbaum & Aghajanian 1977; Dennis et al 1987; Freedman & Aghajanian 1984; Masserano & Weiner 1983; Missale et al 1998; Scheinin et al 1988; Schlicker & Gothert 1998; Schlicker et al 1998; Starke 1972; Starke et al 1975a; b).

1.2 Inactivation of catecholamine neurotransmission

DA and NA signalling in the CNS is primarily terminated by physical removal of the neurotransmitters from the synaptic cleft by catecholamine transporters (Hoffman et al 1998), though this depends on the brain region in question. Following re-uptake, DA and NA are mostly recycled by re-storage into intracellular vesicles. The small proportion of neurotransmitters that escapes vesicular sequestration by VMATs is metabolised by intracellular enzymes (Eisenhofer et al 2004; Eisenhofer et al 1995; Goldstein et al 2003; Kopin 1985). The mechanisms of catecholamine re-uptake and enzymatic inactivation of catecholamine transmission are reviewed here (also see Figure and Figure 3).

1.2.1 Inactivation of neurotransmission by catecholamine transporters

40-60 % of released catecholamines are removed from the extracellular space by one of four catecholamine transporters present in the CNS: DA transporter (DAT), NA transporter (NET), plasma membrane monoamine transporter (PMAT) or organic cation transporter (Engel et al 2004; Grundemann et al 1998b; Guiard et al 2008a; Trendelenburg 1991; Wu et al 1998). These four catecholamine transporters differ in their sub-cellular localisations and affinities for catecholamine substrates:

DAT and NET are presynaptic $uptake_1$ transporters, which have high affinity for NA and DA (Gu et al 1994; Trendelenburg 1986). In contrast, PMAT and OCT 2/3 are postsynaptic $uptake_2$ transporters, which have low affinity, but high capacity (Engel & Wang 2005; Grundemann et al 1998a). Additionally, the local densities of these transporters vary between brain regions (Amphoux et al 2006; Moron et al 2002), and are dynamically regulated (Blakely et al 1994; Carvelli et al 2002; Daws et al 2002; Torres et al 2003).

About 90 % of catecholamines are thought to be removed via rapid $uptake_1$ into neurons, although $uptake_2$ may be particularly important for limiting the spread of catecholamine signalling by clearing catecholamines, which have escaped $uptake_1$, at extraneuronal sites in the CNS (Eisenhofer 2001; Jonker & Schinkel 2004).

1.2.2 Enzymatic inactivation of catecholamine neurotransmission

Catecholamine catabolism may occur via several different enzymatic routes, whereby MAO and COMT act either independently or in combination (see Figure 2; Trendelenburg 1986). This leads to catecholamine metabolites that are deaminated, *O*-methylated, or both. In most aminergic regions the role of COMT in the inactivation of catecholamine neurotransmission is thought to be minor compared to that of monoamine oxidase (e.g. Rivett et al 1982).

Noradrenaline is always converted to 3-methoxy-4-hydroxymandelic acid (VMA), whereas the main metabolite of dopamine is homovanillic acid (HVA; Eisenhofer et al 2004; Goldstein et al 2003). The relative presence of the intermediate products from the two enzymatic routes differs between brain regions (Karoum et al 1994; Rivett et al 1982; Rivett et al 1983). The predominant metabolites in the hippocampus are yet to be determined reliably.

The catecholamines DA and NA will be introduced in more detail below. This introduction will mainly concentrate on the potential functions of DA and NA in the CNS, and the hippocampus in particular.

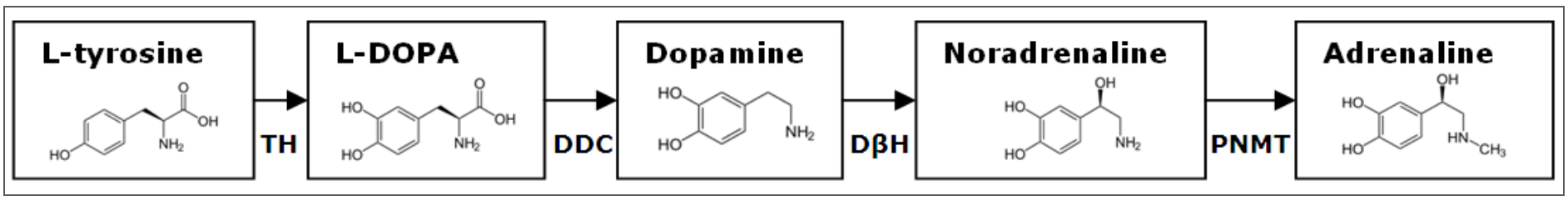


Figure 1 Biosynthesis of catecholamines
TH (tyrosine hydroxylase), which hydroxylates the aromatic amino acid L-tyrosine to yield 3,4-dihydroxyphenylalanine (L-DOPA), is only present in the cytosol of catecholamine-containing cells. L-DOPA is quickly metabolised to dopamine by L-aromatic amino acid decarboxylase (DDC). All noradrenergic neurons contain an additional vesicular enzyme called dopamine β-hydroxylase (DβH), which oxidizes DA to form NA. The presence of DβH differentiates between distinct DA- and NA-containing neurons in the brain. Neurons, which additionally contain the enzyme phenylethanolamine-N-methyltransferase (PNMT), can also form adrenaline by methylation of NA.

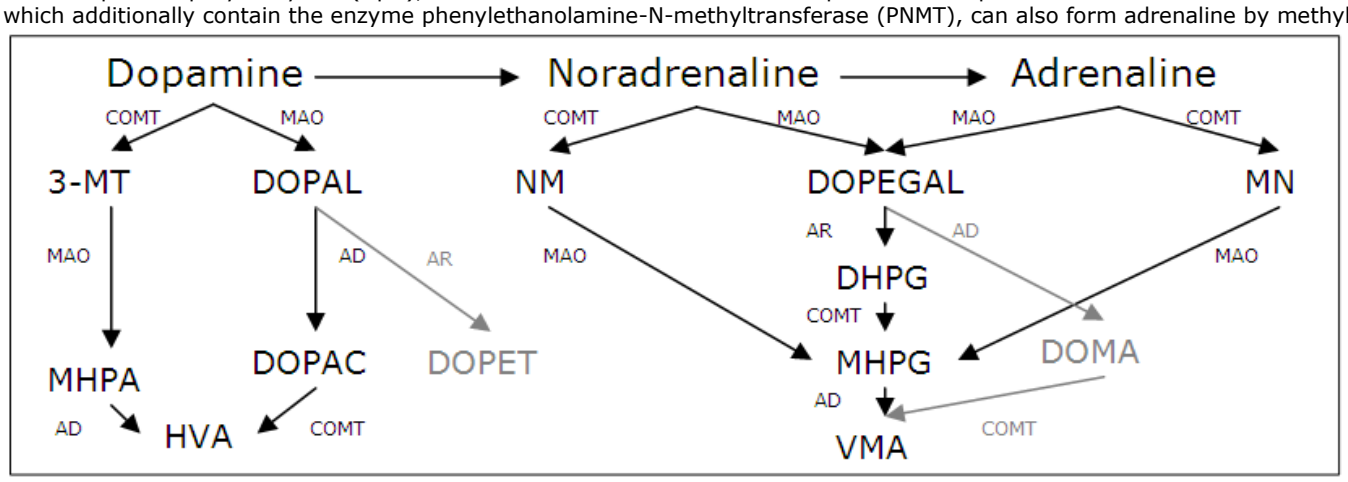


Figure 2 A schematic illustration of the major routes of catecholamine metabolism
Glucuronide and sulfate-conjugates have been excluded from the figure, although they constitute important excreted metabolites (Figure adapted from Eisenhofer et al 2004; Goldstein et al 2003). Pathways marked in grey play a smaller role in catecholamine metabolism than pathways marked in black. As shown, dopamine is preferentially metabolised to the acid derivative (DOPAC) by AD, whereas noradrenaline and adrenaline are preferentially metabolised to the alcohol metabolite (DHPG) by AR. NM, normetanephrine; DOMA (or DHMA), 3,4-dihydroxymandelic acid; MN, metanephrine; VMA, vanillylmandelic acid; 3-MT, 3-methoxytyramine; DOPAC, 3,4-dihydroxyphenylacetic acid; DHPG (or DOPEG), 3,4-dihydroxyphenylglycol; HVA, homovanillic acid; MHPG (or MOPEG or HMPG), 3-methoxy-4-hydroxyphenylglycol; MAO, monoamine oxidase; COMT, catechol-*O*-methyltransferase; ADH, alcohol dehydrogenase; AD aldehyde dehydrogenase; AR, aldehyde reductase; DOPAL, 3,4-dihydroxyphenylacetaldehyde; HV, homovanillin; DOPEGAL, 3,4-dihydroxyphenylglycolaldehyde; MHPA, 3,4-methoxy-4-hydroxyphenylacetaldehyde; DOPET, 3,4-dihydroxyphenylethanol.

1.3 Dopamine

Dopamine (DA) was discovered in 1910 (Barger G). In the CNS, DA acts via four principal dopaminergic pathways to control a number of important physiological processes such as motor functions, arousal, reward-related states, secretion of pituitary hormones and cognitive functions (see Table 1 below). The overlapping role of DA (and COMT) in motivation and memory functions is discussed in further detail below, because the possibility that DA (and COMT) may serve as a link between emotion and cognition is particularly intriguing from the point of view of this thesis (Phillips et al 2008).

DA pathway	Origin	Target regions	Function
Nigrostriatal	Substantia nigra pars compacta	Caudate nucleus and putamen (striatum)	Motor control and some cognitive functions
Mesolimbic	Ventral tegmental area	Nucleus accumbens and the tuberculum olfactorium	Arousal, locomotor activity and reward-related states
Tuberoinfundibular	Hypothalamus	Pituitary gland	Control secretion of prolactin and somatotrophin
Mesocortical	Midbrain neurons	Septum, amygdala, prefrontal cortex, brainstem, hippocampus and other limbic areas	Higher cortical functions

Table 1 Summary of the four principal dopaminergic pathways in the brain

1.3.1 Dopamine receptors

There are five DA receptor subtypes (D1-D5; Sealfon & Olanow 2000), which are all seven transmembrane G-protein coupled metabotropic receptors (GPCRs). Dopamine receptors are divided into two functional classes according to their pharmacological, anatomical and biochemical properties: the D_1-like (D_1 and D_5) and D_2-like receptors (D_2, D_3 and D_4). In addition, several DA receptor genes encode splice variants (Missale et al 1998). The expression of DA receptor subtypes and splice variants appears to be sensitive to a wide range of stimuli and are hence, differentially controlled in different brain regions (e.g. Khan et al 1998; Laurier et al 1994a; Schwartz et al 1995). This explains some of the functional

heterogeneity within the downstream effects of DA (Schwartz et al 1995; Sokoloff & Schwartz 1995). Further molecular evidence for the functional diversity of DA comes from the fact that DA contributes to a wide variety of intracellular signalling cascades:

D_1-like receptors are positively coupled to the adenylate cyclase pathway, whereas D_2-like receptors are negatively coupled to this signalling cascade (see Figure 3; Girault & Greengard 2004; Greengard 2001). The downstream effects of D_1-like receptors are thought to be stimulatory, whereas D_2-like receptors exert inhibitory effects on synaptic transmission (West & Grace 2002). One of the most interesting downstream targets of DA is DARPP-32 (dopamine and cAMP-regulated phosphoprotein of 32kDa), because it is an important point of convergence for lots of signalling pathways (Svenningsson et al 2004), hence allowing DA signalling to interact with other post-synaptic signalling pathways. DA can also modulate other signalling cascades via its actions at synergistic DA receptors (Sokoloff et al 1995), GPCR heteromers (Fuxe et al 2010a; Fuxe et al 2010b), or non-dopaminergic receptors, such as α_2 adrenoceptors (Boyajian & Leslie 1987).

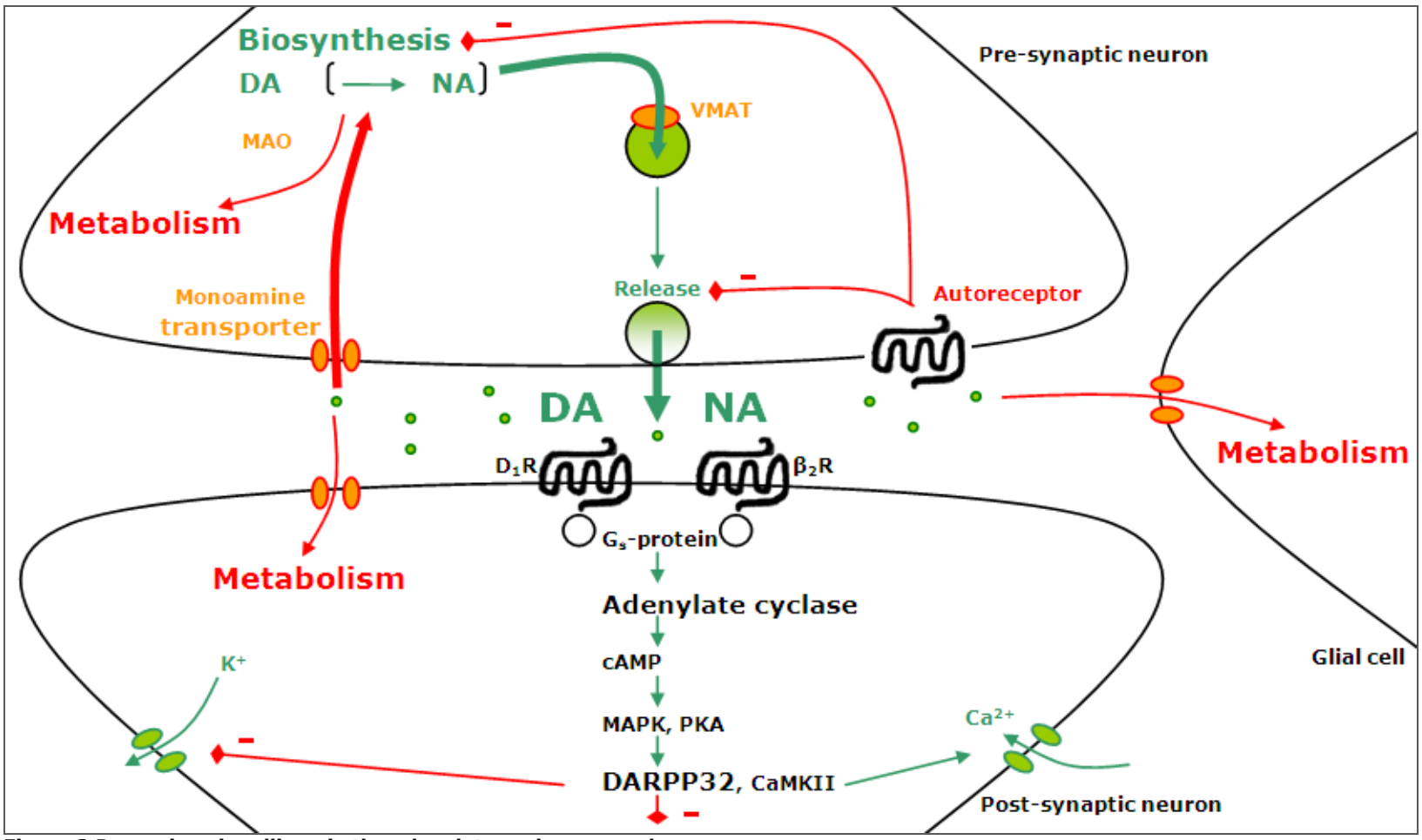


Figure 3 Dopamine signalling via the adenylate cyclase cascade

cAMP, cyclic AMP; COMT, catechol-*O*-methyltransferase; DA, dopamine; MAO, monoamine oxidase; MAPK, mitogen-activated protein kinase; NA, noradrenaline; PKA, protein kinase A; PP1, protein phosphatise 1; VMAT, vesicular monoamine transporter

1.3.2 Evidence for a dopamine system in the hippocampus

The structural and functional evidence for a hippocampal DA system is remarkably complex and inconsistent. As discussed below, the literature encompasses species differences (Berger et al 1991; Gaspar et al 1989; Goldsmith & Joyce 1994; Kohler et al 1991a; Samson et al 1990), ventro-dorsal gradients, as well as major subfield and strata heterogeneity within the hippocampal DA system.

DA terminals are most densely distributed in subiculum and CA1 areas of the ventral hippocampus, with less innervation of the dorsal subregions and very little innervation of the DG (see subchapter 3 for an introduction to hippocampal neuroanatomy; Gasbarri et al 1994a; Gasbarri et al 1996; Gasbarri et al 1997; Gasbarri et al 1994b; Goldsmith & Joyce 1994; Hortnagl et al 1991; Samson et al 1990; Swanson et al 1987; Swanson 1982; Swanson & Hartman 1975; Torack & Morris 1990; Verney et al 1985).

In contrast to the DA innervation data, rat chemoanatomical data suggest that D_2 receptors are particularly densely distributed in the dorsal hippocampus DG, CA1 and subiculum (Brouwer et al 1992; Bruinink & Bischoff 1993; Goldsmith & Joyce 1994; Khan et al 1998; Kohler et al 1991a; Kohler et al 1991b; Otmakhova & Lisman 1999; Scatton 1980; Swanson et al 1987; Yokoyama et al 1994). Hence, the pattern on hippocampal DA innervation does not overlap with the suggested pattern of DA receptor mRNA expression data, which is itself receptor subtype-dependent (see Figure 4 below, Gingrich et al 1992; Goldsmith & Joyce 1994; Khan et al 2000; Kohler et al 1991b; Laurier et al 1994b; Mansour et al 1990; Mansour et al 1992; Mansour et al 1991; Meador-Woodruff et al 1992; Mengod et al 1992). However, even though the exact subregional distribution of DA receptors is unknown, all DA receptors, apart from D_4receptors (Kwon et al 2008; Noain et al 2006), have unequivocally demonstrated some hippocampal expression.

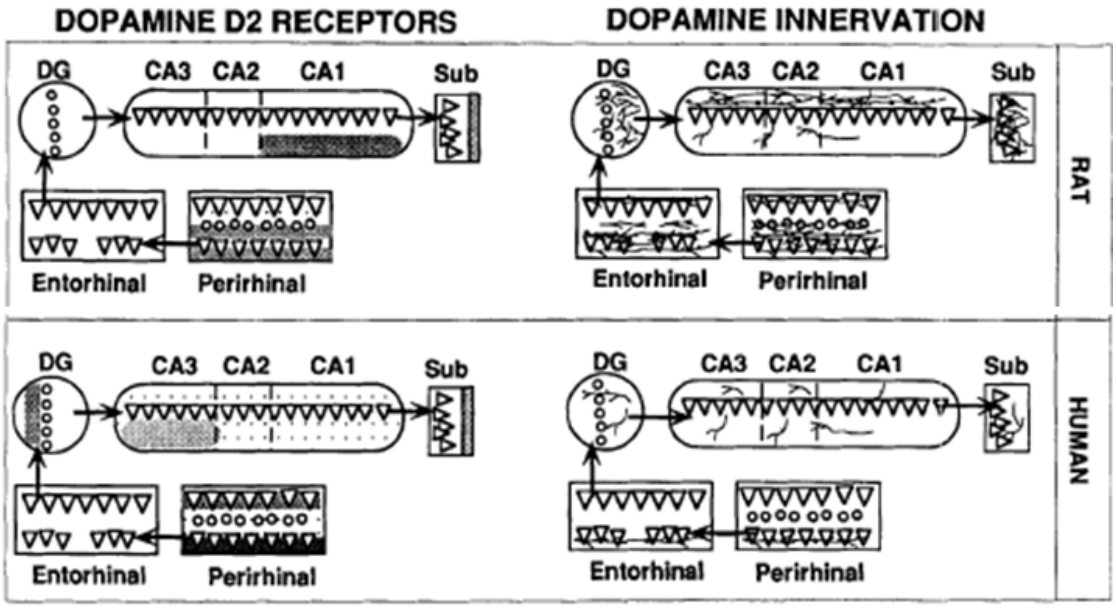


Figure 4 Schematic diagram of the pattern of dopamine (D2) receptor expression and dopamine innervations in the rat and human hippocampus and parahippocampal cortices
(adapted from Goldsmith & Joyce 1994)

In order to have a functional DA system, the hippocampus not only needs dopaminergic projections and receptors, but also molecules to terminate DA neurotransmission. Encouragingly, MAO and COMT (Cristino et al 2008; Grimsby et al 1990; Luque et al 1995; Matsumoto et al 2003a; Saura et al 1992; Saura et al 1994), as well as DAT, NET, PMAT and OCT-3 (Dahlin et al 2007; Gasser et al 2009; Lewis et al 2001; Schroeter et al 2000) have been shown to be expressed in the rodent and human hippocampi. Furthermore, pharmacological and genetic manipulations have suggested that these enzymes and transporters are also functional in the hippocampus (e.g. Morice et al 2007).

In summary, the hippocampus has all the necessary structural components for a functional DA system i.e. dopaminergic fibres, DA receptors, metabolic enzymes and uptake transporters - although there are inconsistencies regarding species differences, ventro-dorsal gradients, and subregional heterogeneity.

In addition to the structural evidence for a hippocampal DA system, microdialysis studies have shown that DA is actively released in the hippocampus (see chapter 4; Bischoff et al 1979; Ihalainen et al 1999) and electrophysiological

studies suggest that DA modulates hippocampal synaptic transmission (see below). Thus, both structural and functional evidence imply the existence of a dopamine system in the hippocampus.

1.3.3 Modulatory role of dopamine at hippocampal synapses

DA can alter acute synaptic transmission in the hippocampus (Benardo & Prince 1982a; b; Berretta et al 1990; Gribkoff & Ashe 1984a; b; Kaphzan et al 2006; Manahan-Vaughan & Kulla 2003; Otmakhova & Lisman 1999; Stanzione et al 1984). However, DA is thought to have a more potent neuromodulatory effect on synaptic plasticity (Jay 2003). Synaptic plasticity is the ability of synaptic connections between two neurons to change in strength, by means of changes in synaptic efficiency, as well as anatomical changes in synaptic dimensions, biochemical content, connectivity and the number of synapses. DA is known to modulate long-term plasticity (LTP), which is a form of synaptic plasticity thought to encode long-term memory and hippocampus-dependent spatial learning in the vertebrate brain (Andersen P 2006; Bliss 1996; Bliss & Collingridge 1993; Frey 2001; Matthies 1989; Morris et al 2003). The best studied form of LTP is associative and input-specific NMDA receptor-dependent LTP. This LTP consists of up to three phases, each characterized by different time courses and cellular properties, as summarised below (Collingridge 1992; Lisman 2003; Otani & Ben-Ari 1993; Reymann et al 1986):

1) Short-term plasticity (STP), a gradually decaying phase, which depends on N-methyl-d-aspartate receptor (NMDAR) activation and Ca^{2+} (Collingridge et al 1992; Malinow et al 1994; Matthies et al 1995; Reymann et al 1988a; Reymann et al 1989).

2) LTP1, an intermediate phase, which depends on metabotropic glutamate receptor activation and phosphorylation of protein kinase (PKA, PKC and CaMKII) substrates (Lisman et al 2002; Lovinger et al 1987; Malinow & Tsien

1990; Matthies et al 1991; Matthies & Reymann 1993; Otani & Connor 1998; Reymann et al 1988b).

3) LTP2, a long-lasting phase, which requires translation of pre-existing mRNA to form regulatory plasticity-related proteins (PRP) locally (Bradshaw et al 2003; Fazeli et al 1993; Fazeli et al 1988; Frey et al 1991a; Frey et al 1988; Krug et al 1984; Otani & Abraham 1989; Otani et al 1989; Steward & Schuman 2001) and potentially also mRNA transcription in the soma (Barco et al 2002; Frey et al 1996).

All afferent information is automatically stored at glutamatergic synapses in the hippocampus by early-LTP (STP, LTP1), amongst other molecular mechanisms. If this information is deemed relevant for the organism, it is stored more permanently by reinforcing early LTP into late LTP (LTP2-3). This process has been shown to require the time-dependent, synergistic activation of glutamatergic and modulatory inputs, which include dopaminergic projections from the ventral tegmental area (Hassan et al 2006; Korz & Frey 2007; Navakkode et al 2007; O'Carroll & Morris 2004; Swanson-Park et al 1999; Uzakov et al 2005). In vivo and in vitro studies suggest that dopaminergic modulation is crucial for late-phase CA1 LTP (LTP2-3; Frey et al 1989; Frey et al 1991b; Frey et al 1990; Gurden et al 2000; Huang & Kandel 1995; Morice et al 2007; Navakkode et al 2007; Swanson-Park et al 1999) and the maintenance of early LTP in the CA1 region of the hippocampus (STP and LTP1; Kusuki et al 1997; Li et al 2003; Otmakhova & Lisman 1996).

In summary, the above findings support a modulatory role for DA in synaptic plasticity and cognitive flexibility in the hippocampus. Notably, pharmacological and lesion studies suggest that the modulatory role of DA on hippocampal LTP is functionally relevant (Gasbarri et al 1996; Korz & Frey 2007; O'Carroll et al 2006). This dopaminergic modulation of LTP is a possible molecular mechanism by which DA might influence cognition, attention and emotion (see below).

1.3.4 Dopamine in cognition

Research into the role of catecholamines in primate cognition began after a landmark finding by Brozoski *et al* (1979) in non-human primates. This study showed impaired working memory after catecholamine depletion, which is a well-replicated observation (Collins et al 2000; Crofts et al 2001; Dias et al 1996a; b; Roberts et al 1994). However, enhanced DA signalling down-stream of pharmacological or environmental manipulations as well as genetic variability has been shown to be both beneficial (e.g. Apud et al 2007; Cools et al 2001; de Frias et al 2004; Egan et al 2001; Knecht et al 2004; Schott et al 2006) and detrimental (e.g. Arnsten & Goldman-Rakic 1998; Cools et al 2001; Sawaguchi & Goldman-Rakic 1991) to primate episodic memory, working memory, and attention.

Taken together, it appears that intermediate levels of DA may be optimal for working memory performance – a hypothesis mainly supported by data on PFC- rather than hippocampus-mediated cognitive function. The leading hypothesis states that DA influences PFC-mediated functions, such as working memory performance and attentional control, in accordance with an inverted U-shaped dose-response curve (see Figure 5; Goldman-Rakic et al 2000). A suggested molecular mechanism for this inverted U-shaped curve is the modulation of PFC pyramidal cell memory field tuning by D_1 receptor occupancy (Goldman-Rakic 1995; Williams & Goldman-Rakic 1995).

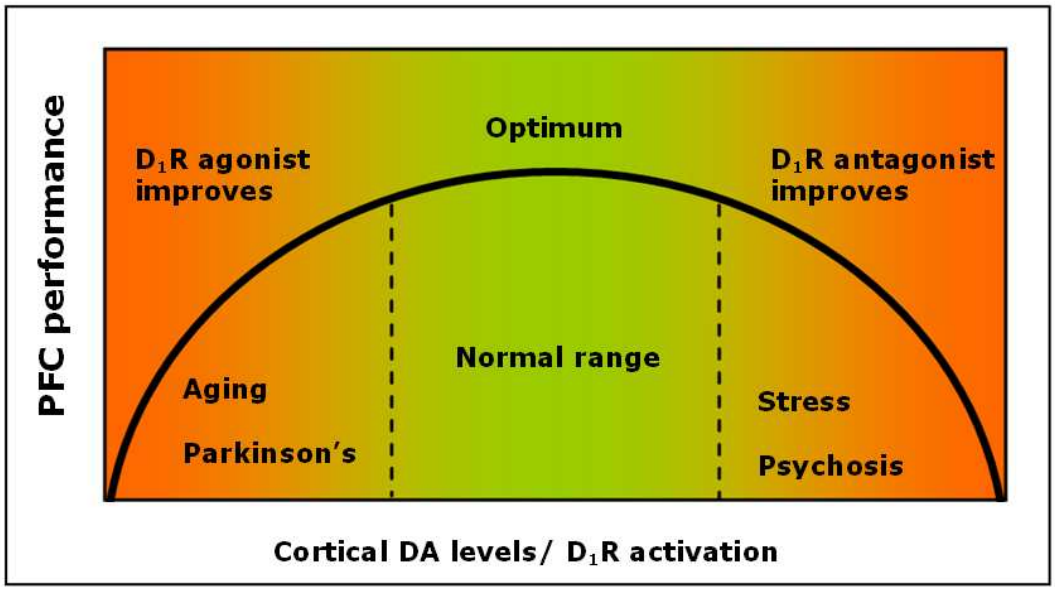


Figure 5 The inverted U-shaped relationship between DA and PFC function
PFC function is optimal at intermediate levels of dopamine (green area). PFC function is impaired in states of hyperdopaminergia, as in stressed animals or during amphetamine-induced psychosis, as well as in hypodopaminergia, as in aged animals and in patients with Parkinson's disease (adapted from Goldman-Rakic et al 2000).

Even though an inverted U-shaped curve is a good model for the relationship between cortical DA levels and cognitive function (Callicott et al 1999), the relationship between hippocampal DA and cognitive function is unknown. However, there are some encouraging findings from functional magnetic resonance imaging (fMRI) studies in humans, which suggest that the activation of dopaminergic midbrain regions may enhance hippocampus-dependent memory formation (e.g. Schott et al 2006; Wittmann et al 2005).

Similarly in rodents, DA receptor agonists improve hippocampus-dependent spatial memory performance, whereas decreasing DA receptor activation leads to hippocampus-dependent memory impairment (Bach et al 1999; Bernabeu et al 1997; Gasbarri et al 1996; Goto & Grace 2008; O'Carroll et al 2006; Packard & White 1991). Hence, DA receptor activation is thought to be necessary during memory encoding for the formation of long-term memory traces in the hippocampus (Packard & White 1989). However, the beneficial effect of DA for working memory performance is not clear-cut, as it may depend on the DA

receptor subtype, the amount of DA stimulation, the type of memory test and the brain region in question (Granon et al 2000; Murphy et al 1996; Sahakian et al 1985; Zahrt et al 1997).

1.3.5 Dopamine in emotion and reward

In addition to the extensive data on the role of DA in cognition, DA is also thought to regulate certain aspects of "emotion" (Berridge & Robinson 2003; Cenci et al 1992; Schultz 2002). Notably, emotion is used here as a non-specific term for emotion-related phenotypes since the traditional view of DA as a mediator of subjective affect and sensory pleasure may no longer hold true. Instead, many researchers now believe that the mesolimbic DA system may be more involved in encoding the motivational, rather than affective, aspects of reward (Berridge & Robinson 2003). Though most of the evidence for this comes from extra-hippocampal regions, there is also a limited amount of evidence that hippocampal DA may contribute to emotion-related phenotypes (the involvement of the hippocampus in emotion is discussed in Chapter 5).

Notably, Lisman and Grace (2005) have proposed a model, which involves hippocampal DA release in encoding novelty, salience and goals (Figure 6). The role of the suggested hippocampal-ventral tegmental loop (HC-VTA loop) may be to use converging network-wide evidence to decide whether incoming information is motivationally relevant. If so, novelty can induce hippocampal DA release (Ihalainen et al 1999; Li et al 2003), which could enable hippocampal late-LTP for long-term storage of the motivationally relevant information.

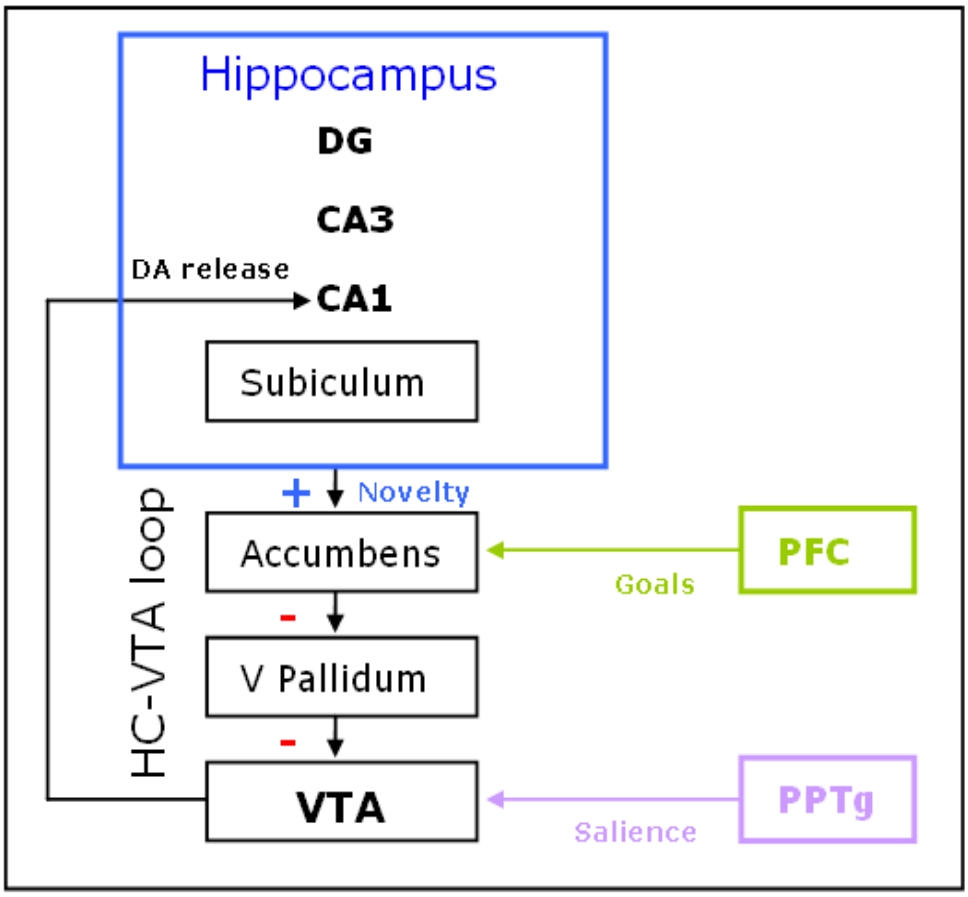


Figure 6 The hippocampal-VTA loop

The suggested 'HC-VTA loop' is activated when the hippocampus detects new information that has not been stored in long-term memory. This hippocampal 'novelty signal' is carried to the ventral tegmental area (VTA) via a polysynaptic pathway, involving the subiculum, nucleus accumbens and ventral pallidum (V Pallidum). As a result, DA cells in the VTA exhibit short-latency novelty-dependent firing (e.g. Horvitz et al 1997). DG, dentate gyrus; CA3 and CA1; cornu ammonis region 1 and 3 of the hippocampus; PFC, prefrontal cortex; PPTg, pedunculopontine tegmental nucleus (adapted from Lisman & Grace 2005).

1.3.6 Dopamine in the pathogenesis of schizophrenia

DA is involved in the pathogenesis of multiple CNS disorders, such as Parkinson's disease, Alzheimer's disease and schizophrenia. Schizophrenia pathogenesis will be used as an example for the role of DA in pathogenesis, since Chapter 2 of this thesis studies the role of COMT in schizophrenia:

Schizophrenia is a chronic brain disorder, which is diagnosed by persistent positive and negative symptoms that are not attributable to substance abuse or other medical conditions (DSM-IV 1994; ICD-10 2007). Positive symptoms include hallucinations, delusions, disorganized speech or thinking as well as grossly

disorganized or catatonic behaviour. Negative symptoms include avolition, affective flattening and alogia. Patients also consistently exhibit cognitive dysfunction, including deficits in attention, executive function and specific forms of memory (e.g. Blanchard & Neale 1994; Censits et al 1997). Furthermore, many schizophrenia patients also have mood symptoms, such as depression or anxiety, and exhibit worse interpersonal skills, self-care, academic and/or occupational achievements than the general population (DSM-IV 1994).

A leading hypothesis for the development of schizophrenia is the "dopamine hypothesis", which proposes that hyperactivity of subcortical DA neurons contribute to the (positive) symptoms in schizophrenia - potentially via inappropriate attribution of salience to unimportant stimuli (Howes & Kapur 2009). The DA hypothesis was originally developed based on the observations that antipsychotic drugs increase dopamine metabolism in mice (Carlsson & Lindqvist 1963), and that dopamine agonists and antagonists affect positive symptoms in schizophrenia (Angrist & Gershon 1970; van Rossum 1966). Also, the clinical efficacy and side-effect profiles of antipsychotics are related to their affinity for the D_2 receptor (Creese et al 1976; Kapur et al 2000; Meltzer 1976; Seeman et al 1976). The first direct evidence for dysregulation of dopamine signalling in schizophrenia came from single photon emission computerized tomography (SPECT) studies, which found that amphetamine-induced striatal dopamine release was associated with emergence or worsening of positive symptoms in drug-free patients with schizophrenia (Abi-Dargham et al 1998; Breier et al 1997; Laruelle et al 1996). However, antipsychotics only treat positive symptoms, and are inefficacious or not tolerated in 20 – 30 % of schizophrenia patients (Angrist et al 1980; Geddes et al 2000; Jones & Pilowsky 2002), which argues somewhat against the dopamine hypothesis.

The traditional dopamine hypothesis has been reformulated to state that positive symptoms are associated with subcortical hyperdopaminergia, whereas negative and cognitive symptoms are associated with cortical DA hypofunction

(Davis et al 1991; Guillin et al 2007; Weinberger 1987). Thus, it has been suggested that schizophrenia involves an imbalance between cortical and subcortical activity (see Figure 7). There is limited evidence for cortical hypodopaminergia *per se* (Keshavan et al 2008; Wong & Van Tol 2003), though rodent lesion studies (Pycock et al 1980a; Pycock et al 1980b), and cortical hypoactivity in schizophrenia (Callicott et al 2003; Callicott & Weinberger 1999; Ingvar & Franzen 1974; Taylor 1996) provide some evidence for the theory.

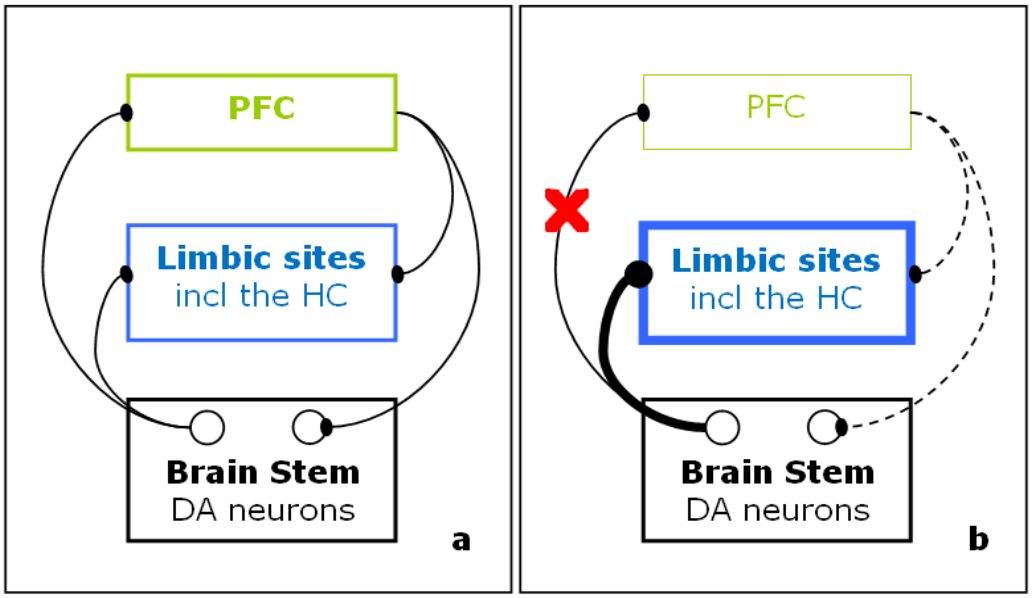


Figure 7 Schematic illustration of the interactions between the mesolimbic and mesocortical dopamine (DA) systems

during (a) normal brain function and (b) after selective lesioning of the DA input to the prefrontal cortex (Pycock et al 1980b). The effects of these lesions may model the mesolimbic hyperdopaminergia and mesocortical hypodopaminergia potentially underlying schizophrenia pathogenesis (Figure adapted from Weinberger 1987)

In addition to the imbalance between cortical and subcortical activity, the prolonged cortical hypoactivity in schizophrenia is hypothesized to reduce sustained, tonic DA release in subcortical regions. This in turn is proposed to cause a compensatory increase in DA responsivity, causing transient or phasic DA release in subcortical regions to elicit abnormally large responses. In other words, there may also be an imbalance between tonic and phasic DA transmission in schizophrenia (Goto et al 2007; Grace 1991; Moore et al 1999). Importantly, this tonic-phasic imbalance could have functional consequences in schizophrenia, since

phasic DA is related to expectation of reward (Schultz 1998), whereas stress may induce high tonic DA in PFC (Deutch et al 1990).

Moreover, according to two studies this tonic-phasic imbalance may be associated with COMT activity. Nolan et al (2004) and Bilder et al (2004) proposed that the COMT Met^{158} allele (see subchapter 2 below) results in increased tonic DA and decreased phasic DA subcortically, as well as increased DA transmission cortically. This in turn is hypothesized to promote cognitive stability, but limit cognitive flexibility of neuronal networks, which underlie working memory and executive function.

In summary, there is evidence for a crucial role of central DA signalling in schizophrenia. However, there is no evidence that changes in DA signalling are the primary cause of schizophrenia symptoms (e.g. a hyperdopaminergic state is not present during periods of remission; Laruelle et al 1999). Instead, DA probably acts in combination with other neurotransmitters to induce schizophrenia pathogenesis. Notably, glutamate, NA, GABA, serotonin and acetylcholine have also been implicated in schizophrenia pathogenesis (e.g. Coyle 2006; Dean et al 2003; Glenthoj & Hemmingsen 1999; Harrison 1999a; Keshavan et al 2008; Lisman et al 2008; Stone & Pilowsky 2007).

1.4 Noradrenaline

1.4.1 Adrenergic receptors

There are two main α-adrenoceptor subclasses (α1 and α2 including α2A, α2B, α2C; Docherty 1998) and three β-subtypes (β_1, β_2 and β_3). The different forms of adrenoceptor GPCRs are distinguished by their distinct pharmacological, biochemical and anatomical properties. α_1-adrenoceptors are positively coupled to phospholipase C and increase both intracellular inositol triphosphate and diacyl glycerol levels. The resulting release of intracellular Ca^{2+} is important for synaptic potentiation in the CNS (Izumi & Zorumski 1999). In contrast, α_2-adrenoceptors and β-adrenergic receptors are coupled to the adenylate cyclase pathway (see Figure 3) and have opposite effects on Ca^{2+}-gated K^+ channels responsible for the slow after-hyperpolarisation, which follows action potentials.

1.4.2 Noradrenergic projections in the CNS

In contrast to the morphologically "local" DA systems (see Section 1.3), noradrenergic cell bodies in the locus coeruleus and throughout the lateral tegmental fields send remarkably diffuse and topologically disorganised projections to most regions of the brain (Moore & Bloom 1979; Nakamura & Iwama 1975). The hippocampus receives ipsilateral NA projections from the locus coeruleus to the DG polymorphic layer, CA3 stratum radiatum and CA3 stratum lucidum, in particular (Loughlin et al 1986a; Loughlin et al 1986b; Loy et al 1980; Moore & Bloom 1979; Pickel et al 1974; Storm-Mathisen 1977; Swanson & Hartman 1975). The endogenous NA content is greater in the temporal than the septal end of the hippocampus, so the ventral hippocampus is thought to receive a greater NA (and DA) input than dorsal hippocampus (Hortnagl et al 1991; Oleskevich et al 1989). Notably, NA innervation of the hippocampus is much more prominent than DA innervation of the hippocampus (see Figure 8).

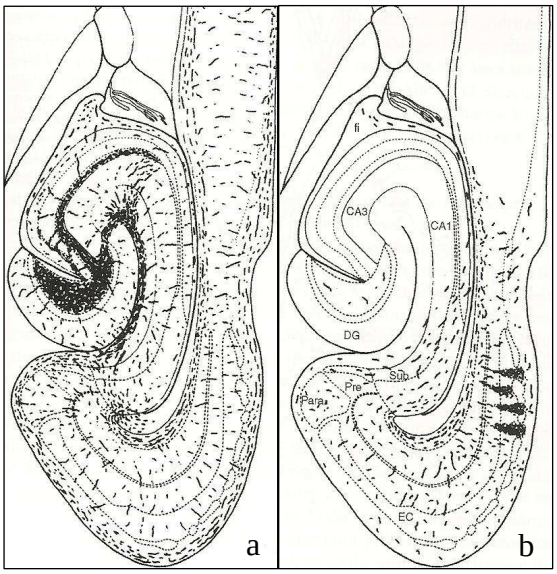


Figure 8 Monoaminergic projections to the hippocampus are sparse

Line drawings with horizontal sections through the rat hippocampus, subicular complex and entorhinal cortex show the distribution of (a) noradrenergic and (b) dopaminergic fibres. Figure adapted from (Andersen P 2006), courtesy of (Swanson et al 1987).

1.4.3 Neuromodulatory role of noradrenaline at hippocampal synapses

Given that NA affects slow after-hyperpolarisation following action potentials, it can alter synaptic transmission by reducing the spike frequency adaptation associated with depolarizing stimuli – resulting in an increase in action potential frequency (Haas & Konnerth 1983; Madison & Nicoll 1982; Pedarzani & Storm 1996). In addition to its effects on acute synaptic transmission, NA can also modulate hippocampal transmission long-term via similar molecular mechanisms to DA (see Section 1.3; also see Stanton & Sarvey 1985a; c).

NA has been shown to modulate synaptic plasticity in the DG: Depletion of the NA input leads to a reduction in the magnitude of LTP in the DG (Bliss et al 1983; Munro et al 2001; Stanton & Sarvey 1985b). Reinforcement of LTP into late-LTP also requires NA in the DG (Almaguer-Melian et al 2005a; Almaguer-Melian et al 2005b; Hopkins & Johnston 1988; Izumi & Zorumski 1999; Korz & Frey 2004; 2005; 2007; Straube et al 2003a; Straube et al 2003b; Walling & Harley 2004).

Importantly, NA-dependent synaptic plasticity may be a molecular mechanism for the involvement of NA in cognition and emotion (Fillenz 1972). Long-term LTP in the hippocampus has been shown to be induced by appetitive and

aversive stimuli, as well as by novelty (Kitchigina et al 1997; Seidenbecher et al 1997).

1.4.4 Role of noradrenaline in cognition and emotion

With respect to arousal, Arnsten has proposed a tonic-phasic NA hypothesis, whereby phasic firing of NA neurons reflects levels of interest in a given stimulus (Rajkowski et al 2004). In contrast, tonic NA levels may mirror levels of wakefulness, alertness, and stress (Foote et al 1980).

Additionally, Amy Arnsten has proposed that NA exhibits an inverted U-shaped dose-response curve, whereby either too little or too much NA impairs working memory performance (Arnsten & Li 2005). The mechanisms for this appears to be that moderate levels of cortical NA release activate α_{2A}-adrenoceptors, whereas high NA levels (e.g. during stressful situations) engage α_1-adrenoceptors and low NA levels activate β_1-adrenoceptors (Arnsten 2000). Hence, moderate levels result in closure of hyperpolarisation-activated cyclic nucleotide-gated cation channels and increased neuronal firing via inhibition of cAMP signalling, which appears to improve PFC-dependent functions (Arnsten 2007). In contrast, suboptimal NA levels increase cAMP or phosphotidyl inositol signalling - leading to impairment in PFC-dependent working memory. Evidence for this hypothesis comes from experiments involving both primates (Arnsten 2004; Arnsten & Goldman-Rakic 1990; Arnsten & Li 2005; Roberts et al 1994) and rodents (Botwinick et al 1977; Freedman et al 1979; Harley 2004; Quartermain et al 1977; Randt et al 1971). This hypothesis may however be somewhat simplistic, because NA appears to affect different aspects of cognition in different brain regions in an adrenoceptor subtype-specific manner (Gibbs et al 2010; Gibbs & Summers 2005; also compare Hopkins & Johnston 1988; Izumi & Zorumski 1999).

1.5 Relationship between hippocampal dopamine and noradrenaline signalling

There is both structural and functional evidence for dopamine and noradrenaline systems in the hippocampus, as discussed above. Given that both DA and NA exhibit an inverted U-shaped relationship between monoamine content and cognitive performance, it appears that optimal levels of both DA and NA are required for proper brain functioning (Arnsten 2009). Interestingly, the beneficial effects of optimal NA transmission on cognitive performance may be due to NA-dependent enhancement of cortical signals. In contrast, optimal DA transmission may improve PFC functions by attenuating noise via a reduction in non-specific firing in non-preferred neuronal inputs (Arnsten 2007). Thus, NA and DA may act synergistically or reciprocally to improve cognition by affecting the signal/noise ratio of neuronal information transfer. A similar relationship is probably true for arousal, since sub-optimal levels of monoaminergic transmission associated with to too little (during fatigue or boredom) or too much (during stress) arousal impair regulation of behaviour an thought (Arnsten 2007).

Moreover, there is prominent crosstalk between the DA and NA signalling pathways (Goldman-Rakic et al 1990; Guiard et al 2008b). For example, DAT and NET are both involved in DA uptake in the hippocampus (Guiard et al 2008a). This implies that DA and NA systems in the hippocampus are probably interlinked and dependent on each other. Given that DA is an intracellular precursor to NA (see Figure 1 and Figure), it is even possible that DA in the hippocampus exists only within noradrenergic neurons. Hence, many effects formerly attributed to hippocampal DA may actually involve both NA and DA, or only noradrenergic transmission in the hippocampus.

The potential synergies between monoaminergic systems of the hippocampus have important implications for interpreting the results of this thesis. Without more detailed information about the hippocampal DA and NA systems, it is

not possible to attribute behavioural and neurochemical effects of COMT inhibition to either DA or NA neurotransmission alone (see further discussion in Chapter 7).

2 Catechol-*O*-methyltransferase (COMT)

COMT is an enzyme which was first identified and characterized by Axelrod and Tomchick in 1958. COMT breaks down catechol-containing compounds by catalysing the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to one of the hydroxyl groups on the catechol-ring of its substrates (Figure 9; Axelrod & Laroche 1959; Axelrod et al 1958; Axelrod & Tomchick 1958; Guldberg & Marsden 1975; Mannisto & Kaakkola 1999; Mannisto et al 1992b). Endogenous substrates include the catecholamine neurotransmitters (DA and NA) and their catechol-containing metabolites, which are the focus of this thesis, as well as L-DOPA and hydroxylated catechol oestrogens. Exogenous xenobiotic catechols, dietary polyphenols, vitamin C and various drugs are also substrates of COMT (e.g. Blaschke & Hertting 1971; Bowers-Komro et al 1982).

2.1 COMT gene structure

The human COMT gene is localised to chromosome 22, band q11.2 (Grossman et al 1992). It consists of six exons, the first two of which are non-coding. The COMT gene encodes two distinct COMT isoforms from two alternative promoters (Lundstrom et al 1991; Tenhunen 1996; Tenhunen et al 1993; Tenhunen et al 1994; Winqvist et al 1992). The P1 promoter guides transcription of the shorter (1.3 kb) mRNA transcript, which encodes soluble COMT (S-COMT). The longer (1.5 kb) mRNA sequence is expressed by the P2 promoter and can encode both membrane-bound COMT (MB-COMT) and S-COMT from alternative start codons. In addition to the two COMT isoforms, the human brain also expresses different COMT splice variants (Tunbridge et al 2007a).

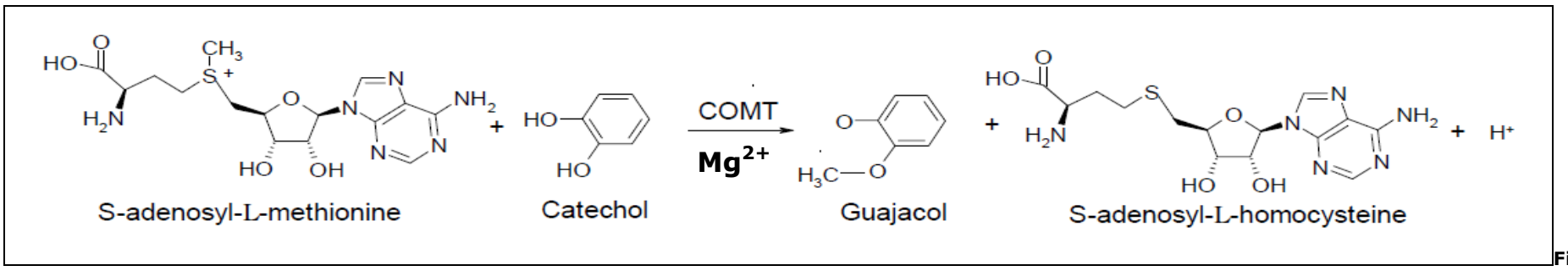


Figure 9 Methyl transfer from SAM to catechol in the COMT-catalysed reaction
The *O*-methylation occurs via a sequentially ordered reaction mechanism, involving a S_N2-like transition state (Jeffery & Roth 1985; Rivett & Roth 1982). The binding and dissociation order is as follows: SAM, Mg^{2+}, substrate, methylated product, Mg^{2+}, demethylated SAM (Lotta et al 1995). Mg^{2+} is necessary for the catalytic activity of COMT, though Cd^{2+}, Hg^{2+}, Mn^{2+} and Cu^{2+} can also promote methylation. In contrast, Ca^{2+} ions, mercaptoethanol, dithiothreitol, various reducing agents as well as products of the COMT-catalysed reaction such as S-adenosyl-L-homocysteine (SAH) can inhibit the reaction (e.g. Jeffery & Roth 1984; Kern & Bernards 1997).

COMT predominantly *O*-methylates the hydroxyl group at the meta (3-) position of the substrate catechol ring. The affinity of exogenous substrates for COMT can be increased by apolar, electron-withdrawing and planar substituents in the catechol ring side chains, presumably to allow more favourable interactions with the hydrophobic residues surrounding the active site (Lautala et al 2001; Sipila & Taskinen 2004; Vidgren et al 1994).

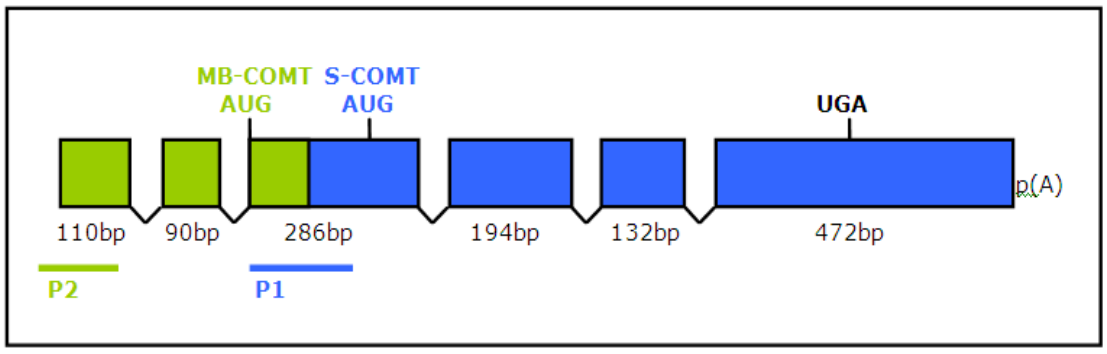


Figure 10 Structure of the COMT gene
Exons are shown as boxes and introns as connecting lines (adapted from Tenhunen et al 1994). The promoters P1 and P2, the AUG translation start sites for MB-COMT and S-COMT, as well as the UGA stop codon are also labelled. The blue portion indicates the exon sequence of the 1.3 kb COMT mRNA transcript, encoding S-COMT. Note that this portion lacks the MB-COMT AUG codon and can hence only encode S-COMT. The green portion indicates the exon sequence unique to the long 1.5 kB COMT mRNA transcript, which encodes MB-COMT (and S-COMT).

2.2 Regulation of COMT gene expression

The promoter regions of COMT have binding sites and hormone-response elements for oestrogens and glucocorticoids, which can regulate COMT expression (Jiang et al 2003; Salama et al 2006; Wentz et al 2006; Xie et al 1999). These regulatory sites may contribute to sexually dimorphic (discussed in Chapter 3), as well as stress- and age-related (e.g. Guldberg & Marsden 1975; Torda & Kvetnansky 1983; Tunbridge et al 2007b) effects of COMT.

COMT gene expression is also under epigenetic regulation (Isles & Wilkinson 2008) via methylation of phoshodiester-linked cytosine and guanine (CpG) islands in the COMT promoters (Iguchi-Ariga & Schaffner 1989; Mill et al 2006), which may be associated with pathogenesis of schizophrenia, bipolar disorder, heavy alcohol use and carcinogenesis (e.g. Abdolmaleky et al 2006; Sasaki et al 2003; Weisz et al 1998).

2.3 Genetic regulation of COMT activity

Low, intermediate and high COMT activities have been shown in populations (Chen et al 2004a; Floderus et al 1981). This trimodal distribution is due to a functional single nucleotide polymorphism (SNP) in the coding sequence of COMT, namely the Val^{158}Met SNP (rs4680; Lachman et al 1996). This G472A SNP results in a valine (Val) for methionine (Met) substitution at codon 158 in MB-COMT (108 in S-COMT). Val^{158}/Val^{158} homozygotes have 3 – 4 fold greater peripheral COMT activity and about 40 % higher central COMT activity compared to Met^{158}/Met^{158} homozygotes (Chen et al 2004a; Lotta et al 1995; Scanlon et al 1979; Weinshilboum & Raymond 1977). Since the Val^{158} and Met^{158} alleles are co-dominant, Val^{158}/Met^{158} heterozygotes have intermediate levels of COMT activity. The mechanism for Val^{158}Met-mediated COMT activity changes is likely due to the increased thermolability of the Met^{158} form, compared with the Val^{158} form of the COMT protein. This thermolability leads to increased degradation of the unfolded

protein (Boudikova et al 1990; Lotta et al 1995; Nackley et al 2006; Scanlon et al 1979; Weinshilboum & Dunnette 1981).

Even though $Val^{158}Met$ is a major genetic determinant of COMT enzyme activity, there are currently 23 other known SNPs within the human COMT coding sequence and at least 107 further SNPs in the non-coding regions (National Center for Biotechnology Information, 2010). 11 of these coding SNPs alter the gene DNA sequence but leave the protein sequence unchanged. In contrast, 12 of these coding SNPs are non-synonymous, i.e. they alter the amino acid sequence and may hence directly influence protein function. However, these COMT SNPs mostly contribute effects of uncertain size and directionality (Chen et al 2004a). Nevertheless, it is crucial to note that Diatchenko et al (2005) have found three major genetic variants (haplotypes) of the COMT gene, divergent in two synonymous and one non-synonymous SNP ($Val^{158}Met$), which determine COMT activity in humans by affecting the stability of COMT mRNA and hence, the translation of COMT protein (Nackley et al 2006).

2.4 COMT protein structure

Soluble COMT contains 221 amino acids, and its molecular mass is about 24 kDa in rat and 26 kDa in human. MB-COMT contains an additional membrane anchor at its N-terminus, consisting of 43 (rat) or 50 (human) hydrophobic amino acids, making its molecular mass 28-32kDa (Bertocci et al 1991; Lundstrom et al 1991; Salminen et al 1990; Tenhunen et al 1994; Ulmanen & Lundstrom 1991; Winqvist et al 1992).

Recombinant COMT proteins were used to obtain the three-dimensional crystal structure of rat S-COMT (Vidgren et al 1994). The COMT enzyme has a single domain structure consisting of a central β-sheet, which is surrounded by eight α-helices. The active site consists of the catechol-binding site and the S-adenosyl-L-methionine (SAM)-binding site. SAM is bound deeper than Mg2+ and the catechol in the hydrophobic binding groove. The residues crucial for the binding

of substrates to the active site are conserved across rat and human cDNA (Lotta et al 1995; Mannisto & Kaakkola 1999; Tenhunen et al 1994; Vidgren 1998; Vidgren et al 1994; Vidgren et al 1991), which suggests rodents and humans have highly similar substrate spectra and O-methylation reactions.

2.5 COMT expression

COMT mRNA is found in both invertebrates and vertebrates (Guldberg & Marsden 1975). The P2 promoter seems to function constitutively and hence, the longer transcript is expressed ubiquitously, whereas the regulation of P1 differs between tissues (Tenhunen 1996). Notably, the human brain only expresses the longer transcript (Hong et al 1998; Matsumoto et al 2003a; Tenhunen et al 1994). Nevertheless, S-COMT comprises around 30 % of brain COMT, indicating that the longer transcript can encode both isoforms, using the leaky scanning mechanism of translation initiation (Tenhunen & Ulmanen 1993). Hence, the expression of the two COMT mRNA transcripts does not entirely correlate with protein levels of MB-COMT and S-COMT.

In the periphery, COMT has an important role in detoxification. This may explain why COMT protein expression and activity is highest in organs related to first-pass metabolism of endogenous catechols, e.g. the liver, kidney and gastrointestinal tract. COMT is also found abundantly in tissues through which exogenous catechols may enter the body, e.g. the skin and lungs (Guldberg & Marsden 1975; Kaplan et al 1980; Karhunen et al 1994; Mannisto 1998; Myohanen et al 2010).

In the CNS, COMT activity (Guldberg and Marsden 1975; Roth 1992), as well as COMT mRNA (Tenhunen and Ulmanen, 1993; Hong *et al.,* 1998; Matsumoto 2004) and protein (Tenhunen and Ulmanen, 1993; Tenhunen *et al.,* 1994) expression vary between different brain regions (Guldberg & Marsden 1975; Hong et al 1998; Kaplan et al 1979; Karhunen et al 1994; Matsumoto et al 2003a; Myohanen et al 2010; Saavedra et al 1976). COMT mRNA is highly expressed in the

prefrontal cortex, hippocampus, spinal cord, cerebellum, medulla, striatum and choroid plexus. Additionally, COMT is expressed at the blood brain barrier, presumably to prevent toxic substances entering the brain (Baranczyk-Kuzma et al 1986; Hardebo et al 1980; Hardebo & Owman 1980; Lai & Spector 1978; Spatz et al 1986). In contrast, amygdala, striatum and thalamus show lower COMT expression.

2.5.1 Cellular localisation of COMT in the CNS

The cellular localisation of COMT in the CNS has not been fully resolved. This is partly due to the lack of isoform-specific COMT antibodies. Despite considerable controversy in this field, immunohistochemistry, Western blotting, lesion studies and in vitro studies suggest COMT is expressed in microglia, astroglia, as well as some neurons (e.g. pyramidal neurons, striatal spiny neurons, cerebellar purkinje and granular cells; Bertocci et al 1991; Broch & Fonnum 1972; Grossman et al 1985; Hansson 1984; Kaakkola et al 1987; Kaplan et al 1979; Karhunen et al 1995; Kastner et al 1994; Matsumoto et al 2003a; Myohanen et al 2010; Rivett et al 1983; Silberstein et al 1972; Tilgmann et al 1992; Ulmanen et al 1997).

S-COMT comprises around 30 % of mouse brain COMT; with highest MB-COMT/S-COMT ratios found in the hippocampus, cerebellum and thalamus (Myohanen et al 2010). There has also been some suggestion of cellular differences between S-COMT and MB-COMT (Kaakkola et al 1987; Rivett et al 1983). This could potentially be related to the fact that MB-COMT is functionally more important than S-COMT in neurons, as it is able to methylate catecholamine neurotransmitters at physiological concentrations due to its higher substrate affinity (Km; Bonifacio et al 2000; Lotta et al 1995; Malherbe et al 1992; Rivett et al 1983; Rivett & Roth 1982; Sipila & Taskinen 2004).

2.5.2 Sub-cellular localisation of COMT

Similar to the cellular localisation of COMT, the sub-cellular localisation of COMT also remains controversial.

MB-COMT was initially thought to be present on the plasma membrane, and therefore possibly protruding into the synapse (Aprille & Malamud 1975; Karhunen et al 1995). However, COMT is unlikely to be functional in the extracellular space as high extracellular Ca^{2+} levels would inhibit MB-COMT enzyme activity (Jeffery & Roth 1984).

An alternative view is that MB-COMT resides on intracellular membranes, particularly the rough endoplasmic reticulum (RER), mitochondria and nucleus, protruding into the cytosol. In contrast, S-COMT is localised in the cytosol and to a lesser extent, in the nucleus (Bertocci et al 1991; Broch & Fonnum 1972; Grossman et al 1985; Karhunen et al 1995; Myohanen et al 2010; Raxworthy et al 1982; Tilgmann et al 1992; Ulmanen & Lundstrom 1991; Ulmanen et al 1997).

Interestingly, it seems that the cellular and subcellular distribution of COMT can be dynamically modified in certain pathophysiological situations (e.g. Reenila et al 1997). For example, it is thought that exposure to catecholestrogens may cause up-regulated S-COMT to be transported into the nucleus in order to protect the DNA from hydroxylated oestrogen-induced oxidative damage (Hirose et al 2007; Weisz et al 1998; Weisz et al 2000).

The cellular and sub-cellular localisation of COMT has important implications for the interpretation of the results of this thesis. For example, if COMT is localised on intracellular membranes in the post-synaptic cell, it is challenging to explain how COMT senses extracellular catecholamine concentrations in order to effectively modulate catecholamine signalling via catecholamine metabolism (see Chapter 7 for further discussion).

2.6 Psychopathophysiology and COMT

Given its role in the inactivation of catecholamine neurotransmitters (Figure), COMT has been extensively studied with respect to psychiatric disorders. There are reports of genetic association of the COMT Val^{158}Met polymorphism with most psychiatric disorders, for example early onset major depressive disorder, anorexia nervosa, substance abuse, alcoholism, attention-deficit hyperactivity disorder, pain-related syndromes and anxiety-related disorders (Craddock et al 2006; Hosak 2007). The best-studied associations of COMT are those with schizophrenia (see Section 2.6.1), bipolar disorder (Farmer et al 2007) and obsessive compulsive disorder (Azzam & Mathews 2003; Pooley et al 2007). However, the evidence for genetic association of COMT with these psychiatric disorders is highly controversial – as exemplified by the studies on the association between COMT and schizophrenia, discussed below.

2.6.1 COMT may be associated with schizophrenia

Family, twin and adoption studies estimate the heritability of schizophrenia at around 80 % (Gottesman & Gould 2003; Gottesman 1991; McGuffin et al 1995; Sullivan et al 2003). Despite the high heritability of schizophrenia, genetic linkage and association studies have not managed to unequivocally associate one gene or one genetic locus with schizophrenia - though meta-analyses have identified some susceptibility loci (Badner & Gershon 2002; Lewis et al 2003; Ng et al 2009) and candidate susceptibility genes (including e.g. COMT, D-amino oxidase [DAO], dysbindin, G72, neuregulin 1 [NRG1], proline dehydrogenase [PRODH], regulator of G-protein signalling 4 [RGS4] and zinc finger binding protein 804A [ZNF804A]; Harrison & Owen 2003; Harrison & Weinberger 2005; O'Donovan et al 2009).

COMT is a particularly attractive candidate schizophrenia susceptibility gene for several reasons. Firstly, COMT metabolises DA (Figure), a neurotransmitter that is involved in schizophrenia pathogenesis (see Chapter 1). Secondly, the COMT gene lies on chromosome 22q11 (Grossman et al 1992; Maynard et al 2002;

Winqvist et al 1992), which has been associated with schizophrenia using genetic linkage studies (Badner & Gershon 2002; Condra et al 2007; Hamilton et al 2002; Hamshere et al 2005; Karayiorgou & Gogos 2004; Lewis et al 2003; Takahashi et al 2003) and research on velo-cardio-facial syndrome (which is caused by hemizygous deletion of 22q11; Bassett et al 2008; Paylor et al 2001). Additionally, one meta-analysis of the genetic association literature on the COMT Val^{158}Met polymorphism suggests that the Val^{158} allele is associated with schizophrenia (Glatt et al 2003).

However, the results of genetic association studies on the COMT Val^{158}Met polymorphism are inconclusive. In contrast to the studies suggesting the Val^{158} allele is associated with schizophrenia, other studies associate the Met^{158} allele with schizophrenia and schizophrenia-like symptoms (e.g. Ohmori et al 1998; Sazci et al 2004). Additionally, a couple of studies suggest that Val^{158}Met heterozygosity may be protective (Liou et al 2001; Tsai et al 2004a). Slightly stronger evidence suggests that a haplotype including the Val^{158} allele may be associated with schizophrenia (Chen et al 2004b; Funke et al 2005; Handoko et al 2005; Shifman et al 2002). However, increasingly more studies find no association between the Val^{158}Met SNP or COMT haplotypes and schizophrenia (see meta-analysis by Munafo et al 2005).

In summary, it is currently unclear whether the Val^{158}Met SNP is associated with schizophrenia (Williams et al 2007). This may reflect that COMT only confers susceptibility to schizophrenia in combination with another genetic polymorphism (e.g. via epistatic interactions; Nicodemus et al 2007) or with environmental factors (Caspi & Moffitt 2006). Alternatively, the lack of robust association may reflect problems involved with the methodology or complexity of the disorder (Sagud et al 2010). However, most researchers now believe that the lack of positive association between the Val^{158}Met SNP is because COMT may not be directly associated with schizophrenia

2.7 COMT is associated with cognitive and emotional phenotypes

Since there are huge controversies within the genetic association literature with respect to psychopathogenesis, researchers have started investigating the associations between susceptibility genes and behavioural phenotypes. Within the COMT literature, this is achieved by studying the behavioural effects of either the human Val^{158}Met SNP or the brain-penetrant pharmacological COMT inhibitor, tolcapone. In rodents, behavioural phenotypes are investigated in either genetically manipulated COMT mice or rodents treated with the COMT inhibitor tolcapone.

2.7.1 COMT Val^{158}Met and cognition

The first human study to investigate the relationship between COMT and cognitive phenotypes was a paper by Egan *et al* (2001), which examined the effects of the Val^{158}Met SNP on prefrontal cortex-dependent cognition and cortical physiology in patients with schizophrenia, unaffected siblings, as well as healthy control subjects.

This seminal study found that the load of the COMT Met^{158} allele is associated with relatively better executive function during the Wisconsin Card Sorting Task (WCST) of prefrontal cortex-dependent executive function: Met^{158}/Met^{158} individuals performed better than Val^{158}/Val^{158} subjects and Val^{158}/Met^{158} showed intermediate levels of performance (see Figure 11). Using functional magnetic resonance imaging (fMRI), Egan *et al* (2001) also demonstrated that Val^{158} allele load predicted greater cortical activation for a given level of performance during the n-back task (a simple working memory task), which the paper interpreted as a relatively less efficient cortical response in Val^{158} allele carriers. Finally, Egan *et al* (2001) also included a family-based association analysis of 104 family trios, which suggested increased transmission of the COMT Val^{158} allele to offspring with schizophrenia.

In summary, the landmark paper by Egan *et al* (2001) concluded the high activity Val^{158} allele of COMT, which results in increased prefrontal DA metabolism, is associated with both poorer executive function and relatively inefficient PFC function (as well as a slightly increased risk for schizophrenia).

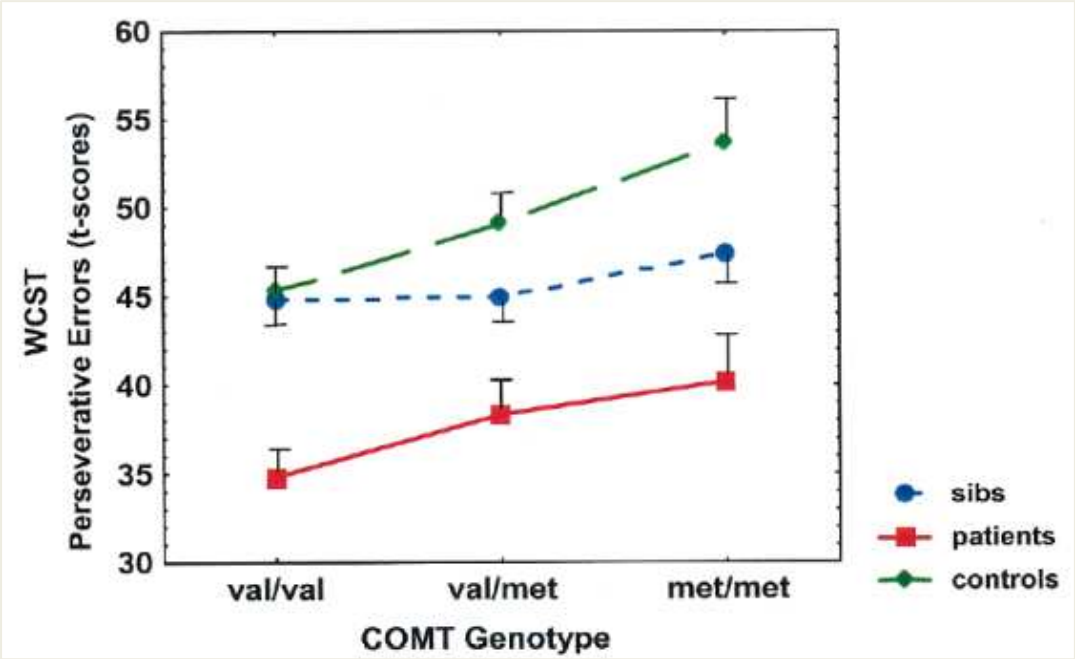


Figure 11 Association of COMT genotype with performance on the Wisconsin Card Sorting Task (WCST)
Higher perseverative error t-scores (±SE) indicate better performance (Egan et al 2001): Val/Val homozygotes perform worse than Met carriers in schizophrenics than in the siblings of schizophrenia patients or controls.

The detrimental effects of the Val^{158} allele on n-back (Bertolino et al 2004; Goldberg et al 2003), WCST performance (Barnett et al 2007; Joober et al 2002; Malhotra et al 2002; Rosa et al 2004) and brain activation patterns during cognitive tasks (Bertolino et al 2006; Blasi et al 2005; Gallinat et al 2003; Krach et al 2010; Tan et al 2007; Tsai et al 2003) have been replicated in several studies. Moreover, the Met^{158} allele may interact with antipsychotic treatment (Bertolino et al 2004; Weickert et al 2004) and other COMT SNPs (Liao et al 2009; Meyer-Lindenberg et al 2006) to improve n-back and WCST performance. In addition, it appears that lower COMT activity (associated with the Met^{158} COMT allele) is associated with improved attention (Bilder et al 2002; Blasi et al 2005), but perhaps impaired

cognitive flexibility (Nolan et al 2004). Meta-analyses of these COMT studies suggest that the two most robust cognitive effects of the Val^{158}Met SNP are on intelligence quotient (IQ; Barnett et al 2008) and prefrontal activation during cognitive processing (Mier et al 2010). However, it is crucial to note that not all cognitive tasks are sensitive to the Val^{158}Met SNP (Tunbridge et al 2006a). This means that meta-analyses including all studies in this field are unlikely to find statistically significant associations of COMT with working memory and executive function (Barnett et al 2008).

As concluded by Egan *et al* (2001), the changes in flexibility and efficiency of cortical signalling appear to occur via changes in DA signalling (Goldman-Rakic et al 2000; Mattay et al 2003; Meyer-Lindenberg et al 2005). The mechanism for this may occur via the effects of different DA receptor subtypes on cortical signal-to-noise ratio (Arnsten 2007; Tunbridge et al 2006a; Winterer et al 2004; Winterer & Goldman 2003), or in accordance with the tonic-phasic model of subcortical DA neurotransmission (Bilder et al 2004).

Given that this thesis investigates the role of COMT in hippocampal function, it is interesting to note that two fMRI studies have found a link between the Val^{158}Met SNP and hippocampal activation or connectivity during memory performance (Bertolino et al 2006; Schott et al 2006). Consistent with the data above, Bertolino et al (2006) found the Met^{158} allele to be associated with more accurate recognition memory performance. This study also found that healthy Val^{158}/Val^{158} subjects have reduced hippocampal activation and increased connection strength between the hippocampus and PFC during recognition memory retrieval. In contrast, Schott et al (2006) found that Met^{158}/Met^{158} subjects show stronger functional coupling between the hippocampus and the PFC during episodic memory encoding.

2.7.2 Tolcapone and cognition

There are three tolcapone studies in humans using the brain-penetrant COMT inhibitor tolcapone (see Chapter 3 for further details on tolcapone). All of these suggest that COMT inhibition improves cognitive performance. Gasparini et al (1997) found that chronic tolcapone treatment for six months improved the performance of eight patients with advanced Parkinson's disease on tests of attention, memory, and executive function. In a larger cohort of healthy subjects, Apud et al (2007) found that one week of tolcapone treatment enhanced executive function and N-back working memory performance. This study also found that for intradimensional shifting (and verbal memory), individuals with Val^{158}/Val^{158} genotype improved after tolcapone, whereas Met^{158}/Met^{158} subjects worsened. A similar result was obtained by Giakoumaki et al (2008), who found that two weekly tolcapone treatments improved performance on two working memory tasks (n-back and letter number sequencing) in 12 healthy individuals with Val^{158}/Val^{158} genotype, but not in 11 Met^{158}/Met^{158} subjects. These results are consistent with the results of the genetic association literature reviewed in subchapter 2.7.1 and appear to agree with the inverted U-shaped curve function between monoamine levels and working memory performance (Figure 5).

The human study by Apud et al (2007) also suggested that tolcapone treatment results in decreased dorsolateral PFC activation during the N-back task – interpreted as improved efficiency of information processing in PFC during working memory performance. This result is consistent with the effects of the COMT $Val^{158}Met$ SNP (Egan et al 2001; see subchapter 2.7.1) and amphetamine, an indirect monoaminergic agonist (Mattay et al 2003).

Pharmacological studies of tolcapone in rats agree well with the human studies. In rats, acute tolcapone improves cognitive performance on an extradimensional set shifting task (Tunbridge et al 2004b), delayed spatial win-shift test (Lapish et al 2009), single-trial passive avoidance paradigm (Khromova et al 1997), and possibly also on radial- and linear-arm mazes (Liljequist et al 1997). In

addition to these pharmacological studies in rats, studies using genetic COMT knockout mice indicate that homozygous male mice show a prominent improvement in spatial learning and working memory, whereas heterozygous mice show improved recognition memory (Babovic et al 2008). In contrast, mice with genetically increased COMT enzyme activity show disrupted working and recognition memory performance, as well as impaired extradimensional set shifting (Papaleo et al 2008).

In summary, the genetic studies on both human and rodent cognition (using human genetic association studies or knockout mice) map well onto pharmacological data across several species. Thus, the beneficial effects of low COMT activity on certain aspects of cognition appear robust. Moreover, these beneficial effects could plausibly be mediated in part by effects on hippocampal catecholamine metabolism. However, most of the improvements in cognitive function associated with low COMT activity are usually ascribed to the effects of DA in the PFC.

2.7.3 COMT Val^{158}Met and emotion

In addition to the robust findings with regards to cognitive phenotypes, COMT has also been associated with phenotypes that are related to emotion. Females homozygous for the Met^{158} allele show elevated neuroticism and increased tendency to become anxious, compared with Val^{158} carriers (Enoch et al 2003; Olsson et al 2005). Also, Met^{158} allele load predicts increased novelty seeking, extraversion and reward dependence both alone (Reuter & Hennig 2005; Stein et al 2005; Tsai et al 2004b), and together with SNPs on other genes (Benjamin et al 2000a; Benjamin et al 2000b). Moreover, the Met^{158} allele may be associated with suicide attempts and outward expression of anger (Kotler et al 1999; Lachman et al 1998; Nolan et al 2000; Rujescu et al 2003; Strous et al 2003; Volavka et al 2004). The Val^{158}Met SNP may also be associated with both the sensory and affective sensitivity to pain, as well as the severity of pain-dependent negative

internal affective states (Diatchenko et al 2005; Zubieta et al 2003). The Met^{158} allele may also be associated with potentially emotion-related disorders, such as depression, anorexia nervosa, alcoholism, substance abuse, OCD, AD/HD and pain-related disorders. Most notably, a meta-analysis by Mier *et al* (2010) found an association between COMT and prefrontal activation during emotional processing, which suggests that the links between COMT and some aspects of emotion are robust.

Given the topic of this thesis, it is crucial to note that three studies have specifically implicated the hippocampal formation in the links between COMT and emotional processing. Smolka *et al* (2005) suggested that increased hippocampal activation elicited by unpleasant stimuli in subjects with genetically lower COMT activity may contribute to their lower emotional resilience against negative mood states. A larger study by Drabant *et al* (2006) confirmed that Met^{158} allele load (associated with low COMT activity) was linked with dose-dependent increase in hippocampal activation during viewing of faces displaying negative emotion (fear or anger). Also, in Met^{158}/Met^{158} homozygotes, limbic and prefrontal regions showed increased functional coupling during the emotional processing task. Finally, Mechelli et al (2009) have shown in 50 children that Met^{158} allele load predicts greater activation of the parahippocampal gyrus during emotional processing and that Met^{158}/Met^{158} homozygotes have greater functional coupling between the medial anterior cingulated cortex and the parahippocampal gyrus than Val^{158}/Val^{158} homozygotes during emotional processing.

There are very few pharmacological studies investigating the emotion-related behavioural effects of decreased or blocked COMT activity. Gogos *et al* (1998) showed that homozygous COMT knockout female (but not male) mice demonstrate increased anxiety in a light/dark box. In contrast, tolcapone administration in rats and mice increases exploratory activity in the open field test (Maj et al 1990). In summary, the role of COMT in rodent anxiety is somewhat

unclear. Nonetheless, the human genetic data reviewed above suggest that there is converging evidence for an association between the COMT Met^{158} allele and impaired emotional processing.

2.7.4 COMT Val^{158}Met and evolution

As discussed above, the COMT Met^{158} allele is associated with improved working memory and overall executive function. However, the COMT Met^{158} allele may also be associated with impaired emotional processing and elevated risk of breast cancer (Ding et al 2010). This apparent trade-off between cognitive and emotional correlates of COMT activity has also been alluded to in the human pharmacological data and the rodent literature discussed above.

The observation that cognitive paradigms favour the Met^{158} allele carriers, whereas emotional paradigms favour the Val^{158} allele carriers could explain why there may be evolutionary pressure for the coexistence of both alleles in humans (the "warrior/worrier" hypothesis; Mier et al 2010). This is in contrast to other species, which do not have the Val^{158}Met SNP (Palmatier et al 1999). Instead, other species only have a form of COMT, which has activity similar to, or slightly higher than, the human Val^{158} form (Chen et al 2004a). This suggests that the high activity allele of COMT is the ancestral allele.

There is substantial variation in the frequency of the Val^{158}Met polymorphism between different ethnic populations (Ameyaw et al 2000; Klemetsdal et al 1994; McLeod et al 1994; McLeod et al 1998; Palmatier et al 1999; Rivera-Calimlim & Reilly 1984): In Caucasian subjects, the frequency of the low-activity Met allele is approximately 50 %, whereas the frequency is much lower in Asians (about 20-30 %) and Africans (e.g. 6 % in Ghana). This variation may have an evolutionary explanation. One hypothesis is that the frequency of the evolutionarily recent Met^{158} allele is very low in African populations, because these populations are evolutionarily very old according to the popular theory on human evolution, which suggests that *Homo sapiens* arose in Africa. Another hypothesis is

that Val^{158} and Met^{158} allele frequencies are evolutionarily determined by which human COMT allele is most advantageous in a specific environmental context.

3 The Hippocampus

The hippocampus is a curved bulb-like structure in the temporal lobe, protruding into the lateral ventricles. For the purposes of this thesis, the hippocampus is defined as consisting of the dentate gyrus (DG), cornu Ammonis regions 1 – 3 (CA1–3) and subiculum. Notably, some studies refer to these structures as the hippocampal formation and include the multi-layered entorhinal cortex (EC), presubiculum and parasubiculum. The hippocampus is also sometimes defined as part of the "(meso)limbic system", which comprises anatomically or functionally related brain structures at the medial brain surface.

3.1 Structure of the hippocampus

In contrast to neocortical regions, the architecture of the phylogenetically old hippocampus is very simple (Forster et al 2006). Principal cells are arranged in a single layer and afferent fibres make *en passage* connections with neuronal dendrites. This forms a three-step feedforward loop called the "tri-synaptic circuitry" between a series of hippocampal subregions (Figure 12; Andersen et al 1966a; Andersen et al 1966b; Ramon y Cajal 1893; 1911). Moreover, the hippocampus is almost perfectly organised in a laminar structure. Hippocampal cells are contained in specific cellular layers, whereas afferent fibres terminate in a highly ordered fashion in distinct cell-free layers (see figure 13; Andersen P 2006).

The hippocampus is a unique brain structure, because it receives highly processed, multimodal information and sends divergent projections to brain structures (e.g. Amaral et al 2007; Gray & McNaughton 2000; Pikkarainen et al 1999; Swanson et al 1987). Each hippocampal subregion has different input and output projections, reflecting their role within the hippocampus: The **DG** plays a crucial role in receiving incoming sensory information, whereas **CA3** is mainly responsible for intra-hippocampal communication. **CA1 and the subiculum** have important roles in sending extrinsic output projections and acting as comparators of

incoming and outgoing information, which allows them to perform functions such as novelty detection (see Figure 6).

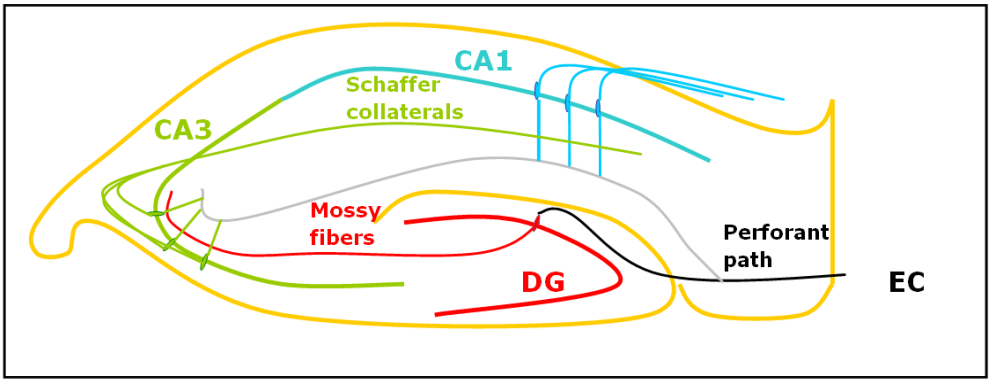


Figure 12 Simplified illustration of the tri-synaptic circuit of the hippocampus
Perforant path fibres (drawn in black) from the EC synapse on DG granule cell dendrites. The second step of the circuit comprises axons from granule cells (named mossy fibres; drawn in red) synapsing on CA3 apical dendrites. Finally, CA3 cells send projections (named Schaffer collaterals; drawn in green; Schaffer 1892) to the apical dendrites of CA1 pyramidal cells (adapted from Collingridge 1992; Enoch et al 2003).

3.2 Role of the hippocampus

Early neuroanatomists thought the hippocampus played a major role in the olfactory system, this theory has now been almost entirely deserted (Brodal 1947). Nonetheless, olfactory information must contribute to the functions of the hippocampus since the hippocampus receives olfactory projections (Shipley & Adamek 1984) and rodents clearly exhibit olfactory learning and memory. More prominent theories on the role of the hippocampus include hypotheses of its involvement in the neuronal circuits responsible for emotion and cognition (see Chapter 5 and Chapter 6, respectively).

3.3 The hippocampus and COMT

There is an emerging literature suggesting a role for COMT in the hippocampus (Table 2). Notably, high hippocampal COMT activity and expression have been demonstrated in rats (Masuda et al 2003; Matsumoto et al 2003a; Saavedra et al 1976). Also, human fMRI data demonstrates that the $Val^{158}Met$ genotype modulates hippocampal activation during cognitive (Schott et al 2006) and emotional processing (Drabant et al 2006; Krach et al 2010; Smolka et al 2005). Finally, the $Val^{158}Met$ genotype affects gray matter volume and density of the hippocampal formation (Cerasa et al 2008; Ehrlich et al 2010; Honea et al 2009; Mechelli et al 2009; e.g. van Amelsvoort et al 2008; Zinkstok et al 2006).

Paper	**Findings**
(Eleftheriou 1975)	Mild stress increases hippocampal COMT activity in mice (open field)
(Saavedra et al 1976)	High COMT activity in rat hippocampus
(Li et al 1998)	COMT inhibition decreases hippocampal HVA levels
(Masuda et al 2003)	High S-COMT activity in rat hippocampus
(Matsumoto et al 2003a)	High COMT mRNA expression in rat hippocampus
(Tunbridge et al 2004c)	COMT inhibition increases hippocampal expression of arc, an immediate early gene that indicates neuronal activation
(Smolka et al 2005)	Met^{158} allele load in humans correlates with increased hippocampal activation during processing of unpleasant stimuli
(Drabant et al 2006)	Met^{158} allele load correlates with increased right hippocampal formation activation during viewing of negative emotional faces as well as increased coupling between hippocampus and orbito-frontal cortex
(Schott et al 2006)	Met^{158}/Met^{158} subjects show stronger functional coupling between hippocampus and PFC than Val^{158} carriers
(Jurata et al 2006)	Hippocampal COMT expression is decreased in "VCFS mice", which display deficits in pre-pulse inhibition, learning and memory
(Redell & Dash 2007)	1) COMT immunoreactivity found in rat hippocampus 2) Hippocampal COMT protein increases after traumatic brain injury
(Cerasa et al 2008)	COMT genotype affects volume of the hippocampus
(Mechelli et al 2009)	
(Honea et al 2009)	
(Ehrlich et al 2010)	
(Krach et al 2010)	COMT genotype influences hippocampal activation

Table 2 Primary literature on COMT in the hippocampus

PFC, prefrontal cortex; VCFS, velocardiofacial syndrome

4 Thesis rationale and aims

The aim of this DPhil thesis is to investigate the role of COMT in hippocampal function. The rationale for this research is based on four main points:

1. The rat hippocampus exhibits high COMT expression and activity (Masuda et al 2003; Matsumoto et al 2003a; Saavedra et al 1976).
2. COMT may play a role in cognition and emotion (Section 2.7), as well as complex psychiatric traits (Section 2.6).
3. The hippocampus is involved in certain aspects of cognition and emotion, as well as their disorders (see Chapter 5 and Chapter 6).
4. DA and NA, which are substrates of COMT, modulate molecular processes underlying memory, and are involved in cognition and emotion (Section 1.3 and Section 1.4).

The above data allude to the possibility that changes in hippocampal COMT activity might alter hippocampus-dependent aspects of cognition and emotion relevant to psychiatric disorders via changes in hippocampal DA and/or NA function.

There have been no studies of hippocampal COMT expression in psychiatric disorders. Hence, **chapter two** studies the effect of genotype and diagnosis of schizophrenia or bipolar disorder on human hippocampal COMT mRNA expression. To date, the link between COMT and hippocampal function is also unexplored. Accordingly, **chapters three and four** investigate the neurochemical consequences of COMT inhibition to see if reduced COMT activity is associated with decreased DA metabolism in the rat hippocampus. **Chapters five and six** investigate the behavioural phenotype of rats treated with a COMT inhibitor using tasks designed to test various hippocampus-dependent functions, which are potentially impaired in schizophrenia and may be associated with the COMT Val^{158}Met polymorphism. Finally, **chapter seven** summarises and discusses the findings of this thesis.

The human and rodent studies presented in this thesis above test the hypothesis that altered COMT activity (due to pharmacological inhibition, a psychiatric disorder and/or genetic polymorphisms) influences hippocampal function.

Oxford University

Chapter 2

COMT expression in the hippocampus; the effect of COMT genotype and psychiatric diagnosis

Linda Maria Laatikainen

DPhil thesis on the role of COMT in hippocampal function

1 Introduction

COMT is highly expressed in the rat hippocampus (Matsumoto et al 2003a), and the COMT Val^{158}Met SNP may be associated with various psychiatric disorders, including schizophrenia (see Chapter 1). However, there have been no studies of hippocampal COMT mRNA expression in psychiatric disorders. Hence, this study investigates the effect of COMT genotype and psychiatric diagnosis on hippocampal COMT expression.

1.1 Structural and functional abnormalities in schizophrenia implicate the hippocampus

The most robust pathophysiological changes in schizophrenia are macroscopic structural changes. These include an enlargement of the lateral and third ventricles as well as reduced grey matter volume in whole brain and in various brain regions (Halliday 2001; Lewis & Lieberman 2000; McCarley et al 1999; Nelson et al 1998; Ward et al 1996; Wright et al 1999; Wright et al 2000). Notably, the magnitude and consistency of grey matter reductions is most robust in the hippocampus. These findings have been replicated in first-episode, unmedicated subjects (Barr et al 1997; Whitworth et al 1998; Zipursky et al 1998); and do not seem to affect subtypes of schizophrenia differentially (Daniel et al 1991). This suggests that they are not merely a consequence of illness or antipsychotic treatment, and hence appear to be involved in the pathogenesis of schizophrenia.

In addition to the macroscopic structural changes, molecular studies have suggested smaller neuronal size, loss of neuronal integrity as well as abnormal synaptic density and plasticity markers in the hippocampus (Harrison 1999b; Heckers & Konradi 2002; Keshavan et al 2008; Knable et al 2004).

Also, functional studies have shown that schizophrenia patients exhibit similar spatial working memory impairments as patients with hippocampal damage (e.g. compare Boyer et al 2007; Kessels et al 2001), though it is necessary to acknowledge that research on human cognitive deficits is full of controversies (see Chapter 6 and Chapter 7). Moreover, schizophrenia patients require intact hippocampal functioning for performance on neuropsychological tests, and display increased (para)hippocampal activation during cognitive tasks (Harrison 2004; Heckers et al 1998), which is dependent on COMT Val^{158}Met genotype (Di Giorgio et al 2010). COMT Val^{158}Met genotype also affects parahippocampal blood flow and volume in schizophrenia patients, but not healthy controls (Eisenberg et al 2010; Ohnishi et al 2006).

In summary, schizophrenia patients show hippocampus-specific structural and functional changes. This implies that the hippocampus is crucially involved in the pathophysiology of schizophrenia and is hence a brain region commonly studied in relation to schizophrenia (Harrison 2004).

1.2 Studying hippocampal COMT expression in bipolar disorder

The present COMT mRNA expression study compares patients with schizophrenia or bipolar disorder with non-psychiatric control subjects. The main reason for studying bipolar disorder patients in addition to schizophrenia patients was pragmatic: the Stanley Medical Research Institute (SMRI) brain cohort used for these studies includes all three subject groups (see Table 4), and SMRI guidelines require that all groups be included in all studies.

However, there are also strong scientific reasons for investigating COMT expression in both schizophrenia and bipolar disorder. First, schizophrenia and bipolar disorder share chromosome 22q as a robust susceptibility locus (Badner & Gershon 2002). Second, COMT has also been implicated as a candidate gene for bipolar disorder (Abdolmaleky et al 2006; Craddock et al 2006; Dickerson et al 2006; Farmer et al 2007; Kirov et al 1998; Papolos et al 1998; Rotondo et al 2002; Shifman et al 2004). Third, schizophrenia and bipolar disorder overlap etiologically

and phenotypically (Robinson & Ferrier 2006; Robinson et al 2006; Schretlen et al 2007). Additionally, structural and functional abnormalities in the hippocampus are similar in schizophrenia and bipolar disorder (Bertolino et al 2003; Frey et al 2007; Torrey et al 2005).

Therefore, it is of interest to know whether any disease-associated changes in hippocampal COMT mRNA expression are specific for schizophrenia, or whether they also generalise to other psychotic disorders, and bipolar disorder in particular.

1.3 Validation of methodology

1.3.1 Use of qRT-PCR

COMT mRNA expression was evaluated using quantitative real-time polymerase chain reaction (qRT-PCR), a technique used to amplify, detect and quantify specific mRNA sequences in real-time (VanGuilder et al 2008). This method is widely used for mRNA quantification, because it is sensitive to low levels of mRNA template, it possesses a large dynamic range, it is fairly resistant to non-specific product formation, and because it is relatively quick to perform. Accordingly, this technique is a reliable and commonly used method to quantify mRNA expression in post-mortem human brain tissue.

Standard PCR is an enzyme-mediated technique, which is used to amplify specific pieces of DNA (Mullis 1990). One PCR cycle involves three main steps identified by different incubation temperatures (see Figure 13). The process shown in Figure 13 is repeated ~35 times in order to exponentially amplify the template.

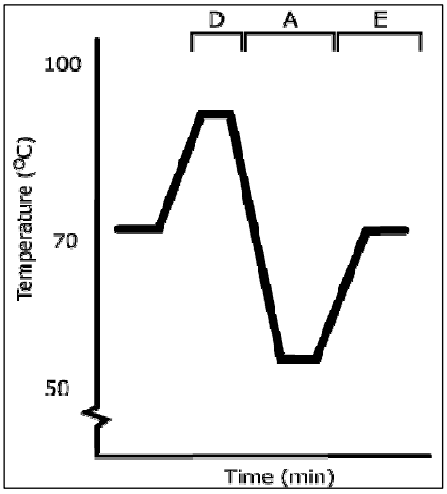


Figure 13 Illustration of a PCR cycle

(D) DNA denaturation: Above ~90 °C, weak hydrogen bonds connecting the two complementary strands of DNA are disrupted.

(A) Primer annealing: At ~50-60 °C, primers hybridise to single-stranded DNA

(E) Primer extension: At the enzyme's ideal operating temperature (~60-75 °C), DNA polymerase binds to the hybridised primer and adds complementary nucleotides in order to unidirectionally extend the DNA chain to form a double-stranded amplicon.

Reverse Transcription PCR (RT-PCR) is a modification of the basic PCR technique, allowing reverse-transcribed mRNA (cDNA) to be used as template. qRT-PCR is a further improvement to RT-PCR, which allows simultaneous amplification and quantification of specific cDNA sequences throughout the course of the PCR reaction. Notably, the amount of PCR product is quantified during the exponential phase of the amplification reaction, when the amount of amplicon is proportional to the amount of cDNA and hence, the amount of mRNA template (see amplification plot in Figure 14). In contrast, during the plateau phase the amount of amplicon may not reflect the initial starting amount of mRNA, because components of the reaction mixture become the limiting reagents (Freeman et al 1999; Pannetier et al 1993).

The two most common qRT-PCR detection methods are non-specific fluorescent dyes (such as SYBR Green), which non-specifically intercalate with double-stranded DNA (dsDNA), and sequence-specific fluorescent reporter probes (e.g. TaqMan assays). The present study used SYBR Green fluorescence to quantify the abundance of COMT mRNA expression, because SYBR Green chemistry allows amplification of longer cDNA sequences than TaqMan probes. The increased amplicon length allowed the design of primers that would successfully detect all known COMT mRNAs expressed in brain (Tunbridge et al 2007a). However, since SYBR Green molecules bind non-specifically to dsDNA, melting curve analysis (see

melting curve in Figure 14) and gel electrophoresis was performed in order to ensure correct amplicon formation (as opposed to primer-dimers or non-specific binding of primers). Also, the binding specificity of (mostly intron-spanning) primers for each transcript was confirmed using the BLAST nucleotide-nucleotide alignment search.

Since multiple SYBR Green molecules bind to the amplicon, the amount of fluorescence produced depends on the starting concentrations and size of the target cDNA. Thus, fluorescence produced is compared to that of a standard curve of serial dilutions of known amounts of pooled cDNA (see standard curve in Figure 14; Livak & Schmittgen 2001; Winer et al 1999). To control for potential pipetting errors, the standard curve, samples and controls[1] were run on the same qRT-PCR plates in triplicate. Pilot studies were performed to optimize the primer annealing temperature and primer concentrations in order to achieve 90-110 % reaction efficiency (standard curve slope between -3.1 and -3.6) and to ensure linearity (standard curve $R^2 > 0.98$).

Furthermore, to control for differences in mRNA quality or quantity resulting from sample collection, storage and preparation procedures, target gene expression is often normalised to the expression of one or more endogenous control genes (also called "housekeeper" or "reference" genes), which are thought to exhibit largely invariant expression (Tunbridge et al 2011). In the present study, pilot studies were performed to select three endogenous control genes: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), transferrin receptor (TFRC) and TATA box-binding protein (TBP).

[1] Controls included non-template controls, containing no cDNA, and reverse-transcriptase negative [RT-] controls, which contained the product of reverse transcription reactions run in the absence of reverse transcriptase (a control which therefore assesses amplification from residual genomic DNA present in mRNA samples).

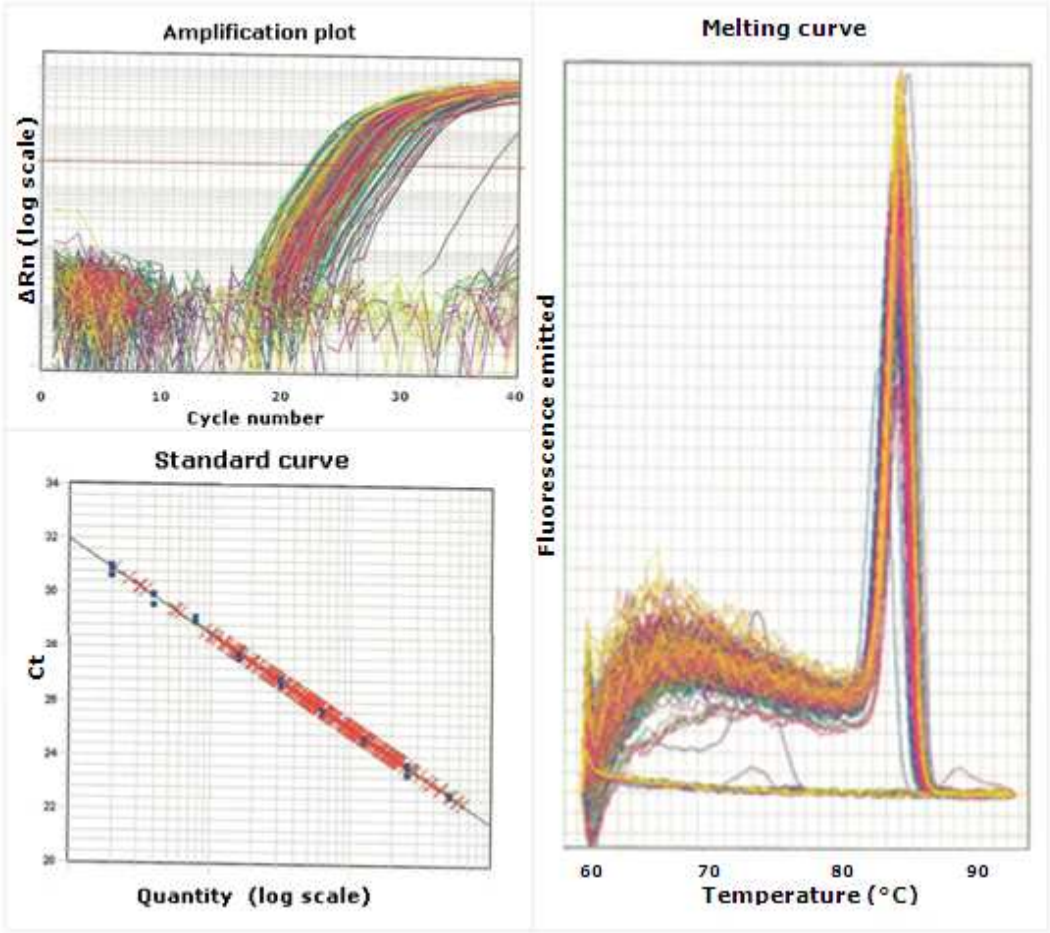


Figure 14 Representative amplification plot, standard curve and dissociation curve from the COMT qRT-PCR experiment

Top left: The amplification curves for triplicate replicas of all 104 individuals. The amount of amplified product (given by the amount of fluorescence emitted on the y-axis) is measured at the end of each PCR cycle (x-axis). The Ct value is the cycle threshold, defined as the cycle number, at which the fluorescence from a sample crosses the threshold for detection of fluorescence above background (red line). The individual outlier amplification curve in purple was not included in the data analysis.
Bottom left: The standard curve of two-fold serial dilutions of pooled cDNA derived from hippocampal mRNA from all 104 subjects. The black squares indicate triplicate samples of pooled DNA at each dilution, with the black line indicating the line of best fit. The red crosses illustrate, where the individual triplicate samples for each experimental subject lie with respect to the line of best fit i.e. how the starting cDNA quantity (x-axis) was estimated from the amplification plot Ct values (y-axis). Any data points lying outside the range of the black squares were not included in the final data analysis, because having very high cDNA concentrations may result in template inhibition.
Right: The melting curve shows a single peak of fluorescence at 84-85 °C. This suggests that primer annealing was specific and hence, only one PCR product was generated. The individual outlier melting curve (in purple), with a peak at about 75 °C, potentially suggests production of primer-dimers, since primer dimers dissociate at lower temperatures than PanCOMT cDNA amplicons due to their smaller size. Hence, this replication of the sample was not included in the data analysis.

However, endogenous control gene expression has been demonstrated to be altered by factors such as postmortem interval, substance use, age, diet, gender, brain area, RNA extraction methodology and various stressors (Derks et al 2008; Harrison 1996; Johansson et al 2007; Preece & Cairns 2003; Tanic et al 2007;

Weickert et al 2010). In the current study, the geometric mean of the three housekeeper genes was altered by brain weight (Harrison et al 2010). Thus, raw COMT mRNA data (not normalised relative to endogenous control gene expression) are also presented for comparison.

1.3.2 Selecting and genotyping COMT SNPs

Genotyping was performed using TaqMan fluorescent reporter probes. These work by emitting fluorescence during the primer extension phase (see Figure 13), when DNA polymerase breaks bonds within the probe so as to release a single fluorescent reporter molecule per amplicon produced. This detection method exhibits increased specificity, and allows accurate genotyping at very high throughput, even in the presence of primer dimers.

Chapter 1 outlines the importance of studying the impact of the Val^{158}Met polymorphism on COMT expression. However, in addition to the Val^{158}Met SNP, other COMT SNPs could also alter the activity of COMT by affecting the expression or stability of its mRNA (Nackley et al 2006). Thus, if disease-associated SNPs are also associated with altered COMT mRNA expression this would manifest as altered COMT mRNA expression in the disease group compared to the control group.

Hence, the range of SNPs to be studied regarding their potential association with COMT mRNA expression is very wide. The choice of SNPs for the investigation was based on promising findings from the existing COMT association literature (see Table 3).

SNP Code	SNP	Literature
rs165599	Intronic (A/G)	(Bray et al 2003; Chen et al 2004b; Diatchenko et al 2005; Funke et al 2005; Handoko et al 2005; Liao et al 2009; Meyer-Lindenberg et al 2006; Shifman et al 2002)
rs4680	Val^{158}Met (G/A)	See Chapter 1
rs4818	Leu^{136}Leu (C/G)	(Diatchenko et al 2005; Nackley et al 2006)
rs4633	His^{62}His (C/T)	(Bray et al 2003; Diatchenko et al 2005; Handoko et al 2005; Nackley et al 2006; Shifman et al 2002)
rs6269	Intronic (A/G)	(Diatchenko et al 2005; Nackley et al 2006; Shifman et al 2002)
rs2075507 prev 2097603	Intronic (A^{-286}G)	(Chen et al 2004a; Funke et al 2005; Meyer-Lindenberg et al 2006; Tunbridge et al 2006b)
rs737865	Intronic (C/T)	(Bray et al 2003; Chen et al 2004b; Funke et al 2005; Handoko et al 2005; Liao et al 2009; Shifman et al 2002)

Table 3 List of commonly studied COMT SNPs

SNPs chosen for the present study are highlighted in bold.

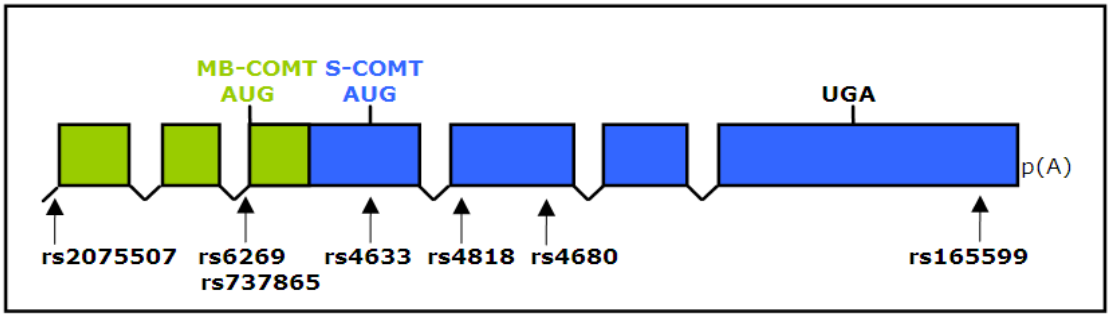


Figure 15 Illustration of COMT SNPs with frequencies >0.15 in the coding region of COMT

(adapted from NCBI reference assembly SNP search function and Diatchenko et al 2005). Exons are shown as boxes and introns as connecting lines. The AUG translation start sites for MB-COMT and S-COMT, as well as the UGA stop codon are also labelled. The blue and green portions indicate the exon sequences of the 1.3 kb and 1.5 kb COMT mRNA transcripts, respectively.

1.4 Aims and hypotheses

The aim of this study was to investigate the effect of genotype and diagnosis on human hippocampal COMT mRNA expression. Previous reports on prefrontal cortical COMT mRNA expression found no differences between diagnostic groups (Matsumoto et al 2003b; Tunbridge et al 2004a), and there is controversy regarding the effect of COMT genotype on expression (Bray et al 2003; Chen et al 2004a; Dempster et al 2006). However, the effects of diagnosis and SNPs on COMT mRNA expression have never been investigated in the hippocampus.

2 Methods

2.1 Subjects

Human hippocampal tissue was obtained from the Stanley Medical Research Institute Microarray Consortium brains ('SMC series'), which comprises brains from 104 adults: 35 controls, 35 with schizophrenia, 34 with bipolar disorder (http://www.stanleyresearch.org/dnn/Portals/0/Stanley/Array%20Collection%20Demographic%20Details%20Chart-Final.pdf). The specimens were collected, with informed consent from next of kin, by participating medical examiners in the USA. The post-mortem tissue has been well-characterised and matched for key confounding factors (see Table 4 for demographic details).

	Controls n=35	**Schizophrenia** n=35	**Bipolar disorder** n=34
Age (years)	44.2±7.6	42.6±8.5	45.4±10.7
Gender (male:female)	26:9	26:9	16:18
Brain pH	6.61±0.27	6.47±0.24	6.43±0.30
RIN	5.03±1.13	4.68±1.36	4.68±1.33
Fresh brain weight (grams)	1444±148	1442±107	1395±142
Side of brain studied (left:right)	16:19	17:18	19:15
Autopsy delay (hours)	29.4±12.9	31.4±15.5	37.9±18.6
Storage time (months)	81±18	87±23	93±26
Smoker at death (yes/no/nk)	9/9/17	23/4/8	15/6/13
Suicide (yes/no)	0/35	7/28	15/19
Onset of illness (years)	-	21.3±1.0	25.3±1.6
Duration of illness (years)	-	21.3±1.7	20.1±1.7
Total inpatient time (years)	-	1.23±0.38	0.53±0.24
Psychotic symptoms (yes/no/nk)	0/35/0	35/35/0	21/10/2
Antipsychotic exposure (kg)	0	85.0±17	10.2±4.0

Table 4 Demographic details of SMC brain series

Values are given as mean ± S.D; RIN, RNA integrity number, whereby higher numbers indicate more intact RNA; nk, not known; Antipsychotic exposure expressed as estimated lifetime exposure in fluphenazine equivalents

2.2 RNA extraction and reverse transcription

In brief, RNA was extracted from frozen hippocampal tissue sections (including parahippocampal cortex) using Tri Reagent (Sigma Aldrich, Poole, UK). RNA integrity (RIN) was measured using an Agilent Bioanalyzer 2100 and RNA 6000 Nano kit (Agilent Technologies, Wokingham, UK). 500 ng RNA was DNase treated for 30 min at 37 °C and 10 min at 70 °C, using 1 unit RNase-free DNase and 24 units RNAsin ribonuclease inhibitor (both Promega, Southampton, UK) and reverse transcribed using 30 ng oligo-dT, 200 units M-MLV reverse transcriptase, 24 units RNAsin, 0.5 mM of each dNTP and reaction buffer (Promega) at 42°C for 1 h and 70°C for 10 min.

2.3 qRT-PCR

qRT-PCR was performed on 10 ng cDNA (assuming 1:1 ratio of cDNA produced from mRNA template during reverse transcription) using powerSYBR Green (Applied Biosystems, Warrington, UK) and PanCOMT, TBP, TFRC or GAPDH primers (see Table 5 for sequences and concentrations). Each 384-well plate included triplicate qPCR reactions for each subject, non-template controls, reverse-transcriptase negative [RT-] controls and a standard curve of serial dilutions of pooled cDNA. Thermal cycling was performed on an Applied Biosystems 7900HT Fast Real-Time PCR system and consisted of an initial denaturing step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s (DNA denaturation), 63°C for 1 min (Primer annealing and extension) and a melting curve (gradual temperature change up to 95 °C over 15s; see Figure 13).

Primer	Sequence 5'→ 3'	Concentration
PanCOMT-F	TGA ACG TGG GCG ACA AGA A	6 µmol/l
PanCOMT-R	CCA TGC GCA CAG CTG AGT A	6 µmol/l
TBP-F	CAC GAA CCA CGG CAC TGA TT	2 µmol/l
TBP-R	TTT TCT TGC TGC CAG TCT GGA C	2 µmol/l
TFRC-F	GGC CTG AGT TTA CAG TGG CTG	3 µmol/l
TFRC-R	GAG TTT CTT CAT GAC AAA TCT GTC TG	3 µmol/l
GAPDH-F	GGG AAA CTG TGG CGT GAT GG	2 µmol/l
GAPDH-R	TGC CAG TGA GCT TCC CGT TC	2 µmol/l

Table 5 Primers used for qRT-PCR experiments

2.4 Genotyping

All subjects were genotyped for five SNPs (see Section 1.3 above) using 5' exonuclease TaqMan® SNP genotyping assays (Applied Biosystems: rs4680, C__25746809_50; rs4818, C___2538750_10; rs4633; C___2538747_20; rs6269: C___2538746_1_). Genotyping of rs2075507 was performed using a custom-made TaqMan® SNP genotyping assay consisting of unlabelled forward (GCC GTG TCT GGA CTG TGA GT) and reverse (GGG TTC AGA ATC ACG GAT GTG) PCR primers and a Tamra probe. The A allele was labelled with FAM™ dye (AAC AGA CAG AAA AGT TTC CCC TTC CCA), and the G allele was labelled with VIC® dye (CAG ACA GAA AAG CTT CCC CTT CCC ATA). In accordance with the information on the Applied Biosystems website, the PCR mix included 4 ng of cDNA, TaqMan Universal PCR Master Mix (2x; Applied Biosystems) and SNP genotyping assay (20x; Applied Biosystems) in a final reaction volume of 5 µl. The thermal cycling protocol set up on the AB7900HT machine consisted of an initial denaturing step at 95 °C for 10 min, followed by 40 cycles of 92 °C for 15 s and 60 °C (62 °C for rs2075507) for 1 min, and a melting curve. All samples were analysed in three independent runs.

2.5 Data analysis

Six subjects were excluded from the final analysis due to unusual amplification curves or data points lying outside the standard curve (see Figure 14). Raw mRNA data for the remaining subjects were read off against a serial dilution calibration curve run for each experiment using SDS v2.2.2 software (Applied Biosystems). For the investigation of total COMT expression, abundance of target mRNA in each sample was calculated from a standard curve and normalised to the geometric mean of the mRNA abundances of GAPDH, TFRC and TBP. The researcher was blind to demographic details of the SMC brain series (summarised in Table 4).

The effects of diagnosis and genotype were analysed using analysis of variance (ANOVA) with Fisher's least significant difference (LSD) post-hoc tests. In case of a statistically significant Mauchly's test of sphericity, a Huynh-Feldt correction was used to alter the degrees of freedom and thereby alter the F-ratio significance level. Data were plotted using GraphPad Prism 5.01 (GraphPad Software Inc, La Jolla, CA, USA) and analysed using SPSS 15.0 statistics software (SPSS Inc, Chicago, IL, USA), using diagnosis and genotype as between-subjects factors and, where applicable, different genes or SNPs as within-subjects factors.

Before performing the ANOVAs, the mRNA data were screened for any significant correlations in the whole sample and in the different diagnostic groups alone. If this analysis yielded any statistically significant correlations for either raw or normalised COMT mRNA abundance, the correlating factors were included as covariates in the ANOVAs.

3 Results

3.1 COMT expression did not differ between diagnostic groups

There was no significant main effect of diagnosis on COMT mRNA expression in the hippocampus (either with or without normalisation by the geometric mean of three housekeeper genes; p's>0.3; Figure 16; Appendix I).

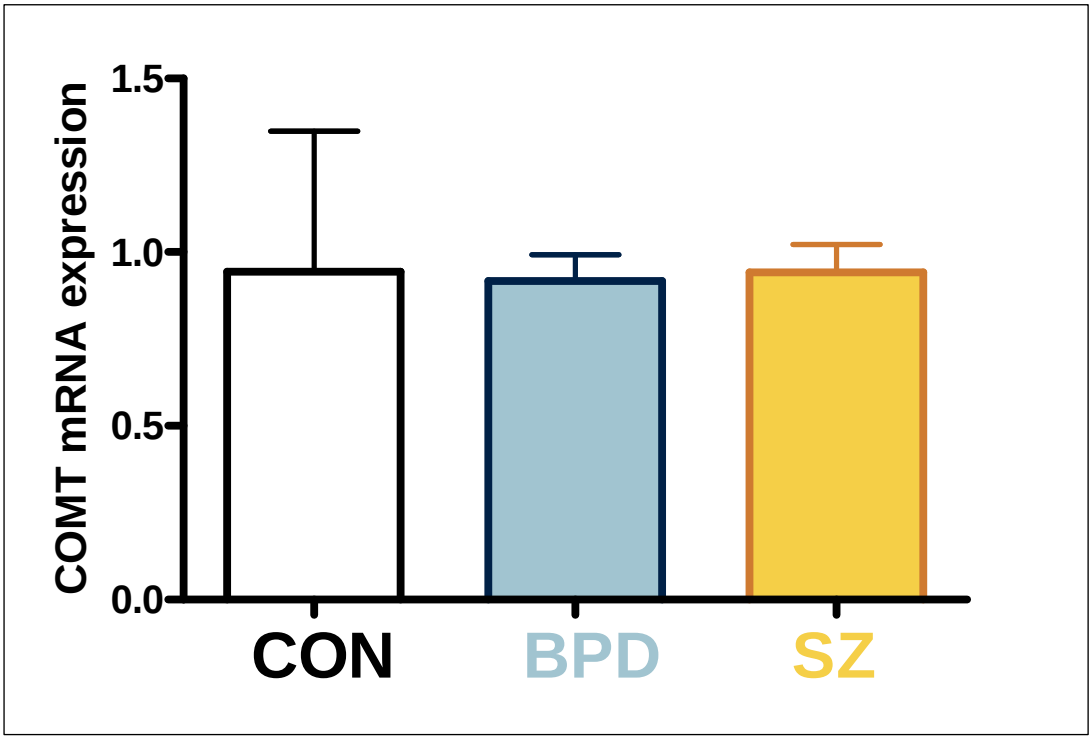


Figure 16 Normalised COMT mRNA expression in control subjects (CON; n=35) **and patients with schizophrenia** (SZ; n=34) **or bipolar disorder** (BPD; n=32)
COMT mRNA data normalised to the geometric mean of GAPDH, TFRC and TBP; Postmortem hippocampal tissue data shown as mean ± SEM.

3.2 COMT expression was unaffected by COMT genotype

One-way ANOVAs did not reveal any significant main effect of COMT genotype on COMT mRNA expression for any of the COMT SNPs studied, namely Val^{158}Met, A-287G, rs4818, rs4633 and rs6269 (F's<1.0; p's>0.3 with or without normalisation; Appendix I). Also, there were no diagnosis*genotype interactions for any of the SNPs, as analysed using two-way ANOVAs (p's≥0.069, Figure 17 and Figure 18).

Furthermore, COMT mRNA expression did not appear to be associated with homozygosity for a haplotype shown to affect COMT protein (Diatchenko et al 2005). However, there was lack of power to perform statistical analyses, because only 34 subjects were homozygous for one of the three forms of the haplotype (APS n=8, LSP n=25, HPS n=1) and there was one APS subject within the schizophrenia group and one HPS subject overall.

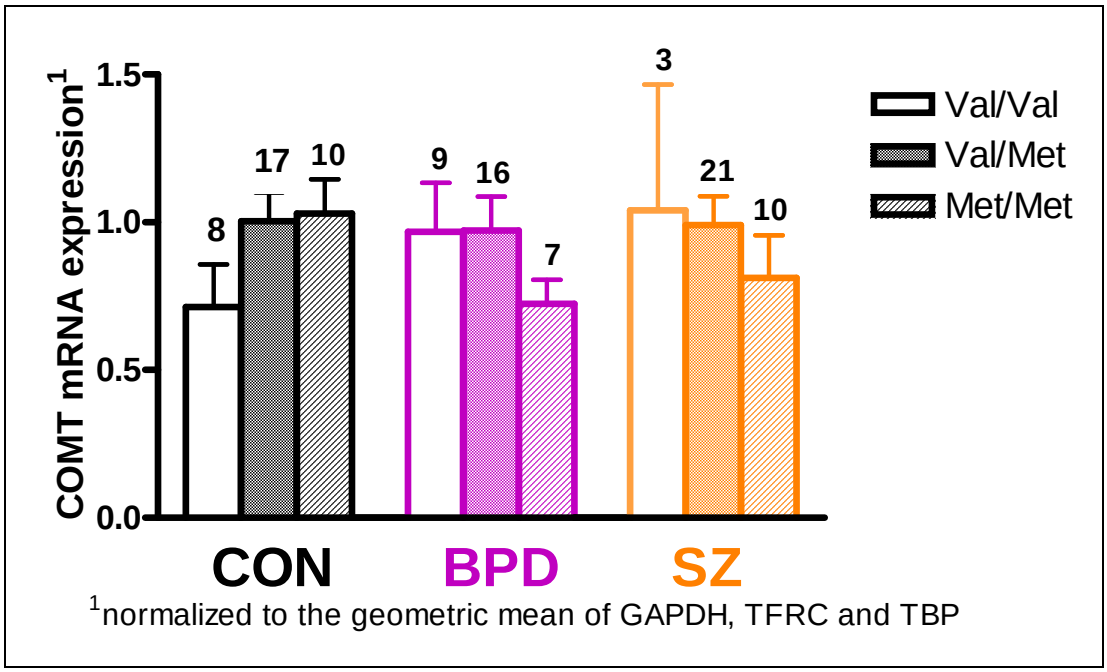
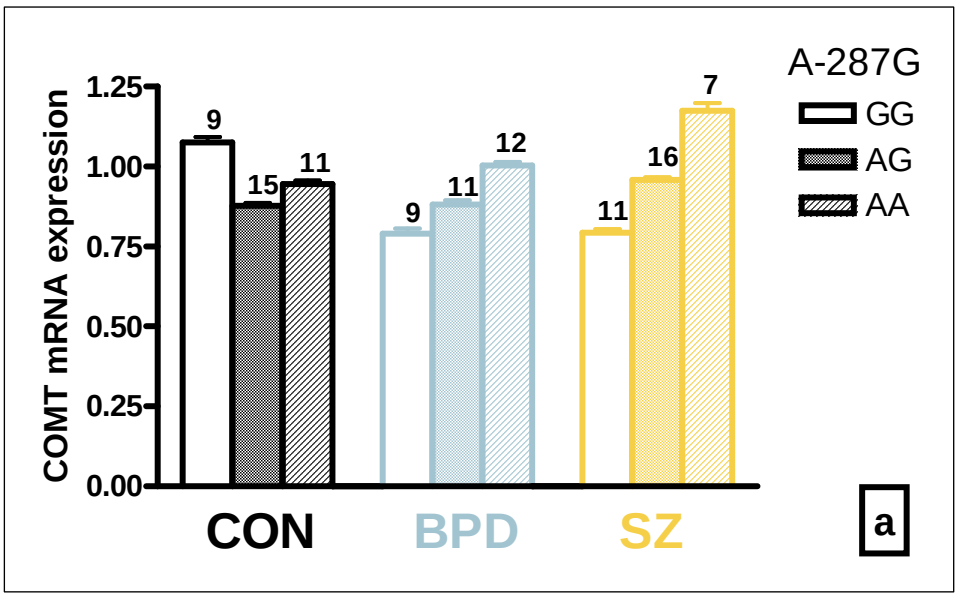
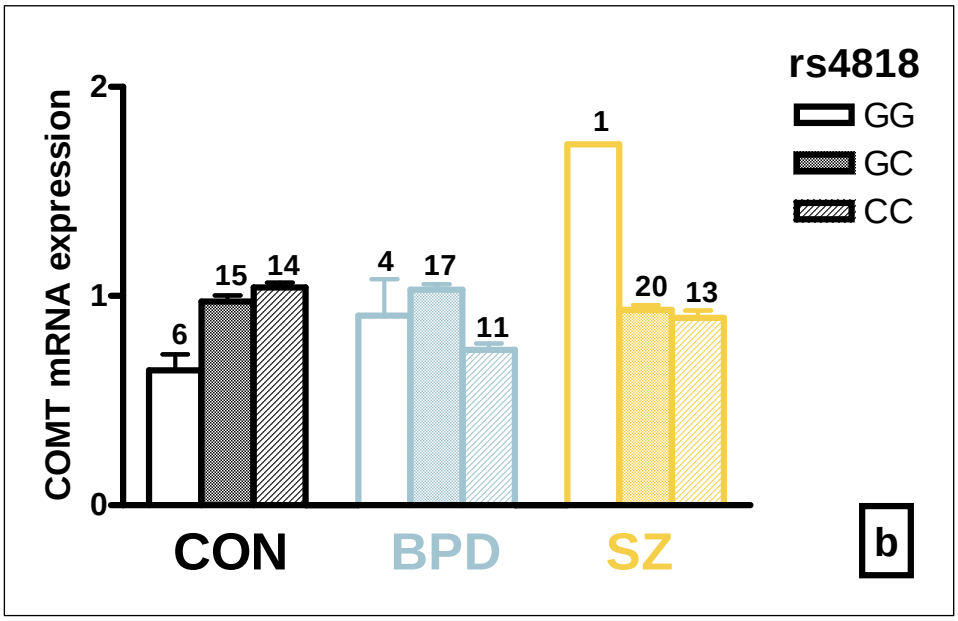
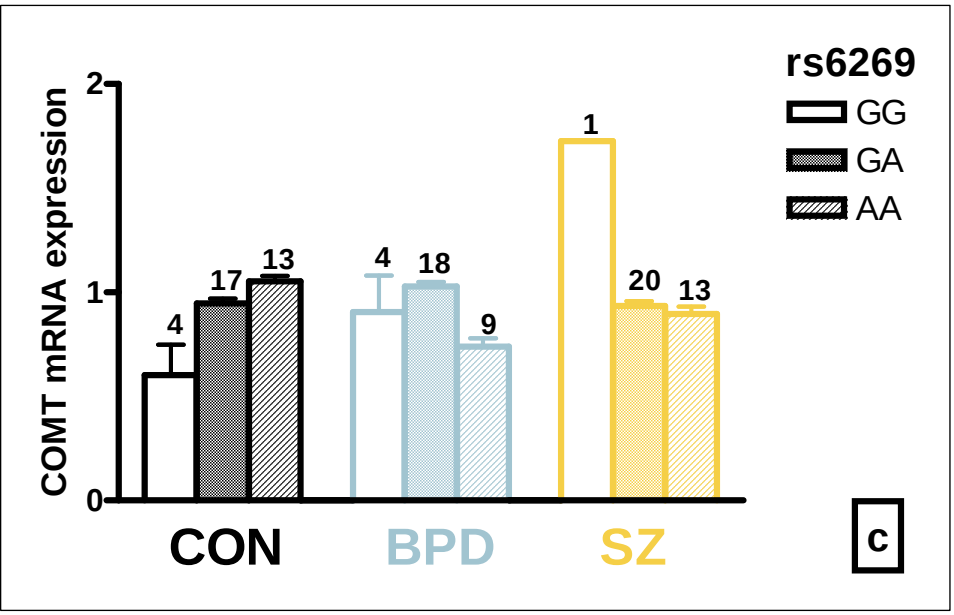


Figure 17 Normalised COMT mRNA expression in control subjects (CON; n=35) and patients with schizophrenia (SZ; n=33) or bipolar disorder (BPD; n=32), by Val^{158}Met genotype
Val/Val or Met/Met, homozygous for the Val^{158} or Met^{158} allele; Val/Met, heterozygous subjects; COMT mRNA data normalised to the geometric mean of GAPDH, TFRC and TBP; Postmortem hippocampal tissue data shown as mean ± SEM. Number of samples in each group are shown in Figure.


A-287G
GG
AG
AA
COMT mRNA expression
1.25
1.00
0.75
0.50
0.25
0.00
9
15
11
9
11
12
11
16
7
CON
BPD
SZ
a


rs4818
GG
GC
CC
COMT mRNA expression
2
1
0
6
15
14
4
17
11
1
20
13
CON
BPD
SZ
b


rs6269
GG
GA
AA
COMT mRNA expression
2
1
0
4
17
13
4
18
9
1
20
13
CON
BPD
SZ
c

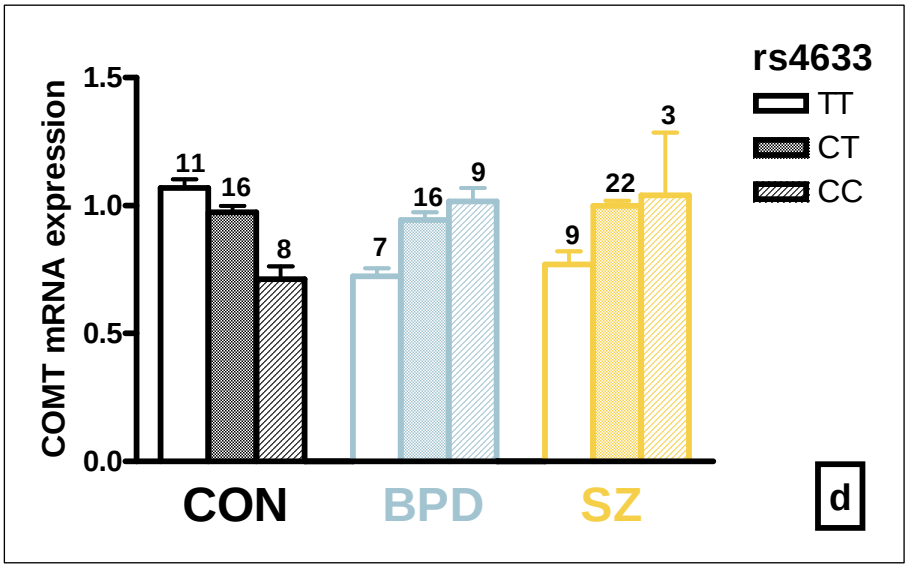


Figure 18 Normalised COMT mRNA expression in control subjects (CON; n=35, 34 for rs6269) **and patients with schizophrenia** (SZ; n=34) **or bipolar disorder** (BPD; n=32, 31 for rs6269)**, by (a) A-287G, (b) rs4818, (c) rs6269 and (d) rs4633 genotype**

GG, AA, CC or TT: homozygous for the allele; AG, GC or CT: heterozygous subjects; COMT mRNA data normalised to the geometric mean of GAPDH, TFRC and TBP; Postmortem hippocampal tissue data shown as mean ± SEM. Number of samples in each group are shown in each Figure.

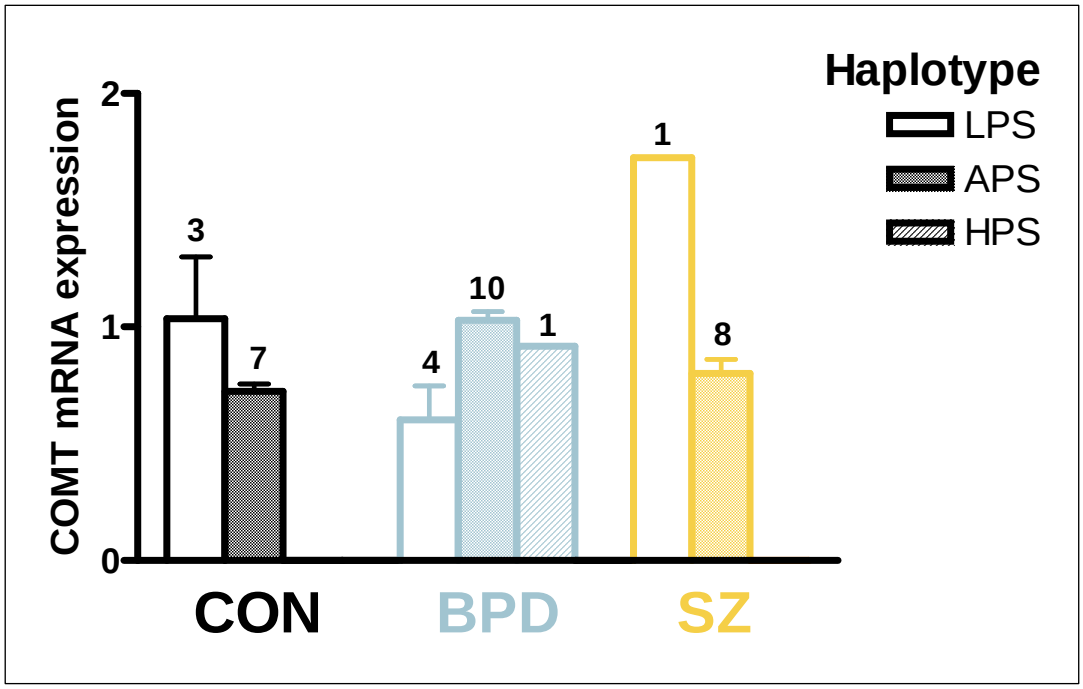


Figure 19 Normalised COMT mRNA expression in control subjects (CON; n=15) **and patients with schizophrenia** (SZ; n=9) **or bipolar disorder** (BPD; n=10)**, according to their haplotype homozygosity**

LPS, APS and HPS are haplotypes associated with low (LPS), average (APS) and high (HPS) pain sensitivity; COMT mRNA data normalised to the geometric mean of GAPDH, TFRC and TBP; Postmortem hippocampal tissue data shown as mean ± SEM. Number of samples in each group are shown in Figure.

3.3 COMT expression was unaffected by demographic variables

The present study did not reveal any sex differences in COMT mRNA expression (Appendix I; Figure 20; t(99)=-0.383, p=0.702). Also, independent samples t-tests did not show any effect of smoking, suicide or side of brain studied (Appendix II; p's>0.4). Moreover, drug or alcohol use do not influence COMT expression, as analysed using one-way ANOVAs (Appendix I; p's≥0.1).

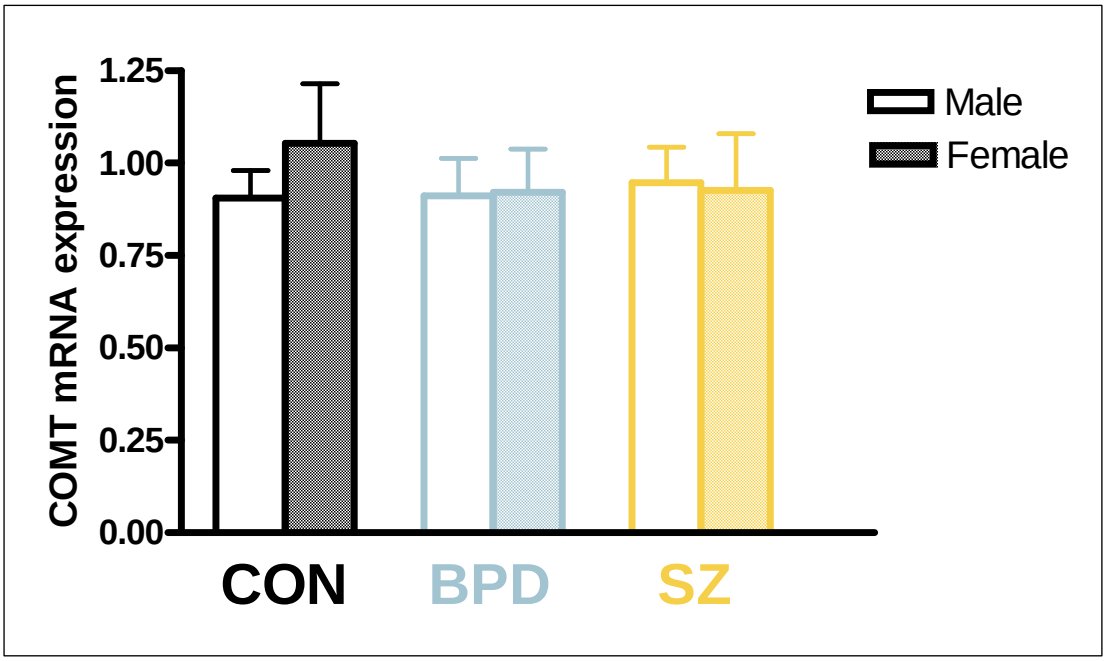


Figure 20 Normalised COMT mRNA expression in control subjects (CON; n=35) **and patients with schizophrenia** (SZ; n=34) **or bipolar disorder** (BPD; n=32) **by sex**
Female n=34, male n=67; COMT mRNA data normalised to the geometric mean of GAPDH, TFRC and TBP; Postmortem hippocampal tissue data shown as mean ± SEM.

Furthermore, COMT expression within the schizophrenia group did not correlate with clinical or therapeutic variables such as antipsychotic exposure (estimated lifetime exposure converted to fluphenazine equivalents; r(34)=-0.036, p=0.841), age of disease onset (r(34)=-0.279, p=0.109), total inpatient time (r(34)=-0.211, p=0.231) or duration of illness (r(34)=0.176, p=0.320). Across all subjects, non-parametric correlations did not show any correlation between normalised COMT mRNA expression and refrigerator interval (r(97)=-0.128, p=0.212), storage time (r(101)=-0.052, p=0.606), age at death (r(101)=-0.031,

p=0.760), post-mortem interval (PMI; r(101)=-0.068, p=0.500), brain pH (r(101)=0.041, p=0.686) or brain weight (r(101)=-0.097, p=0.336).

However, brain weight was significantly correlated with TBP (r(99)=0.320, p=0.001) and GAPDH (r(103)=0.274, p=0.005) expression, as well as the geometric mean of the endogenous control genes (r(101)=0.306, p=0.002). Hence, brain weight was included as a co-variate in all ANOVAs (Harrison et al 2010). However, the inclusion of brain weight as a co-variate did not significantly influence any of the diagnosis- or genotype-related results.

4 Discussion

4.1 COMT expression was unaffected by psychiatric diagnosis and COMT genotype

The present study concluded that overall hippocampal COMT mRNA is not altered in schizophrenia or bipolar disorder, nor affected by COMT genotype. This finding is consistent with an earlier RT-PCR study comparing COMT mRNA expression in post-mortem dorsolateral prefrontal cortex (dlPFC) tissue from 77 normal controls and 31 individuals with schizophrenia, which found no effect of schizophrenia diagnosis or Val^{158}Met, rs1665599 or rs737865 genotype (Chen et al 2004a). Similarly, Matsumoto *et al.* (2003b) found no diagnosis- or Val^{158}Met genotype-related differences in COMT mRNA expression in post-mortem dlPFC tissue from 14 patients with schizophrenia and 15 normal subjects. However, further studies are required to investigate whether there are diagnostic or genotype-related differences in COMT mRNA expression within hippocampal subfields or strata, since in situ hybridisation studies by Matsumoto *et al.* (2003b) revealed layer-specific differences in cortical COMT expression in schizophrenics. Also, the data presented in this chapter do not rule out the possibility that diagnostic or genotype-related differences in COMT expression could be specific to COMT isoforms or any of the recently-reported COMT splice variants (Tunbridge et al 2007a).

The discovery that overall hippocampal COMT mRNA is not affected by COMT genotype supports data suggesting that functional SNPs within the COMT gene do not operate by altering mRNA expression (Scanlon et al., 1979; Boudikova et al., 1990; Lotta et al., 1995; Matsumoto et al., 2003b; Chen et al., 2004). Hence, it appears that the effects of the Val^{158}Met SNP on COMT function and psychopathogenesis are not mediated by changes in COMT mRNA expression.

However, it is important to note that the current qRT-PCR study lacked power to unequivocally prove no effect of genotype on mRNA expression for most

SNPs and certainly for the pain sensitivity haplotype studied. Also, many of the SNPs chosen for investigation have only yielded positive findings as part of functional COMT haplotypes (Bray et al 2003; Chen et al 2004b; Diatchenko et al 2005; Funke et al 2005; Handoko et al 2005; Nackley et al 2006; Shifman et al 2002). Hence, future research may find that certain COMT haplotypes affect hippocampal COMT expression, especially if expression studies are carried out using less variable preparations than post-mortem tissue, such as idealised cell systems (see e.g. Nackley et al 2006).

4.2 COMT expression was unaffected by demographic variables

The present study did not find any sex differences within the normalised COMT data. This result is consistent with a previous paper by Matsumoto et al (2003b), which did not find any relationship between cortical COMT mRNA levels and gender. In addition to the negative results regarding sex, this study did not find any correlation between COMT mRNA levels and confounding variables such as pH of the post-mortem tissue, brain weight, RIN, autopsy delay, storage time and cause of death; all of which can potentially influence mRNA expression in qRT-PCR studies (e.g. Barton et al 1993; Durrenberger et al 2010; Lipska et al 2006).

Moreover, lifetime antipsychotic exposure, total inpatient time, duration of illness and age of disease onset had no impact on COMT mRNA expression in patients with schizophrenia. However, the clinical and therapeutic data available for the SMC brain series was fairly limited. Hence, COMT mRNA expression could for instance differ within schizophrenia between patients with specific types (see DSM-IV 1994; ICD-10 2007) or severity of symptoms.

Finally, COMT mRNA expression was not influenced by smoking or recreational drug or alcohol use. However, this data cannot be extrapolated to mean that COMT mRNA expression is unaffected by environmental risk factors. Instead, future studies are likely to find that hippocampal COMT mRNA expression is affected by non-genetic environmental factors (such as adverse life events or

pre- and peri-natal developmental risk factor), ethnic background (see Chapter 1) and gene-environment interactions (van Os & Marcelis 1998).

4.3 Summary of Chapter 2

In summary, the present study found no diagnosis-, demographic- or genotype-related changes in hippocampal COMT mRNA expression. Hence, this study did not provide any evidence for the involvement of COMT or the hippocampus in schizophrenia. However, this qRT-PCR study could not fully rule out the possibility that hippocampal COMT may be associated with schizophrenia. Importantly, the present study confirmed the presence of COMT mRNA in the human hippocampus.

Oxford University

Chapter 3

Effect of COMT inhibition on tissue catecholamine and metabolite levels in rat brain regions

Linda Maria Laatikainen

DPhil thesis on the role of COMT in hippocampal function

1 Introduction

Given that DA and NA are substrates of COMT (Chapter 1), COMT is expressed in the hippocampus (Chapter 2 and Matsumoto et al 2003a) and the hippocampus displays a functional DA and NA system (Chapter 1), it is likely that pharmacological inhibition of COMT decreases hippocampal catecholamine metabolism. This hypothesis will be tested in Chapter 3 and Chapter 4 using *ex vivo* and *in vivo* neurochemical methods, respectively. Even though central catecholamine metabolism has been extensively studied both *ex vivo* and *in vivo,* there are no studies on the neurochemical effects of COMT inhibition (and sex) in the hippocampus.

1.1 Sexually dimorphic effects of COMT

There is a wide range of data suggesting that sex affects mRNA expression (Berchtold et al 2008; Derks et al 2008; Vawter et al 2004), brain structure (Xu et al 2000), brain function (Albus et al 1997; Bell et al 2006; Davies & Wilkinson 2006; Ghi et al 1999; Gur et al 1995; Gur & Gur 1990; Sutcliffe et al 2007) and psychopathology (e.g. schizophrenia: Aleman et al 2003; Gur et al 1996; Leung & Chue 2000; Szymanski et al 1995). More importantly for this thesis, there is robust evidence that sexually dimorphic effects are important in the field of COMT research.

First, numerous human and rodent studies have shown that females have 15–40 % lower peripheral COMT activity than males (e.g. Boudikova et al 1990; Briggs & Briggs 1973; Cohn & Axelrod 1971; Floderus et al 1981; Huotari et al 2002; Kaenmaki et al 2009; Philippu et al 1981). This is potentially due to the effects of oestrogens on oestrogen response elements (EREs), which may result in downregulation of the COMT gene (see Chapter 1; Briggs & Briggs 1973; Cohn & Axelrod 1971; Jiang et al 2003; Salama et al 2006; Xie et al 1999). Interestingly,

these sexually dimorphic effects have mostly been demonstrated in the periphery, so the effect of oestrogens on COMT expression in the brain is unclear.

Second, studies on COMT knockout mice have shown sexually dimorphic effects on behaviour (Babovic et al 2008; Gogos et al 1998; Haasio et al 2003; Tammimaki et al 2008) and neurochemistry (Gogos et al 1998; Huotari et al 2002; Kaenmaki et al 2009; Tammimaki et al 2010). With respect to this thesis, the most important such study is by Gogos et al (1998). This paper demonstrated that homozygous COMT knockout female, but not male, mice show increased anxiety on a light/dark exploration task, and that heterozygous COMT-deficient male, but not female, mice display increased aggressive behaviour. In addition to the behavioural results, Gogos et al (1998) also showed that homozygous knockout male, but not female, mice have a 2- to 3-fold increase in the levels of dopamine in frontal cortex (but not hypothalamus or striatal) tissue extracts.

Third, there are reports of sexually dimorphic genetic associations of COMT polymorphisms with a wide range of psychiatric disorders (including OCD, schizophrenia and AD/HD) and related phenotypes. However, as reviewed by Harrison and Tunbridge (2008), many of these findings are not statistically robust: only the gender-specific association of COMT with OCD in males is robust (though also see Alsobrook et al 2002).

In summary, sex differences appear to influence COMT activity (at least in the periphery), COMT-dependent behaviour and the genetic association of COMT with OCD. However, the details of these sexual dimorphisms are yet to be elucidated. Hence, it is important to investigate sex differences in the influence of COMT on brain function. The present study investigates whether there are sex differences in the effect of COMT on neurochemical indices in the hippocampus and other brain regions.

1.2 Moving from humans to rats

There is a marked advantage to studying certain aspects of brain function and psychopathology in rodents. One advantage is that environmental influences, which can interact with genes to alter their effects (Isles & Wilkinson 2008; Rutter et al 2006), can be carefully controlled in an animal laboratory. Another advantage is that it is easier to validate neurochemical results from rodents than from humans, given that most of the existing neurochemical literature on COMT is based on rodents, mainly rats.

The present thesis used rats, because most of the directly relevant COMT literature (e.g. Tunbridge 2004b) was obtained in rats. Also, *in vivo* neurochemical experiments (see Chapter 4) are easier to perform in rats. Finally, there is a wider range of behavioural tasks that can be performed in rats than mice.

Sprague Dawley rats have been previously used in some COMT-related research (Matsumoto et al 2003a; Tunbridge et al 2004b). However, this thesis used pigmented Lister hooded rats, because pigmented rat strains have greater visual acuity than albino strains due to anatomical differences in their visual pathways (Lund 1965). Also, Lister hooded rats display higher locomotor activity than albino rats (Broersen & Uylings 1999; McDermott & Kelly 2008). The greater visual acuity and higher locomotor activity probably decreases the total training time of Lister hooded rats compared to albino rats on behavioural tasks involving visual cues – though this may be somewhat age-, sex- and task-dependent (Andrews 1996). Also, the issue of strain-dependent effects should be borne in mind, especially since different rodent strains can have different baseline COMT activities (Goldstein et al 1980; Roth et al 1990; Weinshilboum & Raymond 1977).

1.3 Using tolcapone to inhibit COMT activity

Since rodents lack the Val^{158}Met SNP and have COMT activity similar to, or higer than, the high activity human Val^{158} form, naturally occurring variations in COMT activity due to the human Val^{158}Met polymorphism could be "modelled" in animal experiments by either generating genetically manipulated mice or by administering COMT inhibitors. Chapters 3-6 use the COMT inhibitor tolcapone (Ro 40-7592) to inhibit COMT activity. Tolcapone (Ro 40-7592) was used, because it is the only centrally acting COMT inhibitor, that is licensed for use in humans (Bonifati & Meco 1999; Mannisto & Kaakkola 1999) and available for purchase.

Since tolcapone is used in the clinic, it is the most extensively studied brain-penetrant COMT inhibitor. Thus, it is known that 10-30 mg/kg tolcapone (given intraperitoneally or orally) significantly inhibits S- and MB-COMT in both rodents and humans (Ceravolo et al 2002; Forsberg et al 2003; Mannisto et al 1992b; Zurcher et al 1990). Neurochemical studies have shown that 10–30 mg/kg tolcapone (given intraperitoneally, intravenously or orally) efficiently blocks both central and peripheral COMT. In mice, a single 30 mg/kg dose of tolcapone causes 99 % inhibition of central COMT after 1 h and inhibition remains 86 % after 6 h (Learmonth et al 2004). Inhibition of central COMT following 30 mg/kg tolcapone (given i.p. to rats) is maximal after 2 h (Tunbridge et al 2004b). This amounts to a half-life of 2–3 h (Dingemanse et al 1995a; Dingemanse et al 1995b; Forsberg et al 2003).

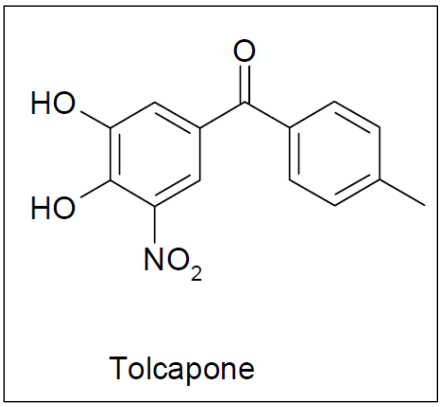


Figure 21 Structure and function of tolcapone
Tolcapone is a highly selective second-generation COMT inhibitor, with virtually no action on other enzymes involved in catecholamine synthesis or metabolism (Backstrom et al 1989; Zurcher et al 1990). Tolcapone binds competitively to the COMT active site and hereby, prevents binding of the substrate to the active site (Borges et al 1997). The selectivity and duration of action of tolcapone are determined by its nitrocatechol structure. The electronegative -NO2 group at the 5-position of its catechol ring decreases the nucleophilicity of the ionised catechol hydroxyls and strongly stabilises the ionised catechol-COMT complex, such that the inhibitor is poorly methylated and gets released very slowly (Ovaska & Yliniemela 1998).

1.4 High performance liquid chromatography coupled with electrochemical detection (HPLC-EC)

High-performance liquid chromatography (also named high pressure liquid chromatography; HPLC) was developed as a reliable and high-sensitivity tool to separate chemical compounds. The technique makes use of the chemical and physical interactions between the compounds in a sample and the chromatography column (Horvath & Lipsky 1966; Horvath et al 1967), as shown using a simplistic diagram in Figure 22.

The present study uses reversed-phase ion-pair chromatography columns to separate compounds of interest in brain tissue extracts (e.g. Pennings & Van Kempen 1979). Following separation, compounds elute from the HPLC column and are detected by their oxidation at an electrochemical detector (e.g. Borchardt et al 1978; Koh et al 1981; Nissinen & Mannisto 1984; Schultz et al 1989; Shoup et al 1980). The more a compound is oxidised, the greater the current produced at the electrode, and therefore, the larger the peak height measured by the external integrator. Chapter 3 and Chapter 4 use a single glassy-carbon electrode and an ISAAC flow cell respectively.

The chemicals measured in the present study were DA and NA, as well as the DA metabolites DOPAC and HVA. The measurement of catecholamine metabolite levels to estimate processes such as metabolism, release and turnover of catecholamines as well as functional activity of central catecholaminergic neurons has been common practice for over 30 years in behavioural pharmacology and in clinical research of psychiatric disorders (e.g. Roth et al 1976; Westerink 1985).

Since COMT converts DOPAC to HVA (Figure), the present study also calculated DOPAC/HVA ratios to give a measure of COMT activity – a method commonly used in the COMT literature (e.g. Tunbridge et al 2004b). Furthermore, DA turnover was estimated by calculating DOPAC/DA ratios (Sharman 1981). Even though this measure is commonly used, it is not the only method of estimating DA

turnover. Other measures used in the literature include calculation of HVA/DA ratios and measurement of DA depletion after inhibition of DA synthesis using alpha-methyl-p-tyrosine (AMPT), an inhibitor of tyrosine hydroxylase (e.g. Hallman & Jonsson 1984).

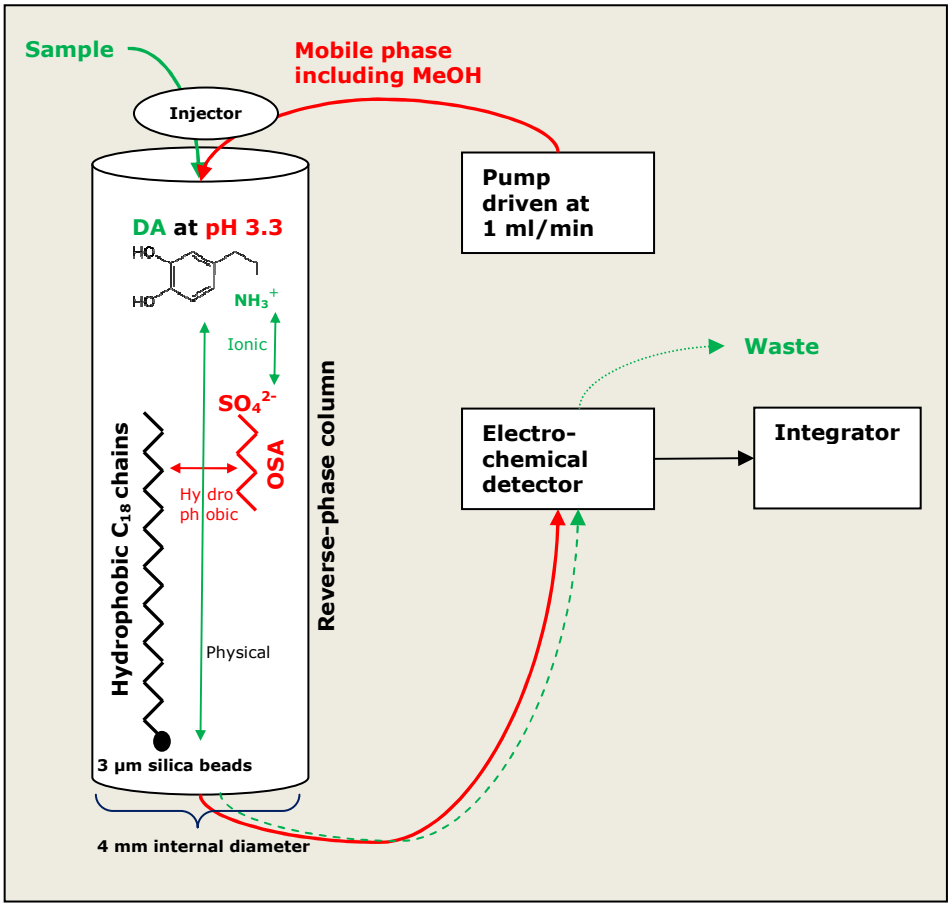


Figure 22 Diagram illustrating the components of a HPLC-EC system and the principles of HPLC using dopamine (DA) as an example compound

The injected sample containing DA is transported to the narrow reverse-phase column (i.e. the column is non-polar, whilst the samples and eluants are polar) by a mobile phase containing 16% methanol (v/v; MeOH; Static parts of the HPLC system are drawn in black, whereas components of the mobile phase and the sample are marked in red and green, respectively.) A decrease in pH (i.e. an increase in availability of H^+) increases retention of acids such as DOPAC and HVA, but has little effect on the amines DA and NA. In contrast, an increase in the concentration of detergent ion-pairing agent 1-Octanesulphonic acid (OSA) increases the retention time of the monoamines, but not that of their acid metabolites. Finally, decreasing the amount of the organic solvent MeOH increases the retention of all molecules. Changes in the ionic strength of the mobile phase, column length, column packing and temperature also impact on the retention of monoamines and their metabolites (see Horvath et al 1967; Sharp 1992).

1.5 Aims and hypotheses

The aims of this study were two-fold:

1. To verify that the neurochemical effects of COMT inhibition in the hippocampus mirror the effects of COMT inhibition in non-hippocampal regions. The hypothesis is that COMT inhibition affects hippocampal catecholamine metabolism such that DOPAC levels increase and HVA levels decrease in hippocampal extracts from tolcapone-treated rats. This has not been previously tested.

2. To investigate whether effects of COMT inhibition on neurochemical indices of catecholamine function are sexually dimorphic. The results of these studies will clarify the biological importance of COMT in the hippocampus in males vs. females.

2 Methods

2.1 Animals

All animal procedures were carried out in the laboratory of Dr Trevor Sharp in the Oxford University Department of Pharmacology in accordance with the Animals (Scientific Procedures) Act 1986 and associated Home Office guidelines. Twenty-four age-matched female (128-154 g) and male (202-230 g) Lister Hooded rats (Harlan, Bicester, UK) were housed in groups of three under standard conditions (lights on 7.30 – 19.30, 21 ± 1 °C temperature, 50 % humidity, ad libitum food and water).

2.2 Tissue collection and sample preparation

Rats were sacrificed 2 h after a single i.p. injection of tolcapone or vehicle (since 2 h was previously shown to be the time of maximum COMT inhibition by tolcapone in the prefrontal cortex; Tunbridge 2004). Prefrontal cortex, striatum, ventral hippocampus, dorsal hippocampus and cerebellum were rapidly dissected out and snap frozen in isopentane and stored at -80 °C.

On the day of HPLC analysis, tissue from the left hemisphere was thawed on ice and weighed. Weighed tissue extracts were homogenized in 750 µl (3000 µl for cerebellum) of 0.06 M perchloric acid (Sigma-Aldrich Company Ltd, Dorset, UK) for 10 s and centrifuged at 15000 rpm for 10 min. 50 µl samples of the supernatants were injected for HPLC analysis using a Rheodyne 7725i manual injector (HiChrom, UK).

2.3 Drugs

Tolcapone (Roche Products Ltd, Welwyn, UK) is a yellow, odourless, non-hygroscopic, crystalline compound. Since tolcapone does not dissolve well, it was made up fresh daily by suspending it in 0.9 % saline with a few drops of Tween-80. To improve suspension of tolcapone, the tolcapone-containing solution was

vortexed and sonicated briefly prior to injections to ensure as accurate dosing as possible. Tolcapone was administered intraperitoneally at a dose of 30 mg/kg. The vehicle was 0.9 % saline with a few drops of Tween-80.

2.4 HPLC-EC analysis

DA, NA, HVA, HMPG, DOPAC and 5HIAA were separated using a Microsorb C_{18} column (3 µm C_{18} Microsorb particles packed in a 100 x 4.6 mm column; Varian Inc, Oxford, UK). The column was eluted isocratically with a degassed mobile phase consisting of 16 % (v/v) methanol, 3mM 1-Octanesulphonic acid (OSA), 1.07mM EDTA and 0.12 M $NaH_2PO_4.H_2O$ at pH 3.3. The column was eluted at a flow rate of 1 ml/min, driven by a 2150 HPLC pump (LKB, Bromma, Sweden). A glassy carbon electrode working at +0.7 V and a salt-bridge Ag/AgCl reference electrode (BAS Instruments, Kenilworth, UK) was used for electrochemical detection (BAS LC-48 amperometric detector, BAS Instruments). An external standard solution containing 0.1 M perchloric acid and 5 pmol of each substance of interest was used to calibrate the HPLC apparatus, converting peak heights to units of concentration on a Milton Roy CI-4100 integrator (Bright Instruments Company Ltd, Huntingdon, UK; see Figure 23). The pmol concentrations were further converted to ng/mg protein units using the wet tissue weights and homogenisation volumes of the tissue samples.

2.5 Data analysis

NA/DA ratios were calculated to give an indication of the relative content of the two catecholamines within each brain region studied. Additionally, DOPAC/HVA and DOPAC/DA ratios were calculated to provide indices of COMT enzyme activity and DA turnover, respectively (see Section 1).

Data are plotted using GraphPad Prism 5.01 (GraphPad Software Inc, La Jolla, CA, USA) and analysed using SPSS 15.0 statistics software (SPSS Inc, Chicago, IL, USA), with brain region as a within-subjects factor, and with sex and

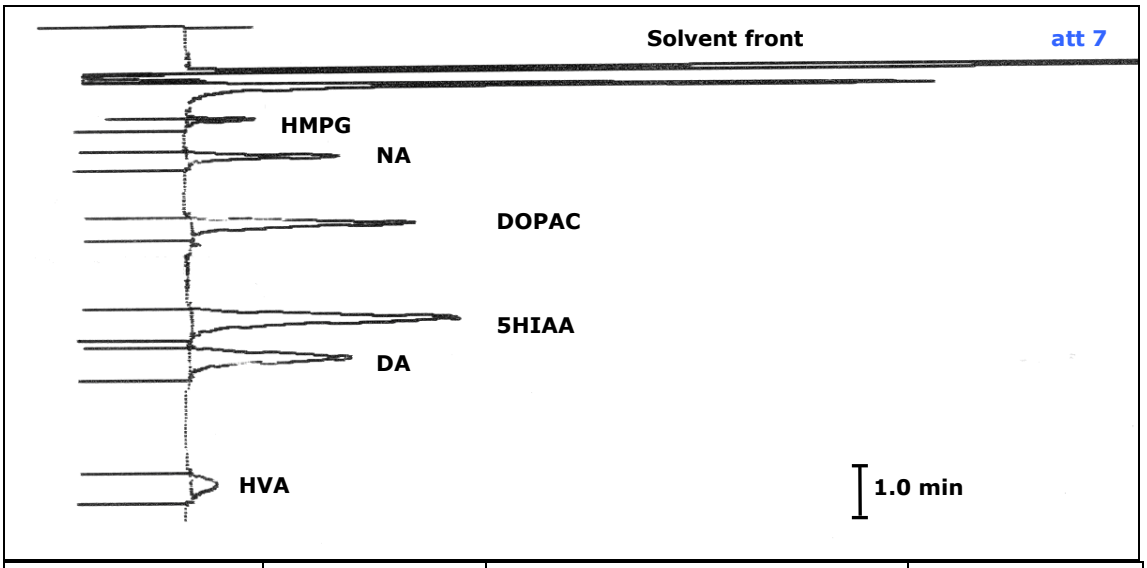
treatment as between-subjects factors. In case of a statistically significant Mauchly's test of sphericity, a Huynh-Feldt correction was used to alter the degrees of freedom and thereby alter the F-ratio significance level.

Three tissue extracts were excluded from the final analysis due to experimental errors. Furthermore, ten individual data points were replaced by group means, because their values were at least two standard deviations from the group mean and hence, may have been contaminants. Notably, this did not influence the results significantly.

Data were initially analysed using repeated-measures analysis of variance (ANOVA) with five levels of the within-subjects factor, corresponding to striatum, prefrontal cortex, cerebellum, dorsal and ventral hippocampus. Statistically significant ($p's<0.05$) and trend-level results ($p's\leq0.1$) were followed up with LSD post-hoc tests. However, statistically significant post-hoc tests following trend-level results were treated with caution.

Following the statistically significant effects of region in all the above ANOVAs, the initial ANOVAs were rerun to conclude what happens in the hippocampus specifically. The new repeated-measures ANOVAs still used brain region as the within-subjects factor, but this time with only two levels corresponding to ventral and dorsal hippocampus tissue extracts. LSD post-hoc tests were carried out as above.

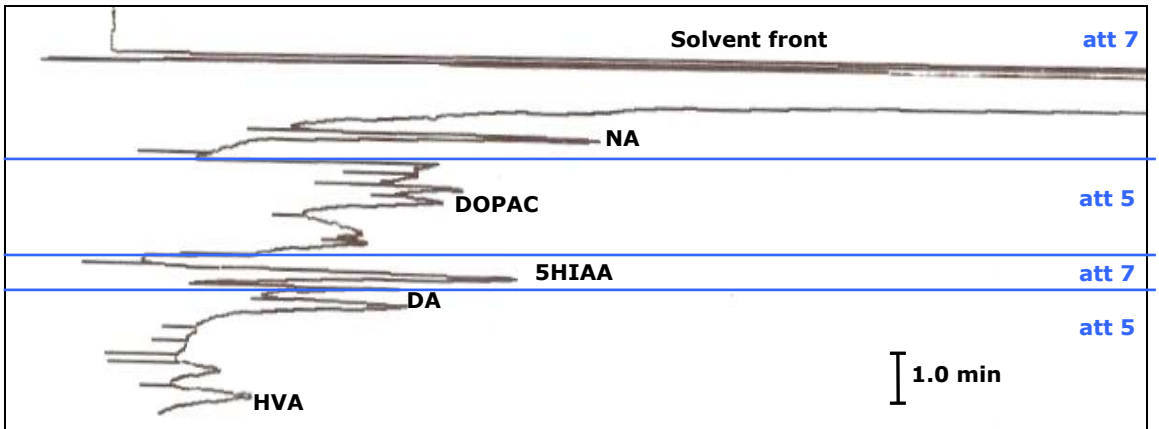
Following repeated-measures ANOVAs, data from individual brain regions were analysed using univariate ANOVAs.



Time (min)	Height (mV)	Concentration (pmol)	Molecule
1.85	3979	5.00	HMPG
2.51	8976	5.00	NA
3.73	12938	5.00	DOPAC
5.44	15517	5.00	5HIAA
6.13	9419	5.00	DA
8.41	1699	5.00	HVA

Figure 23 Example of a HPLC chromatogram corresponding to a standard calibration solution

Peaks of interest are labelled. The retention times and peak height, as well as the corresponding concentrations (5 pmol in standard solutions) are also indicated. The attenuation (att) was set at 7 on the Milton Roy CI-4100 integrator.



Time (min)	Height (mV)	Concentration (pmol)	Molecule
2.23	27700	2.63	NA
2.98	1919	0.20	DOPAC
4.27	30220	2.43	5HIAA
4.69	3565	0.51	DA
6.02	1783	0.25	HVA

Figure 24 Example of a HPLC-EC chromatogram

This prefrontal cortex tissue homogenate sample is taken from a male rat treated with vehicle. The peaks of interest, retention times and peak heights, as well as the corresponding concentrations are indicated. The attenuation (att) was changed on the Spectra Physics SP4400 integrator, as indicated by the blue lines, in order to increase sensitivity for catecholamine detection.

3 Results

3.1 Tissue levels of dopamine and noradrenaline across the rat brain

DA and NA levels were measured in hippocampal, striatal, cortical and cerebellar extracts of vehicle-injected rats. Levels of DA and NA were readily detectable in all regions studied (see Figure 25 and Appendix III for full set of data). Importantly, DA and NA levels were consistently detectable in both dorsal and ventral hippocampus extracts. According to paired sample t-tests, dorsal and ventral hippocampus show similar NA/DA ratios, DA and NA levels (p's>0.5).

Repeated-measures ANOVAs yielded a main effect of region for both DA and NA (p's<0.001; see Appendix III). However, DA levels varied considerably more than NA levels across different brain structures (Figure 25 and Table 6): Hippocampal DA content (~9.8 ng/g tissue) was significantly lower than in striatum (nearly 500-fold; p's<0.001) and prefrontal cortex (3-fold; p's<0.001). In contrast, NA levels were consistently 100-250 ng/g tissue. Nonetheless, hippocampal NA content was significantly higher than in striatum (2-fold; p's=0.001) and prefrontal cortex (~20 %; p's<0.05).

The hippocampal NA/DA ratio was ~24, which was significantly higher than the NA/DA ratio in prefrontal cortex and striatum (p's<0.001), as shown by paired-sample t-tests.

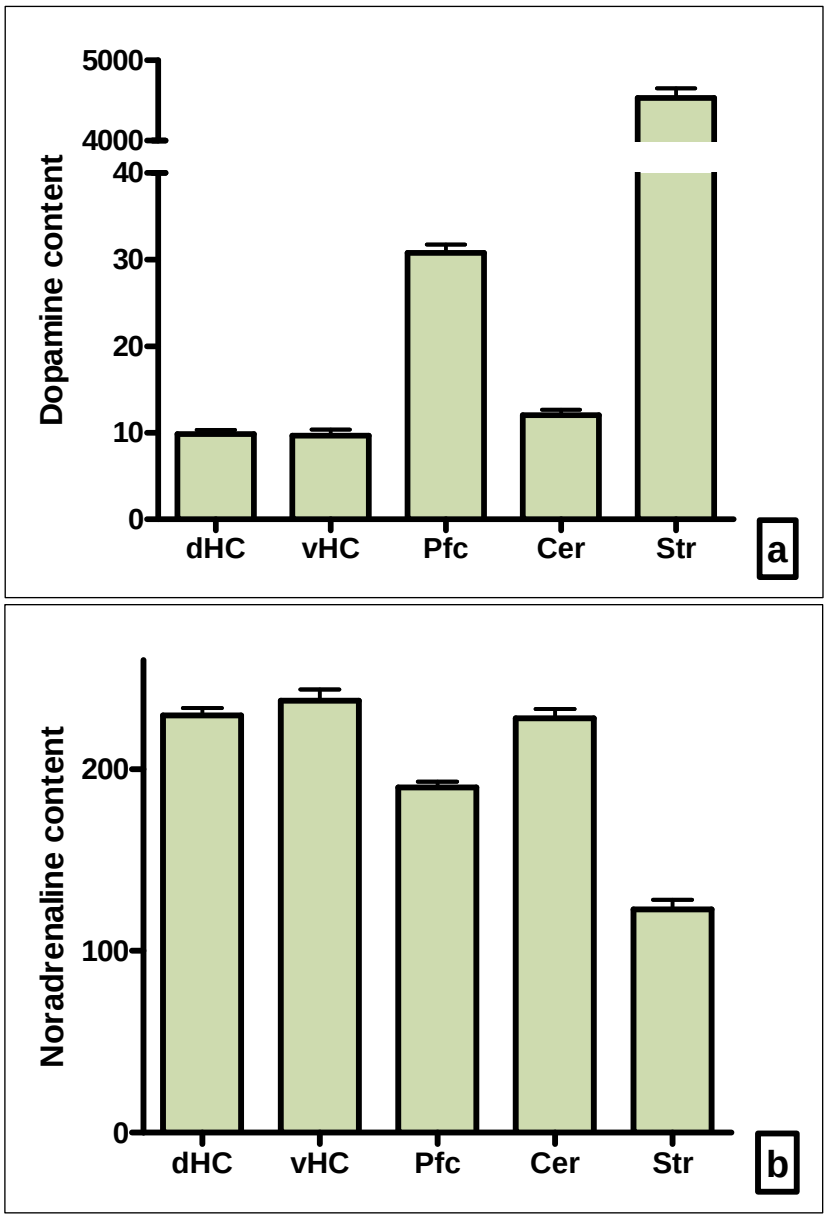


Figure 25 (a) Dopamine and (b) Noradrenaline content in rat brain tissue extracts

Data given in ng/g wet tissue; mean ± SEM; rat n=11 including n=5 females and n=6 males (Cer, cerebellum; dHC, dorsal hippocampus; PFC, prefrontal cortex; Str, striatum; vHC, ventral hippocampus).

Brain region	DA (ng/g)	NA(ng/g)	NA/DA ratio
Cerebellum	12.0±1.9	228.0±16.4	20.4±2.2
Dorsal hippocampus	**9.9±1.4**	**229.7±12.7**	**26.1±3.1**
Prefrontal cortex	30.8±3.1	189.9±10.3	6.5±0.3
Striatum	4535.6±368.3	122.8±17.3	<0.1
Ventral hippocampus	**9.7±2.2**	**237.7±19.1**	**26.1±2.9**

Table 6 Dopamine (DA) **and noradrenaline** (NA) **content in tissue extracts from vehicle-treated rats**

Data given as mean values ± SEM. The NA/DA ratios do not agree with the DA and NA data (in ng/g wet tissue) due to rounding. Total n=11 including n=5 female and n=6 male rats.

3.2 Effect of tolcapone on regional brain levels of dopamine and noradrenaline

DA and NA levels were measured in hippocampal, striatal, cortical and cerebellar extracts from either tolcapone- or vehicle-injected rats. No main effect of tolcapone was present for either DA or NA in repeated-measures ANOVAs including all brain regions (p's>0.4; see Appendix III). Neither were there any statistically significant region*treatment, sex*treatment or region*gender*treatment interactions (p's>0.5). The NA/DA ratio showed a trend-level main effect of treatment ($F_{1,18}$=3.264; p=0.088), whereby NA/DA ratios were slightly lower following tolcapone treatment.

In contrast, the hippocampus-only ANOVA exposed a main effect of treatment, which is statistically significant for DA ($F_{1,19}$=4.826; p=0.041) and at trend-level for NA/DA ($F_{1,19}$=3.778; p=0.067). Compared to vehicle-treated controls, tolcapone-treated rats had increased DA levels by 35 % (10.2 ± 1.2 vs 13.8 ± 1.1 ng/g tissue) and decreased NA/DA ratios by 20% (25.2 ± 1.9 vs 20.1 ± 1.8) in the whole hippocampus, whereas NA levels were only decreased by 4 % (232.9 ± 11.6 vs 222.5 ± 11.0 ng/g tissue). The repeated-measures ANOVAs for DA, NA and NA/DA including dorsal and ventral hippocampus did not reveal any region*treatment interactions (p's>0.1). However, univariate ANOVAs looking at each hippocampal subregion individually suggested that DA levels were increased by tolcapone compared to vehicle-treated controls in the ventral hippocampus ($F_{1,18}$=5.203, p=0.035), but not the dorsal hippocampus ($F_{1,21}$=0.254, p=0.620; Figure 26).

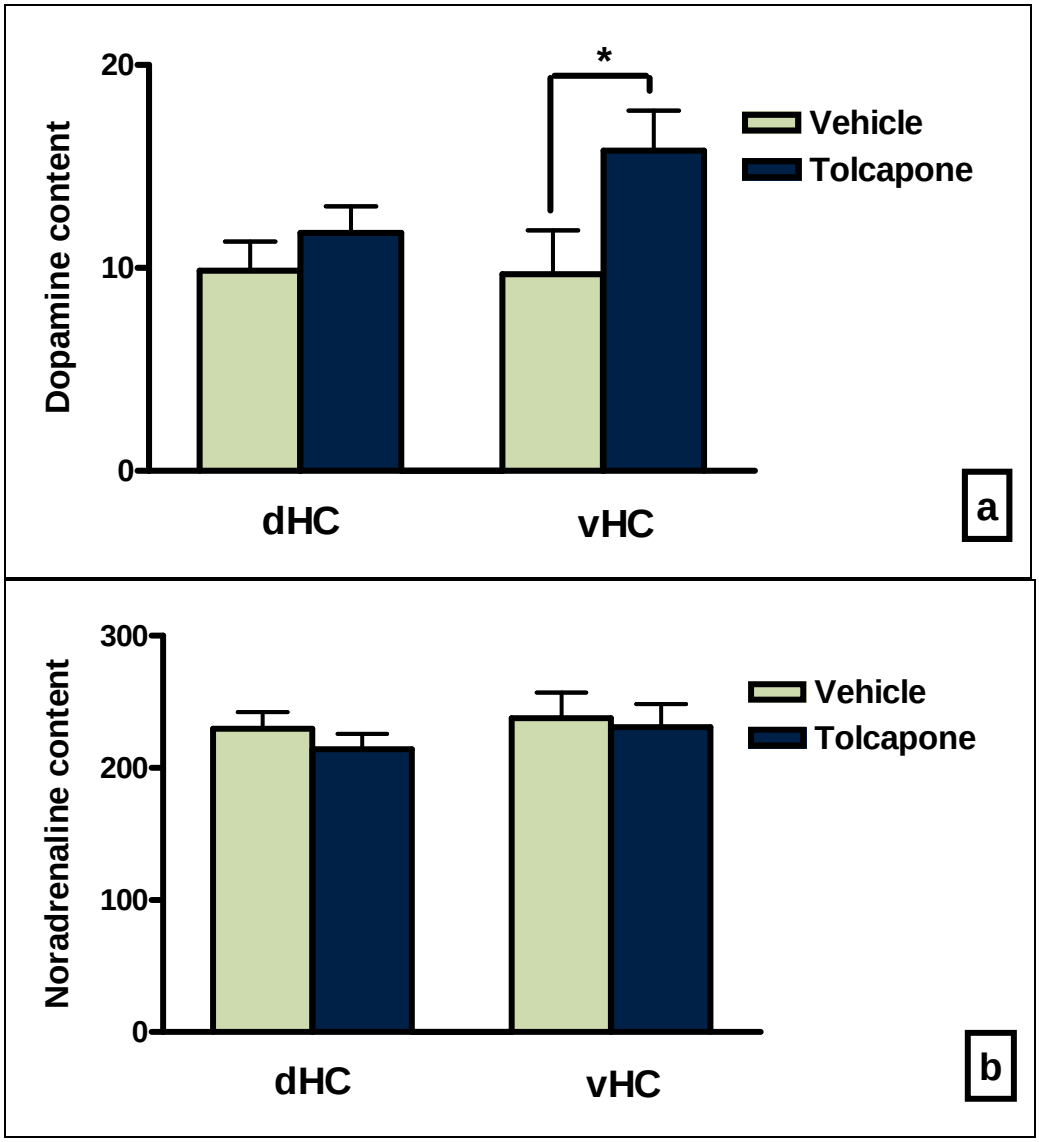


Figure 26 (a) Dopamine and (b) Noradrenaline content in dorsal (dHC) **and ventral** (vHC) **hippocampal extracts**

Data (mean ± SEM in units of ng/g tissue) from tolcapone (n=12) or vehicle-treated (n=11) rats pooled from n=11 female and n=12 male rats (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

3.3 Tissue levels of DOPAC and HVA across the rat brain

Tissue levels of DOPAC and HVA were measured in hippocampal, striatal, cortical and cerebellar extracts from vehicle-injected rats. DOPAC and HVA levels were readily detected in all brain regions studied. ANOVAs on DOPAC, HVA and DOPAC/HVA data revealed main effects of region (p's<0.001; see Appendix III), indicating that the DOPAC and HVA contents vary across the rat brain: Striatal tissue extracts contained highest levels of DOPAC and HVA, which were significantly higher than levels found in hippocampal tissue extracts (p's<0.001). Levels of HVA were generally higher than DOPAC levels, though striatal extracts contained about two-fold higher DOPAC levels than HVA levels (see Table 7). Correspondingly, striatal extracts showed the highest DOPAC/HVA ratios (seven-fold higher than hippocampal DOPAC/HVA ratios).

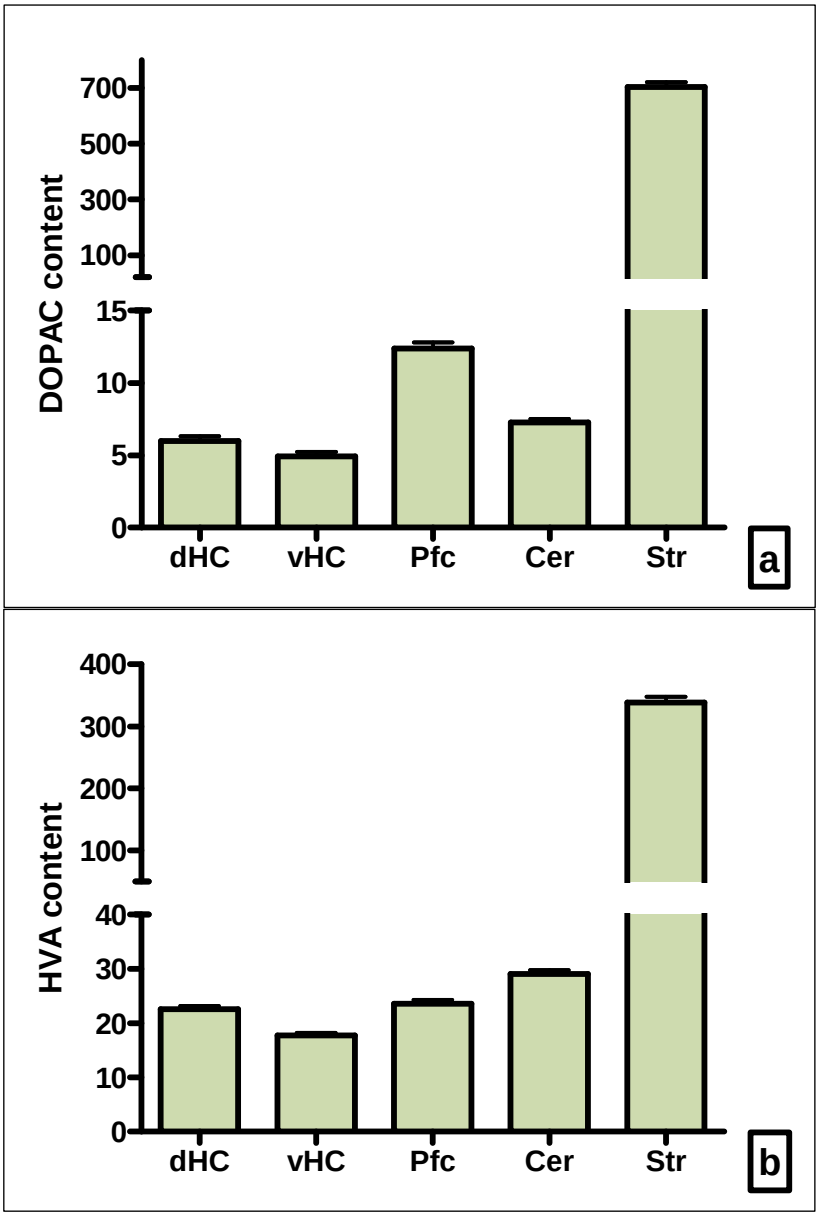


Figure 27 (a) DOPAC and (b) HVA content in rat brain tissue extracts

Mean data ± SEM given in ng/g wet tissue for n=11 rats including n=5 females and n=6 males (Cer, cerebellum; dHC, dorsal hippocampus; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; PFC, prefrontal cortex; Str, striatum; vHC, ventral hippocampus)

Brain region	DOPAC (ng/g)	HVA (ng/g)	DOPAC/HVA ratio
Cerebellum	7.3±0.8	29.0±2.2	0.3±0.1
Dorsal hippocampus	**6.0±1.1**	**22.6±1.8**	**0.3± <0.1**
Prefrontal cortex	12.4±1.3	23.6±2.2	0.5±0.4
Striatum	704.0±53.7	338.2±30.6	2.1±1.6
Ventral hippocampus	**4.9±0.9**	**17.8±**	**0.3±0.1**

Table 7 DOPAC and HVA content, as well as DOPAC/HVA ratios in striatal, cortical, hippocampal and cerebellar tissue extracts from vehicle-treated rats

Data (in ng/g wet tissue) given as mean values ± SEM for n=11 rats, including n=5 females and n=6 males (DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid)

3.4 Effect of tolcapone on regional brain levels of DOPAC and HVA

Tissue levels of DOPAC and HVA were measured in hippocampal, striatal, cortical and cerebellar extracts from tolcapone- and vehicle-injected rats. COMT inhibition using tolcapone yielded an increase in DOPAC levels and a decrease in HVA levels compared to vehicle-treated controls in all regions studied.

Repeated-measures ANOVAs indicated a significant main effect of treatment for DOPAC, HVA and DOPAC/HVA (Appendix III): Tolcapone significantly increased DOPAC levels (21 %; $F_{1,18}$=4.5; p=0.048), decreased HVA levels (50 %; $F_{1,18}$=23.0; p<0.001) and increased DOPAC/HVA ratios compared to vehicle-treated controls across all brain regions examined (195 %; $F_{1,18}$=25.3; p<0.001).

Additionally, the above ANOVAs also revealed significant region*treatment interactions for DOPAC, HVA and DOPAC/HVA (p's<0.05; Appendix III). According to LSD post-hoc tests, tolcapone significantly increased DOPAC levels compared to vehicle controls in all areas, except the dorsal hippocampus (where p=0.1). HVA levels were significantly decreased only in striatum and prefrontal cortex (p's<0.01), whereas DOPAC/HVA ratios were significantly increased compared to vehicle controls throughout the rat brain (p's≤0.005; see Figure 28).

Moreover, the above results relating to DOPAC and DOPAC/HVA were replicated in the hippocampus-only ANOVAs: Tolcapone enhanced DOPAC/HVA (163 %; $F_{1,19}$=6.968; p=0.016) and DOPAC levels significantly in the total hippocampal tissue extracts (by 183 % from 6.3 ± 0.9 to 11.5 ± 0.9 ng/g tissue; $F_{1,19}$=17.578; p<0.001).

3.5 Estimates of dopamine turnover across the rat brain

DOPAC/DA ratios were calculated from tissue DA and tissue DOPAC levels (see above) as an estimate of DA turnover. DA turnover varied three-fold across the rat brain, and was increased in tolcapone-treated rats compared to vehicle-treated controls.

Repeated-measures ANOVAs on DOPAC/DA ratios demonstrated a main effects of region (p's<0.001). Highest levels of DA turnover were observed in cerebellum, closely followed by hippocampal DA turnover. Lowest DA turnover was observed in the striatum (Table 8). Additionally, repeated-measures ANOVA on DOPAC/DA identified a significant main effect of treatment and a region*treatment interaction (p's≤0.003). LSD post-hoc tests show that tolcapone increases DOPAC/DA ratios compared to vehicle-treated controls in all tissue extracts (p's≤0.006), apart from ventral hippocampus extracts (p=0.473). LSD post-hoc tests following a statistically significant region*treatment interaction in an ANOVA on hippocampus-only DOPAC/DA data (p=0.002) suggests that tolcapone significantly impacted DOPAC/DA in the dorsal (p=0.001), but not the ventral hippocampus (p=0.956; see below).

Brain region	DOPAC/DA
Cerebellum	0.68±0.23
Dorsal hippocampus	**0.62±0.15**
Prefrontal cortex	0.44±0.08
Striatum	0.16±0.01
Ventral hippocampus	**0.53±0.09**

Table 8 DOPAC/DA ratios in striatal, cortical, hippocampal and cerebellar tissue extracts from vehicle-treated rats

Data given as mean values ± SEM for rat n=11, including n=5 females and n=6 males (DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid).

3.6 Hippocampal subregion-specific effects

The present study was designed to study catecholamine levels and DA metabolism in hippocampal extracts in comparison with striatal, cerebellar and cortical extracts. Hence, this study was also interested in potential dorso-ventral dissociations in DA metabolism within the hippocampus. One such dissociation was that the dorsal hippocampus had more DOPAC and HVA than the ventral hippocampus. Also, tolcapone-treated rats had higher DOPAC and DOPAC/HVA compared to vehicle-treated controls in the dorsal, but not ventral hippocampus.

Repeated-measures ANOVAs on hippocampal DOPAC and HVA data showed that the dorsal hippocampus had significantly more DOPAC and HVA than the ventral hippocampus (p's≤0.001). There was also a drug*hippocampal region interaction for DOPAC ($F_{1,19}$=10.543; p=0.004) and, at trend-level, DOPAC/HVA ($F_{1,19}$=3.212; p=0.089). LSD post-hoc tests indicate that both DOPAC and DOPAC/HVA were increased by tolcapone compared to vehicle in the dorsal but not the ventral hippocampus (p's>0.1; see Figure 29).

As discussed above, tolcapone also had subregion specific effects on DOPAC/DA and DA. Namely, within the dorsal hippocampus, tolcapone increased DOPAC/DA over two-fold compared to vehicle-treated controls (from 0.6 ± 0.1 to 1.4 ± 0.1), whereas DOPAC/DA ratios were unchanged in the ventral hippocampus (0.6 ± 0.1; Table 8). In contrast, DA levels were increased in ventral, but not dorsal hippocampus (see above).

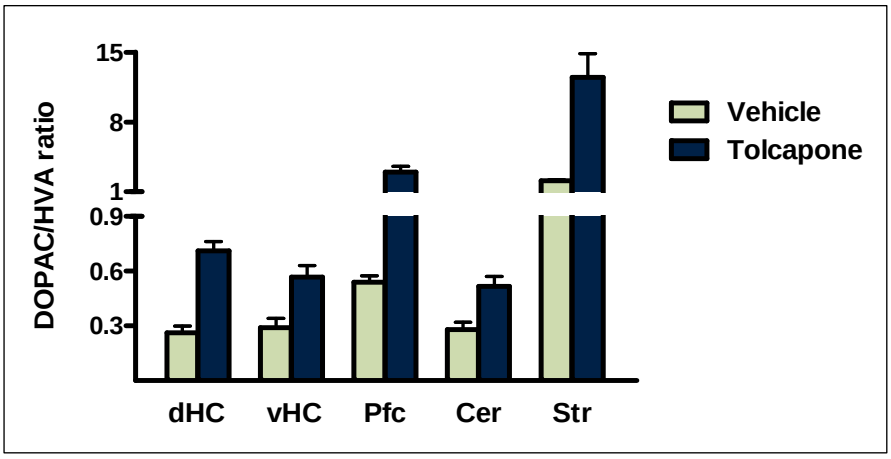


Figure 28 DOPAC/HVA ratio in rat brain tissue extracts

Mean ± SEM data given for tolcapone (n=12) or vehicle-treated (n=11) rats including n=11 females and n=12 males (Cer, cerebellum; dHC, dorsal hippocampus; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; PFC, prefrontal cortex; Str, striatum; vHC, ventral hippocampus).

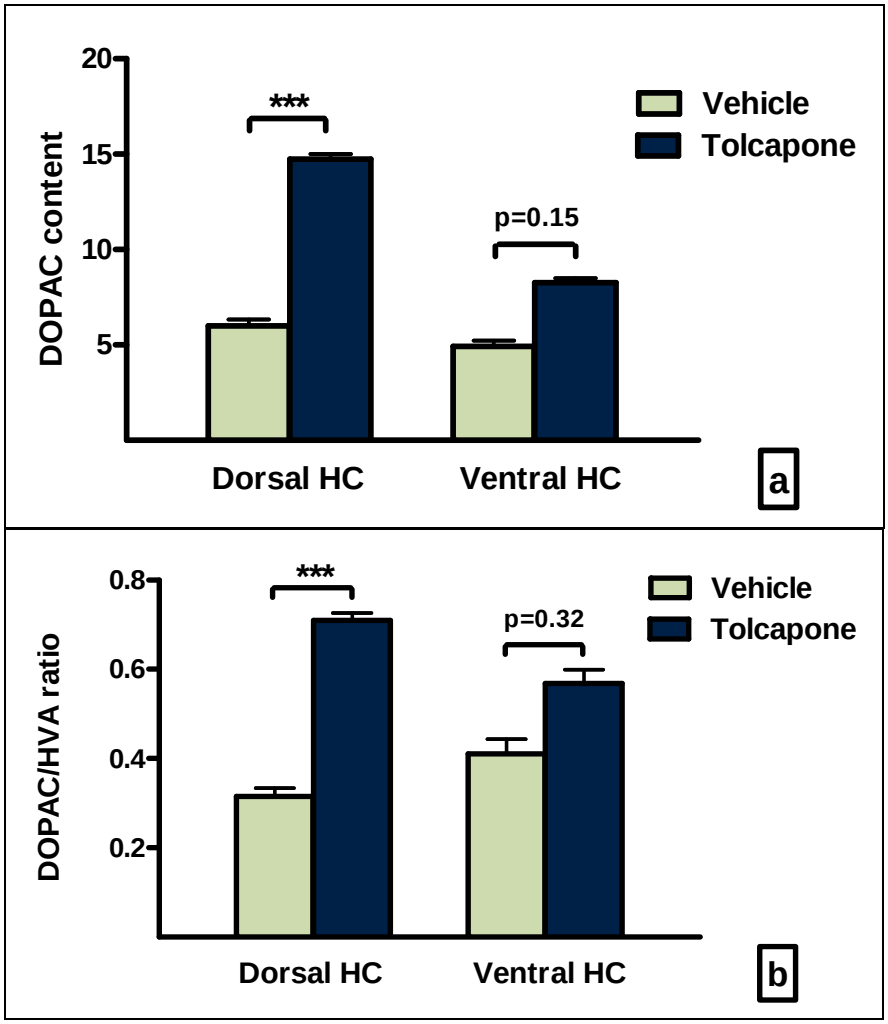


Figure 29 (a) DOPAC content and (b) DOPAC/HVA in the dorsal and ventral hippocampus

Mean ± SEM data given in ng/g tissue for tolcapone (n=12) or vehicle-treated (n=11) rats including n=11 females and n=12 males (* p<0.05, ** p<0.01, *** p<0.001; HC, hippocampus; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid).

3.7 Sex-specific effects

The second aim of this study (see Section 1.5) was to investigate whether effects of COMT inhibition on neurochemical indices of catecholamine function are sexually dimorphic. This was done by comparing levels of DA, NA, DOPAC and HVA in tissue extracts from female rats with levels present in extracts from male rats. The main results of this analysis were that females had higher DOPAC/HVA ratios than males, and that tolcapone-treated female, but not male, rats had higher the DOPAC/HVA ratio compared to vehicle-treated rats.

A main effect of sex was revealed by repeated-measures ANOVAs of DOPAC, HVA and DOPAC/HVA data across all brain regions (p's<0.1; see Appendix III). DOPAC/HVA ratios were significantly higher in females than in males ($F_{1,18}$=4.619; p=0.045). Correspondingly, DOPAC levels across all brain regions were 20 % greater in females than males ($F_{1,18}$=3.811; p=0.067), whereas HVA levels were 22 % lower in females than males ($F_{1,18}$=3.058; p=0.097).

ANOVAs including data from all brain regions studied showed a sex*treatment interaction for the DOPAC/HVA data ($F_{1,18}$=4.458; p=0.049). LSD post-hoc tests suggested that tolcapone affects females more than males, since tolcapone significantly increased the DOPAC/HVA ratio in females ($F_{1,18}$=25.486; p<0.001) – but not in males ($F_{1,18}$=4.253; p=0.054). LSD post-hoc tests on the whole brain HVA data also suggested that tolcapone influences females (p<0.001) more than males (p=0.056), though the sex*treatment interaction for the HVA data was only at trend-level ($F_{1,18}$=3.649; p=0.072).

Additionally, the repeated-measures ANOVAs across all brain regions also yielded region*sex interactions for DOPAC/HVA ($F_{4,72}$=5.208; p=0.026), HVA ($F_{4,72}$=4.263; p=0.045), and at trend level for DOPAC ($F_{4,72}$=3.370; p=0.075). LSD post-hoc tests indicate that the overall region*sex interactions are driven by non-hippocampal regions. Namely, sex effects on HVA, DOPAC and/or DOPAC/HVA levels are present in prefrontal cortex, striatum and cerebellum (p's<0.1), whereas dorsal and ventral hippocampal extracts do not show an effect of sex (p's>0.1).

Finally, region*sex*treatment interactions were observed for DOPAC/HVA ($F_{4,72}$=4.476; p=0.039) and, at trend-level, for HVA across all brain regions ($F_{1,18}$=3.256; p=0.079). LSD post-hoc tests indicated that gender differences for DOPAC/HVA and HVA were only observed in tolcapone-treated striatal and cortical extracts (p's<0.025).

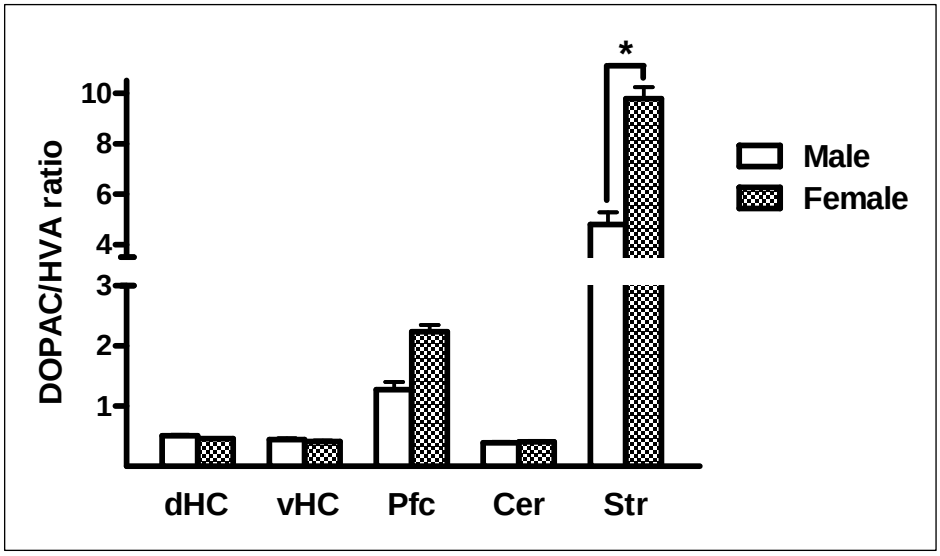


Figure 30 DOPAC/HVA ratio in female (n=11) and male (n=12) rat brain tissue extracts
Mean data ± SEM pooled from n=11 vehicle and n=12 tolcapone-treated rats (* p<0.05, ** p<0.01, *** p<0.001; Cer, cerebellum; dHC, dorsal hippocampus; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; PFC, prefrontal cortex; Str, striatum; vHC, ventral hippocampus).

The region-specific results of the analysis across all brain regions were followed up by investigating the effects of sex separately in each brain region using univariate ANOVAs. These showed an effect of sex and a sex*treatment interaction on striatal DOPAC/HVA (p's<0.05; see Figure 30). Similarly, cortical extract DOPAC levels were significantly affected by sex and a sex*treatment interaction (p's<0.01). In cerebellum, the DOPAC/HVA and DOPAC data yielded sex*treatment interactions (p's<0.05), whereas the HVA data only revealed a main effect of sex (p=0.025). LSD post-hoc tests indicated that sex differences in DOPAC/HVA and HVA were only observed in tolcapone-treated striatal and cortical extracts. Notably, the dorsal and ventral hippocampus did not show any effect of sex or a sex*treatment interaction (p's>0.05).

4 Discussion

This *ex vivo* neurochemical study is the first study to investigate the effects of tolcapone on catecholamine levels and DA metabolism in the hippocampus. This study demonstrated that hippocampal extracts of tolcapone-treated rats have higher DOPAC levels and DOPAC/HVA ratios compared to vehicle-treated rats. A more detailed analysis revealed that tolcapone-treated rats had higher DOPAC and DOPAC/HVA than vehicle-treated controls in the dorsal but not ventral hippocampus. The present study also highlighted some sexually dimorphic effects of tolcapone. However, these effects were driven by data from striatal, cortical and cerebellar extracts, and are hence of limited interest to the hypotheses tested within this thesis.

4.1 COMT plays a role in regulating dopamine metabolism in the hippocampus

The present study demonstrates significant neurochemical effects of COMT inhibition on indices of DA metabolism in all rat brain regions tested, including the hippocampus. Tolcapone increased DOPAC/HVA ratios throughout the rat brain, and enhanced DOPAC levels in all areas except the striatum. Tolcapone also decreased HVA levels – though only in prefrontal cortex and striatum. These results agree well with published ex vivo tissue studies and in vivo microdialysis experiments, which have shown tolcapone-induced (either alone or in combination with other drug treatments) DOPAC accumulation and HVA depletion in striatum, nucleus accumbens, caudate nucleus and prefrontal cortex (Acquas et al 1992; Brannan et al 1992; Budygin et al 1997; Kaakkola & Wurtman 1993; Li et al 1998; Maj et al 1990; Mannisto et al 1992a; Napolitano et al 2003; Tunbridge et al 2004b).

Importantly, ANOVAs including only data from hippocampal extracts showed that tolcapone-treated rats had significantly increased DOPAC and slightly higher DOPAC/HVA than vehicle-treated controls. This provides evidence that COMT is

involved in DA metabolism in the hippocampus. This result also provides evidence that 30 mg/kg tolcapone administered i.p. is a sufficient dose to achieve a significant change in DOPAC/HVA, which suggests that 30 mg/kg tolcapone resulted in significant COMT inhibition in the hippocampus. Hence, this dose was used for the studies described in chapters 4-6. In order to validate this result and gain insight into the timecourse of tolcapone-induced changes in hippocampal dopamine metabolite levels, Chapter 4 uses 30 mg/kg in an *in vivo* microdialysis experiment.

4.2 Tolcapone increases dopamine, but not noradrenaline levels, in the hippocampus

ANOVAs including data from all brain regions tested revealed that tolcapone had no effect on catecholamine levels. This result was somewhat in agreement with *in vivo* microdialysis studies (Lapish et al 2009; Tunbridge et al 2004b), which have found an effect of tolcapone on stimulated, but not basal, DA levels in the extracellular fluid of the prefrontal cortex.

In contrast, ANOVAs on data from dorsal and ventral hippocampal extracts suggested that tolcapone significantly increased DA levels by 35 % and decreased NA/DA ratios by 20 % - whereas NA levels were unaffected by treatment. Such dissociation between the effects of decreased COMT activity on DA and NA levels is consistent with two *in vivo* neurochemical studies in rodents (Gogos et al 1998; Tunbridge et al 2004b). However, the effect of tolcapone on DA is perhaps surprising, given that only one previous neurochemical study has found tolcapone to impact baseline catecholamine levels – though also this study was done *in vivo* (Huotari et al 2001). Other neurochemical experiments, which mostly use microdialysis for sample collection, have not found effects of tolcapone on DA or NA (Acquas et al 1992; Brannan et al 1992; Budygin et al 1997; Janhunen et al 2005; Kaakkola & Wurtman 1993; Li et al 1998; Maj et al 1990; Mannisto et al 1992a; Napolitano et al 2003; Tunbridge et al 2004b). Thus, tolcapone-induced changes in DA may be specific to hippocampal neurochemistry. However, the possibility that

tolcapone-treated rats had higher DA levels before injection than vehicle-treated rats cannot be excluded based on this study. Hence, in order to gather more data on the effects of tolcapone on hippocampal DA metabolism, Chapter 4 studies the effects of tolcapone on DA levels in hippocampal microdialysis samples.

4.3 Dorso-ventral differences in hippocampal neurochemistry

The present study found that in vehicle-treated rats, dorsal hippocampal tissue extracts had higher DOPAC content than ventral hippocampal tissue extracts (Figure 29a). This is consistent with an *ex vivo* neurochemical study by Ishikawa et al (1982), which also found significantly higher DOPAC levels in rat tissue extracts from the dorsal quarter of the hippocampus than other parts of the hippocampus.

The present study also illustrated that tolcapone increases DOPAC to a greater extent in dorsal than ventral hippocampus extracts. Similarly, DOPAC/HVA was increased by tolcapone in the dorsal, but not the ventral hippocampus. Moreover, tolcapone affected DOPAC/DA ratios (which are thought to estimate DA turnover) in the dorsal, but not ventral hippocampus (when analysed using one-way ANOVAs but not using a repeated-measures ANOVA). Hence, COMT might play a greater role in dopamine metabolism in the dorsal hippocampus. However, this conclusion may not be consistent with the observation that tolcapone affected DA levels in ventral but not dorsal hippocampal extracts. Also, the mechanisms underlying these dorso-ventral differences are unclear.

One possibility is that the clearance or transportation of DOPAC from the site of COMT inhibition is more efficient in the ventral than the dorsal hippocampus. A potential mechanism for this may be that tolcapone treatment amplifies the naturally higher DOPAC content in the dorsal than the ventral hippocampus, which saturates the pathways for DOPAC breakdown or transport in the dorsal but not the ventral hippocampus.

Another possibility is that the accumulating DOPAC levels may be involved in a negative feedback loop, which significantly decreases DA synthesis in the

ventral but not the dorsal hippocampus. However, this later option may not be in agreement with the finding that tolcapone causes accumulation of DA in ventral but not dorsal hippocampal extracts. In order to study these options further, Chapter 4 investigates whether tolcapone influences extracellular DOPAC levels in the same subregion-specific manner as tolcapone impacts intracellular DOPAC levels in the present study.

4.4 Sex-specific effects of tolcapone are limited to non-hippocampal brain regions

DOPAC/HVA ratios are used as a measure of COMT activity. Hence, it is interesting to note that DOPAC/HVA ratios were significantly higher in vehicle-treated females than in vehicle-treated males. This baseline variability in central COMT activity is in agreement with human and rodent studies, which have found lower peripheral COMT activity in females than males (e.g. Boudikova et al 1990; Floderus et al 1981; Huotari et al 2002; Kaenmaki et al 2009; Philippu et al 1981). Furthermore, tolcapone affected DOPAC/HVA ratios to a greater extent in females than males. This appears to be somewhat consistent with a neurochemical study by Käenmäki et al (2009), which found increased striatal DOPAC levels in female, but not male, S-COMT knockout mice.

The sex effects on HVA, DOPAC and/or DOPAC/HVA levels revealed by this study are present in prefrontal cortex, striatum and cerebellum, whereas hippocampal extracts do not show any effect of sex. On one hand, this result suggests that the striatum, prefrontal cortex and cerebellum are candidate brain regions to explain the reported sexually dimorphic effects of COMT. Hence, targeted COMT research on prefrontal cortex-, cerebellum- and striatum-dependent functions may yield a more consistent body of evidence regarding COMT-related sex differences (seeHarrison & Tunbridge 2008 for a summary of the controversies to date). On the other hand, this result suggests that the hippocampus is unlikely to yield interesting sex dimorphisms of COMT. Thus, other studies in this thesis will

only study rats of one sex. Male rats are chosen, because the existing COMT literature focuses on male rodents, and because female studies must account for confounding factors related to the menstrual cycle.

4.5 Summary of Chapter 3

This chapter described the first *ex vivo* neurochemical study investigating the effects of tolcapone on hippocampal catecholamine levels and DA metabolism. The study demonstrated that tolcapone also influences hippocampal neurochemistry by showing that hippocampal extracts of tolcapone-treated rats have higher DOPAC levels and DOPAC/HVA ratios compared to vehicle-treated rats. Perhaps somewhat paradoxically hippocampal NA and HVA levels were not affected by COMT inhibition. Interestingly, the observation that tolcapone affects tissue levels of DA, but not NA, perhaps implies that COMT is more relevant for controlling hippocampal DA than NA transmission (see Chapter 7 for further discussion).

A more detailed analysis revealed that tolcapone-treated rats had higher DOPAC, DOPAC/HVA and DOPAC/DA than vehicle-treated controls in the dorsal but not ventral hippocampus. Conversely, DA levels appeared to be more affected by tolcapone in ventral than dorsal hippocampal extracts. The mechanisms for these dorso-ventral dissociations remain to be elucidated and may involve anatomical differences, or pharmacodynamic and pharmacokinetic differences between hippocampal regions. These dorso-ventral differences are further investigated and discussed in chapters 4 – 7.

Finally, this tissue extract study showed sexually dimorphic effects of tolcapone on DOPAC, HVA and DOPAC/HVA in striatal, cerebellar and cortical extracts. These agree well with reports of natural variation between female and male peripheral COMT activities in humans (Section 1.1), though the mechanisms for this effect in the CNS are unknown (see Chapter 7). Since no sex differences were observed in the hippocampus, this thesis will not study sex-specific effects of COMT further.

Taken together, the above results suggest a sex- and region-specific role for COMT in the regulation of DA metabolism throughout the rat brain. Importantly, this study provides novel evidence for the involvement of COMT in hippocampal DA metabolism.

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Chapter 4

Impact of COMT inhibition on extracellular levels of catecholamines in the hippocampus

Linda Maria Laatikainen

DPhil thesis on the role of COMT in hippocampal function

1 Introduction.

There are almost no HPLC studies of hippocampal DA metabolism *in vivo* due to the low levels of DA in this brain region (Table 11). Thus, the present study was designed to investigating the effects of tolcapone on both basal and stimulated DA, NA, DOPAC and HVA levels in dorsal and ventral hippocampal perfusates. The measurement of DA and NA was unsuccessful, because hippocampal catecholamine levels were very close to the detection limit of the HPLC assay. Nonetheless, the present study provides valuable information on the effects of tolcapone on extracellular DOPAC and HVA levels in the hippocampus.

In addition to providing valuable *in vivo* data on the involvement of COMT in hippocampal neurochemistry, the present study investigates hippocampal dorso-ventral dissociations and elucidates the dynamics of pharmacological COMT inhibition induced by an i.p. injection of 30 mg/kg tolcapone in rats (in order to inform the design of the behavioural experiments discussed in Chapter 5 and Chapter 6).

1.1 *In vivo* microdialysis

The study described in Chapter 3 studied tolcapone-induced changes in hippocampal neurochemistry using homogenated tissue preparations. In order to improve temporal and spatial resolution, as well as to avoid post- and peri-mortem effects, an *in vivo* method was used in Chapter 4 to complement the tissue extract data from Chapter 3. Given that hippocampal catecholamine levels are very low, microdialysis was the *in vivo* method of choice for investigating the effect of COMT inhibition on extracellular levels of catecholamines and their metabolites in the hippocampus.

In vivo microdialysis has been used for over 30 years to measure molecules in the extracellular space in a wide range of behavioural, pharmacological and physiological studies (Bourne 2003). Intracerebral microdialysis involves

stereotaxically implanting a perfused microdialysis probe containing a semi-permeable membrane into the brain (Benveniste & Huttemeier 1990; Sharp 1992; Westerink & Tuinte 1986). Depending on their size, molecules from the extracellular space can selectively diffuse across the membrane into the probe, allowing them to be quantified in the perfusate. The use of a semi-permeable membrane prevents the direct washing of brain tissue.

Microdialysis has previously been used to measure extracellular concentrations of DA, NA and their metabolites in the rat brain (e.g. Carlsson et al 1986; Cenci et al 1992; Sharp et al 1987; Westerink & Tuinte 1986). However, there are relatively few studies in which *in vivo* microdialysis has been used to study catecholamine metabolism in the hippocampus (Table 11). Furthermore, there has only been one microdialysis study investigating the effects of tolcapone on hippocampal catecholamines. However, this study did not investigate DA metabolism and was not performed in awake animals, but instead only looked at NA and MHPG levels in the hippocampi of anaesthetised rats (Li et al 1998). Therefore, it is relevant to use *in* vivo microdialysis, coupled with HPLC-EC, to examine the effect of tolcapone on hippocampal catecholamine metabolism in awake, behaving rats.

1.2 Dissociations along the septotemporal axis of the hippocampus

Due to the labour-intensive nature of microdialysis, it is not feasible to study the neurochemical effects of COMT inhibition in all the brain regions studied in Chapter 3. Hence, the present study only dialysed the hippocampus - a region in which tolcapone-induced neurochemical changes have not been explored *in vivo* in awake animals. Microdialysis experiments were performed separately in ventral and dorsal hippocampus, because there is evidence for functional, anatomical and molecular differentiation along the dorso-lateral axis of the hippocampus.

The main functional evidence for dorso-ventral dissociations in the hippocampus comes from animal lesion studies (e.g. Bannerman et al 2004b;

Bannerman et al 1999; also see Deacon et al 2002a; McHugh et al 2004). These suggest that the dorsal (septal) part of the hippocampus is important for learning memory (see Chapter 6 for further details). In contrast, the ventral (temporal) hippocampus is proposed to underlie anxiety-type behaviours (see Chapter 5 for further details).

The preferential involvement of the ventral hippocampus in anxiety and dorsal hippocampus in spatial information processing is consistent with their anatomical connectivity (Barkus et al 2010). The ventral hippocampus is well connected to the PFC, HPA axis and amygdala, all of which are known to be involved in the neural networks underlying responses to stress, arousal, fear, anxiety and reward. In contrast, the dorsal hippocampus receives visuo-spatial information from the sensory cortices (Moser & Moser 1998). Moreover, DA and NA innervation (Gasbarri et al 1994b; Oleskevich et al 1989; Verney et al 1985), DA receptor affinities (Bruinink & Bischoff 1993) and DA metabolite levels (see Chapter 3) may exhibit dorso-ventral differences in the hippocampus (see Chapter 1). This provides further anatomical evidence that dorso-ventral dissociations in the hippocampus could have functional consequences via differences in catecholamine signalling.

Finally, in addition to the anatomical and behavioural differentiation along the dorso-lateral axis of the hippocampus, there is also molecular evidence for dorso-ventral differences within the hippocampus. In a recent microarray study, Christensen et al (2010) found 229 genes that were differentially expressed between dorsal and ventral granular cell layer of the hippocampus.

1.3 Aims and hypotheses

The aim of the present microdialysis study was to support the *ex vivo* neurochemical results presented in Chapter 3 by measuring the effects of COMT inhibition on extracellular DA, NA, DOPAC and HVA levels in dorsal and ventral hippocampal perfusates. In addition to providing valuable *in vivo* data on the

involvement of COMT in hippocampal neurochemistry, the present study aimed to investigate further hippocampal dorso-ventral dissociations and to provide information on the time course of tolcapone-induced changes in catecholamine metabolism. The hypothesis was that COMT inhibition enhances extracellular DOPAC levels and attenuates extracellular HVA levels in the hippocampus, similar to the results obtained from tissue homogenates in Chapter 3.

The present study also aimed to examine the effects of tolcapone on catecholamine levels both at baseline and when their efflux is locally stimulated. Perfusion of the probe with high potassium causes local depolarisation and is thus a physiological method of causing DA and NA efflux (Sharp 1992). Both basal and stimulated catecholamine levels can be determined within the same rats, allowing a reduction in the number of animals required and increasing the statistical power of the study, since within-subjects approaches can be used. If the hippocampal catecholamine systems are more similar to the prefrontal cortical than the striatal systems, tolcapone may be expected to affect DA, but not NA levels in the hippocampus (given previous neurochemical studies in the rodent prefrontal cortex; Gogos et al 1998; Kaenmaki et al 2010; Tunbridge et al 2004b; Yavich et al 2007).

2 Methods

The microdialysis and HPLC-EC procedures were developed and optimised over a period of 18 months in order to increase the margin between the levels of DA in the hippocampus and the detection limit of HPLC-EC (for a summary, see Appendix IV). The method used for the final cohort of 20 rats is described here.

2.1 Animals

All animal procedures were carried out in the laboratory of Dr Trevor Sharp in accordance with the Animals (Scientific Procedures) Act 1986 and associated Home Office guidelines.

Twenty male Lister Hooded rats (192 – 268 g, Harlan, Bicester, UK) were housed in groups of three at standard conditions (lights on 7.30 – 19.30, 21 ± 1 °C temperature, 50 % humidity, ad libitum food and water) for minimum three days prior to surgery.

2.2 Surgery

The rats were anaesthetised with isoflurane through a rat anaesthesia mask (David Kopf Instruments, Tujunga, CA, USA) and fixed in a stereotaxic apparatus (David Kopf Instruments). Body temperature was maintained at 36 ± 1 °C using a homeothermic blanket attached to a 3mm flexible rectal probe (Harvard Apparatus Ltd, Edenbridge, UK). Lidocaine was administered subcutaneously and epicutaneously for local anaesthesia (see below for dosage).

The middle scalp was removed and an intracerebral MAB 6.14.IC guide cannula with a plug (Royem Scientific Ltd, Luton, Ltd) was cemented in place (Refobacin bone cement R; Biomet Europe, Germany) above the hippocampus at coordinates AP -5.0, ML -5.0 for ventral hippocampus or AP -4.3, ML -2.5 for dorsal hippocampus (relative to bregma, Paxinos & Watson 1997). A removable metal guide cap (Royem Scientific Ltd) was attached to the guide cannula to protect it

from chewing by the rat's cagemates. All surgical equipment as well as the guide cap were sterilised prior to surgery using an autoclave, and during surgery using a glass bead steriliser. The duration of surgery (and hence, isoflurane anaesthesia) was kept to a maximum duration of 40min.

After surgery, buprenorphine was administered intramuscularly (see below for dosage) and removed to a recovery cage. During the recovery period standard food pellets were replaced with Readybrek porridge (The Weetabix Food Company, Kettering, UK) and animal welfare was monitored daily.

2.3 Awake microdialysis

After a minimum 3-day recovery period, a single cannula concentric MAB 6.14.3[2] microdialysis probe (Royem Scientific Ltd, Luton, UK) was inserted at coordinates DV -6.0 (ventral hippocampus) or DV -4 (dorsal hippocampus; relative to the dura surface, see Figure 31 below) under brief isoflurane anaesthesia.

After cementing the probe in place, rats were placed in the microdialysis test chamber (hemispherical Perspex bowls that measure 115 cm in diameter and 35 cm in height, covered with a black material; Carnegie Medicin, Stockholm, Sweden) and attached to a liquid swivel system for freely moving animals (Instech Laboratories Inc, Plymouth, PA, USA; Figure 32) using a plastic collar (Royem Scientific Ltd). The floor of the test chamber was covered with shaving chips used in the rat home cage. Readybrek porridge (Weetabix Food Company) was available *ad libitum* during the microdialysis procedure.

The inlet arm of the probe was connected by fluorinated ethylene propylene tubing (FEP; internal diameter 0.12 mm) and polyurethane tubing adapters (both from Royem Scientific) to a CMA/100 perfusion pump (CMA/100, Carnegie Medicin, Stockholm, Sweden) via the liquid swivel system (Instech). Probes were

[2] 14 mm shaft consisting of PI tubing, inlet and outlet tubing consisting of PEEK tubing and a 3 mm tip of exposed 15 K Dalton cut-off PES dialysis membrane of 0.6 mm diameter; relative recovery of approximately 20 % at a perfusion rate of 2 µl/min

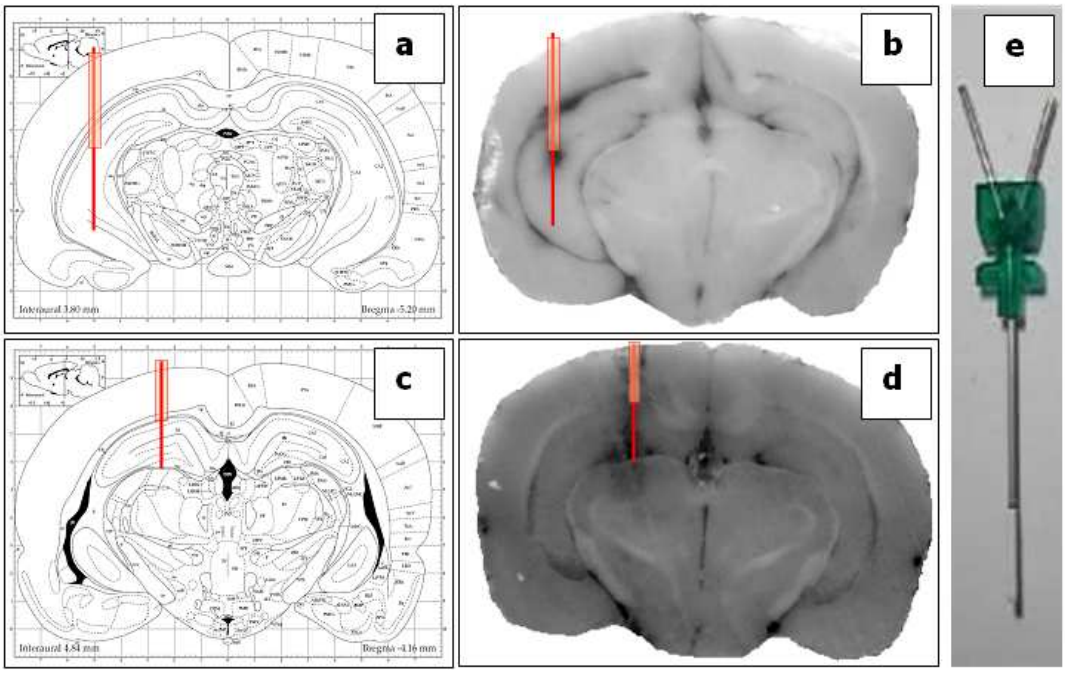


Figure 31 Diagrams (a, c) **and example brain sections** (b, d) **illustrating the location of the MAB6.14.3 microdialysis probes** (red line; probe shown in Figure 30e) **and guide cannulae** (red rectangle) **in the ventral** (a, b) **and dorsal** (c,d) **hippocampus**
Some tissue damage and blood clotting is visible below and around the guide cannula in Figure b.

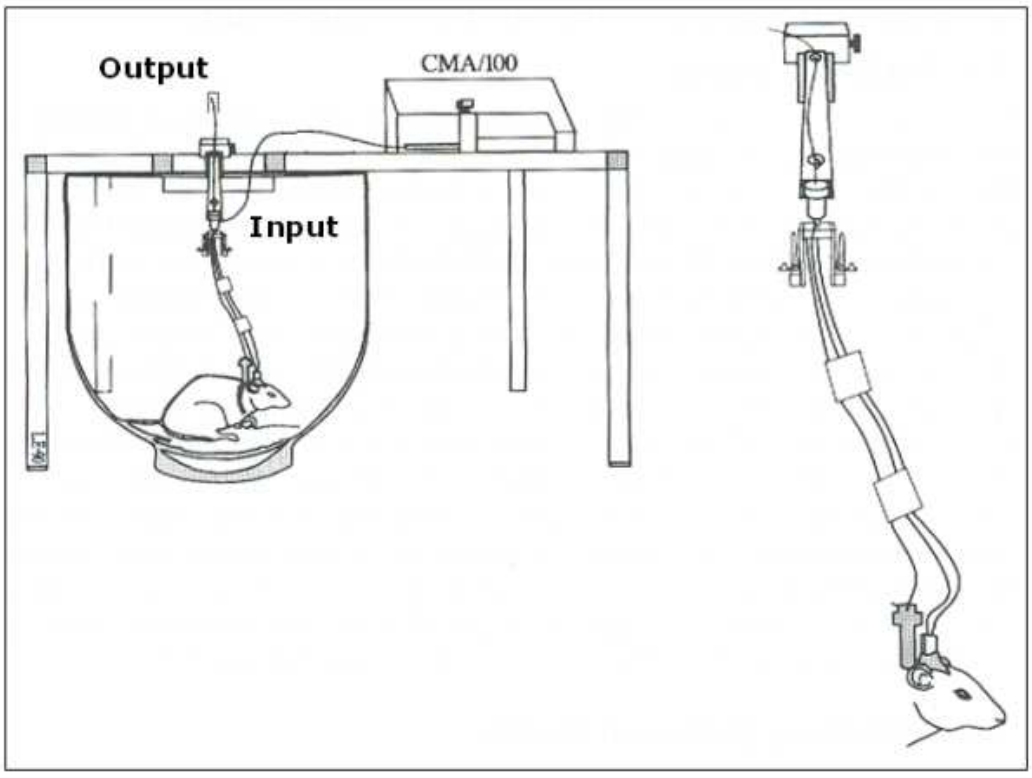


Figure 32 Illustration of equipment used for an awake, freely moving animal
Figure modified from (Sharp 1992); courtesy of Carnegie Medicin, Stockholm: aCSF is delivered to the microdialysis probe implanted in the hippocampus at 2 µl/min via a liquid swivel system (shown on the right) attached to a CMA/100 microinfusion pump. Microdialysis perfusates are manually collected into a collection tube (at a position labelled 'output' in the left-hand figure).

continuously perfused at 2 µl/min with artificial cerebrospinal fluid (aCSF) consisting of 140 mM NaCl, 3 mM KCl, 1.2 mM $Na_2HPO_4.2H_2O$, 0.27 mM $NaH_2PO_4.1H_2O$, 1 mM $MgCl_2.6H_2O$, 2.4 mM $CaCl_2$ and 7.2 mM D-Glucose (based on Sharp and Zetterstrom 1992). The outlet arm of the probe was connected by FEP tubing to collection vials for manual sample collection every 20 min.

After a 1 h stabilisation period, perfusates were collected every 20 min (yielding 40 µl sample volumes) and analysed immediately for DA, HVA, DOPAC and, where possible, NA levels using HPLC-EC (see below). In case of minor delays between sample collection and analysis, samples were stored in the dark at 4 °C. Tolcapone or vehicle (see Section 2.5 below) was injected after 2 h of collecting baseline samples. After a further 120 min (when the effects of tolcapone were shown to be maximal in the prefrontal cortex; Tunbridge et al 2004b), the effects of a 20 min high potassium challenge (aCSF with 56 mM KCl and 87 mM NaCl) were recorded for 80 min. After termination of each experiment, rats were sacrificed and brains were snap-frozen at -80 °C for histological verification of probe placement (Figure 31).

2.4 HPLC-EC

40 µl microdialysis samples were loaded onto a 100 µl loop, which was fixed to a Rheodyne 7725i manual injector (HiChrom Ltd, Theale, UK), using the partial filling method (see manual injector operating instructions; Rheodyne LLC, CA, USA) and a 50 µl SGE syringe (SGE Europe Ltd, Milton Keynes, UK). DA, NA, HVA, DOPAC and 5HIAA were separated with a Microsorb C_{18} column (3 µm C_{18} Microsorb particles packed in a 100 x 4.6 mm column; Varian Inc, Oxford, UK). The column was eluted isocratically at 1 ml/min with a 0.12 M $NaH_2PO_4.H_2O$ buffer solution at pH 2.8 – 4.3 containing 16 % (v/v) methanol, 0.5-4.9 mM 1-Octanesulphonic acid (OSA), 1.07 or 100 mM EDTA and 2 mM NaCl. The constitution of the mobile phase was altered depending on whether NA concentrations were also recorded.

The HPLC system consisted of a PU-2085 HPLC Pump (Jasco Ltd, Great Dunmow, UK), a 120527 Pulse Dampener (Arc Sciences Ltd, Alton, UK), a Degasys Populaire degasser (Jasco) and an ANTEC Decade II amperometric detector set at 35 – 45 °C (Antec Leyden, Zoeterwoude, The Netherlands), all connected using the appropriate diameter PEEK tubing (Sigma-Aldrich Company Ltd, Dorset, England). The electrochemical detector was equipped with a 50 µm spacer and an ISAAC flow cell (Antec Leyden) operated at +0.6 – 0.65 mV (as determined by plotting voltammograms for DA, NA and noise peak heights against voltage).

A conservative estimate of the dopamine limit of detection is 2 fmol / 40 µl injection (achieving a 2:1 or 3:1 signal to noise ratio). An external standard solution containing 0.1 M perchloric acid and 5 pmol of each chemical of interest was used to calibrate the HPLC system on each day, converting peak heights to units of concentration (pmol/20 min sample) on the computerised data acquisition system (ChromPass software; Jasco), which was connected to the HPLC system through a LC Net-II ADC box (Jasco).

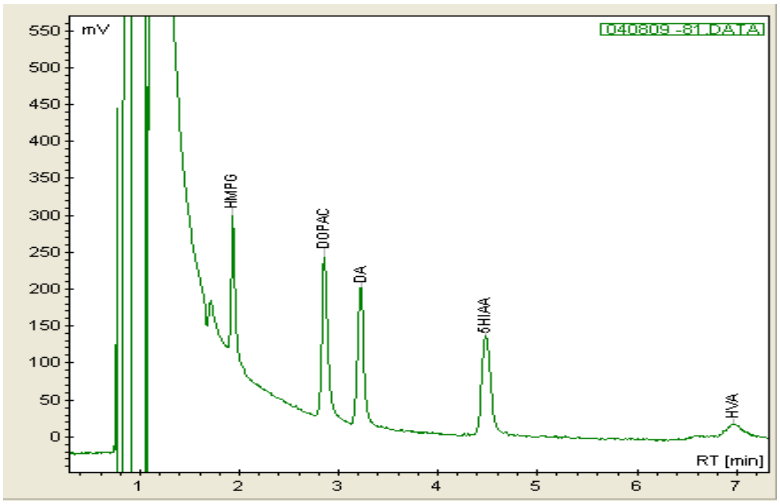


Figure 33 Example of a HPLC chromatogram corresponding to a standard calibration solution
Peaks of interest are labelled. The x-axis indicates retention times (RT) in min and the y-axis indicates peak heights in millivolts (mV). Each component of the standard is present at 5 pmol concentration.

2.5 Drugs

Each rat undergoing surgery received a total of 0.5 ml of 2 % lidocaine (20 mg/ml, Hameln Pharmaceuticals Ltd, Gloucester, UK), which was administered subcutaneously and epicutaneously to each rat undergoing surgery. Also, after surgery buprenorphine (Vetergesic; Alstoe Ltd, Sheriff Hutton, York, UK) was administered intramuscularly at a dose of 0.3 mg/kg. Please refer to Chapter 3 Section 2.3 for details regarding the preparation of the tolcapone suspension and vehicle solution.

2.6 Data analysis

DOPAC/HVA ratios were calculated to provide an index of COMT enzyme activity (given that COMT converts DOPAC to HVA, see Figure). Raw data for individual samples were replaced by a mean of the concentrations 20 min before and 20 min after this sample when the values were at least twice as high as both of the flanking values. Also, DOPAC data from two animals (one vehicle and one tolcapone-treated animal) were discarded due to co-elution of DOPAC with a contaminant on the chromatogram.

Data were analysed with PASW statistics 18.0 software (SPSS Inc, Chicago, IL, USA) using three-way repeated-measures analysis of variance (ANOVA) with two between-subjects factors, namely treatment (vehicle or tolcapone) and brain region (dorsal or ventral hippocampus), and one within-subjects factor (sample number). The within-subjects factor had nine levels, corresponding to the 4^{th} to 12^{th} microdialysis sample from any given animal. Univariate ANOVAs with two between-subjects factors (as above) were used to study mean baseline concentrations of DOPAC and HVA. Statistically significant ($p's<0.05$) and trend-level results ($p's\leq0.1$) were followed up with LSD post-hoc tests. However, statistically significant post-hoc tests following trend-level results were treated with caution. In case of a statistically significant Mauchly's test of sphericity, a Huynh-Feldt correction was used to alter the degrees of freedom and thereby alter the F-ratio significance level.

Following the initial raw data analysis, the above analyses were repeated after DOPAC and HVA data were expressed as a percentage relative to baseline, which is the mean concentration of the last three baseline samples preceding injection of tolcapone or vehicle. Data were plotted using GraphPad Prism 5.01 (GraphPad Software Inc, La Jolla, CA, USA). DOPAC and HVA data are presented as percentage of baseline concentration. Rat weight did not influence either raw or percentage DOPAC and HVA data, so weight was not included as a covariate in the final analyses.

The DA and NA data from 8 and 5 animals, respectively, were discarded by the experimenter. The catecholamine data from these rats was either obscured by the presence of co-eluting contaminant peaks or the levels were no higher than baseline noise - and were hence deemed below the detection limit of the HPLC method. Additional criteria used were reproducibility of the peak position over time as well as the shape of the peak. The same DA and NA data were discarded by a second independent and blinded investigator (TS).

NA/DA ratios were calculated to give a rough indication of the relative extracellular content of the two catecholamines within the hippocampus. There was insufficient power to perform statistical analyses on the NA and DA data. Finally, DA turnover (see Chapter 3) was not investigated in this study as DA and its metabolites are distributed unevenly between extracellular and intracellular compartments. This microdialysis experiment measured neurochemical levels in the extracellular compartment, which is estimated to account for 1 % and 50 % of total DA and DOPAC, respectively. Hence, measuring extracellular DA, DOPAC and HVA levels cannot be indicative of intracellular pathway activity or DA turnover.

3 Results

3.1 Dopamine and noradrenaline were detected in hippocampal perfusates

DA and NA were measured in hippocampal microdialysis perfusates (as illustrated by the sample chromatogram below; Figure 34). NA and DA levels were measured in about 60 % of dialysed rats. However, measurement success rates were compromised by attempting to detect both catecholamines simultaneously on the same HPLC method. Hence, in order to optimize DA peak position, NA was no longer measured for the last eight rats in this study. DA levels were measured in all of these eight rats.

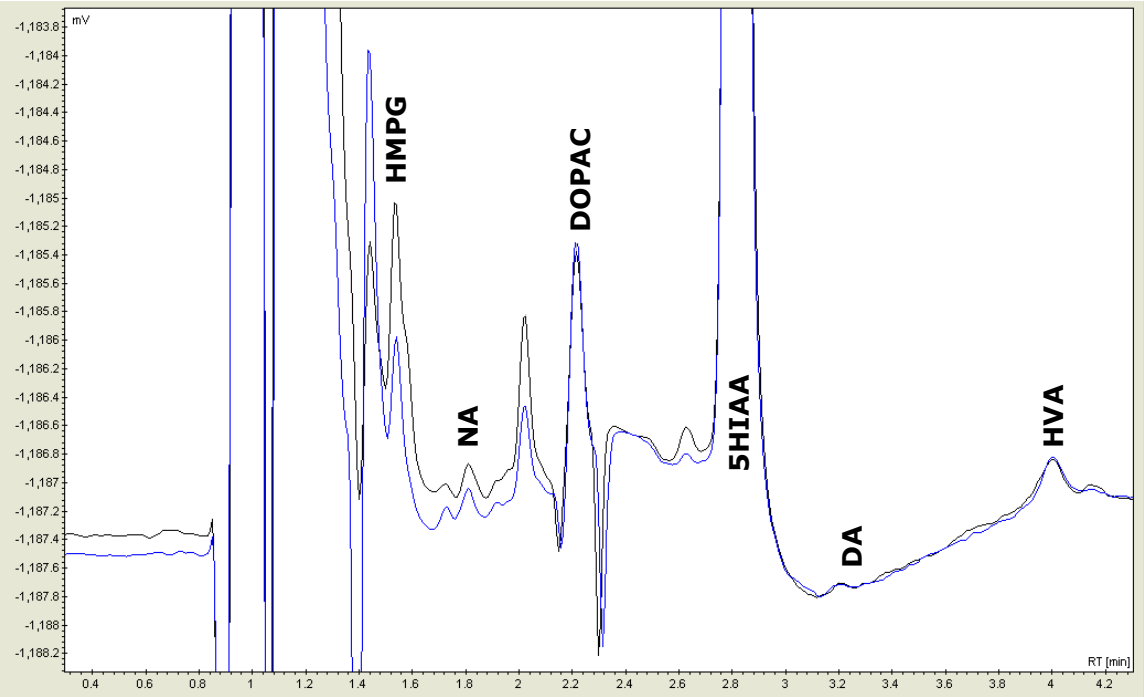


Figure 34 Example of two HPLC chromatograms indicating consistency in peak positions and the detection of both NA and DA in hippocampal dialysates

These chromatograms are 20 min samples taken from a rat immediately after tolcapone administration (black line) and during potassium administration (blue line). Peaks of interest are labelled. The x-axis indicates retention times (RT) in min and the y-axis indicates peak heights in millivolts (mV), from which the concentrations are calculated as follows: NA~20 fmol, DOPAC~200 fmol, DA~10 fmol and HVA~30 fmol.

As summarised in Table 9, basal NA and DA levels across the whole hippocampus were 60 ± 8 and 6 ± 0.6 fmol / 20 min sample, respectively.

Correspondingly, NA/DA ratios were ~10 in this microdialysis study. Basal DA and NA levels in dorsal and ventral hippocampus were pooled for analysis due to the scarcity of dorsal hippocampus DA and NA data.

Dopamine	**vHC (n=5)**	**dHC (n=7)**	**Treatment**
Vehicle (n=7)	12±4	9±5	P=0.275
Tolcapone (n=5)	6±5	4±4	
Region	P=0.534		P=0.926
Noradrenaline	**vHC (n=6)**	**dHC (n=1)**	**Treatment**
Vehicle (n=3)	93±22	64±32	P=0.101
Tolcapone (n=4)	35±16	N/A	
Region	P=0.501		N/A

Table 9 Dopamine and noradrenaline data in ventral and dorsal hippocampal dialysates prior to either vehicle or tolcapone treatment

All data (in fmol/40 µl sample collected over 20 min using a microdialysis probe with a 3 mm membrane) given as mean raw data ± SEM of last three samples prior to injection; p-values from univariate ANOVAs investigating the mean baseline data are also given**.**

3.2 Effect of tolcapone on basal and evoked dopamine and noradrenaline

Figure 35 shows the effect of tolcapone on basal and evoked hippocampal DA and NA levels: Visually there is no difference between the DA and NA levels of tolcapone- and vehicle-treated rats. In contrast, raw DA and NA levels in each individual rat appear noticeably increased for at least one of the timepoints post-potassium. However, statistical investigation of the raw DA data, percentage baseline DA data and average percentage DA data over each hour did not reveal any statistically significant results (data not shown; p's>0.05). However, there was a trend-level effect of tolcapone on the percentage DA data on an univariate ANOVA 20 min before the potassium challenge ($F_{1,10}$=3.851; p=0.078). This suggests that the effect of tolcapone started before administration of high potassium aCSF. Due to a lack of power, NA data were not statistically analysed.

In accordance with the Home Office 3Rs (replacement, refinement and reduction of animal use in research) guidelines, no further rats were sacrificed in

order to increase the power of the present study since even after sacrificing 20 rats, the DA and NA data were too variable to make any meaningful conclusions.

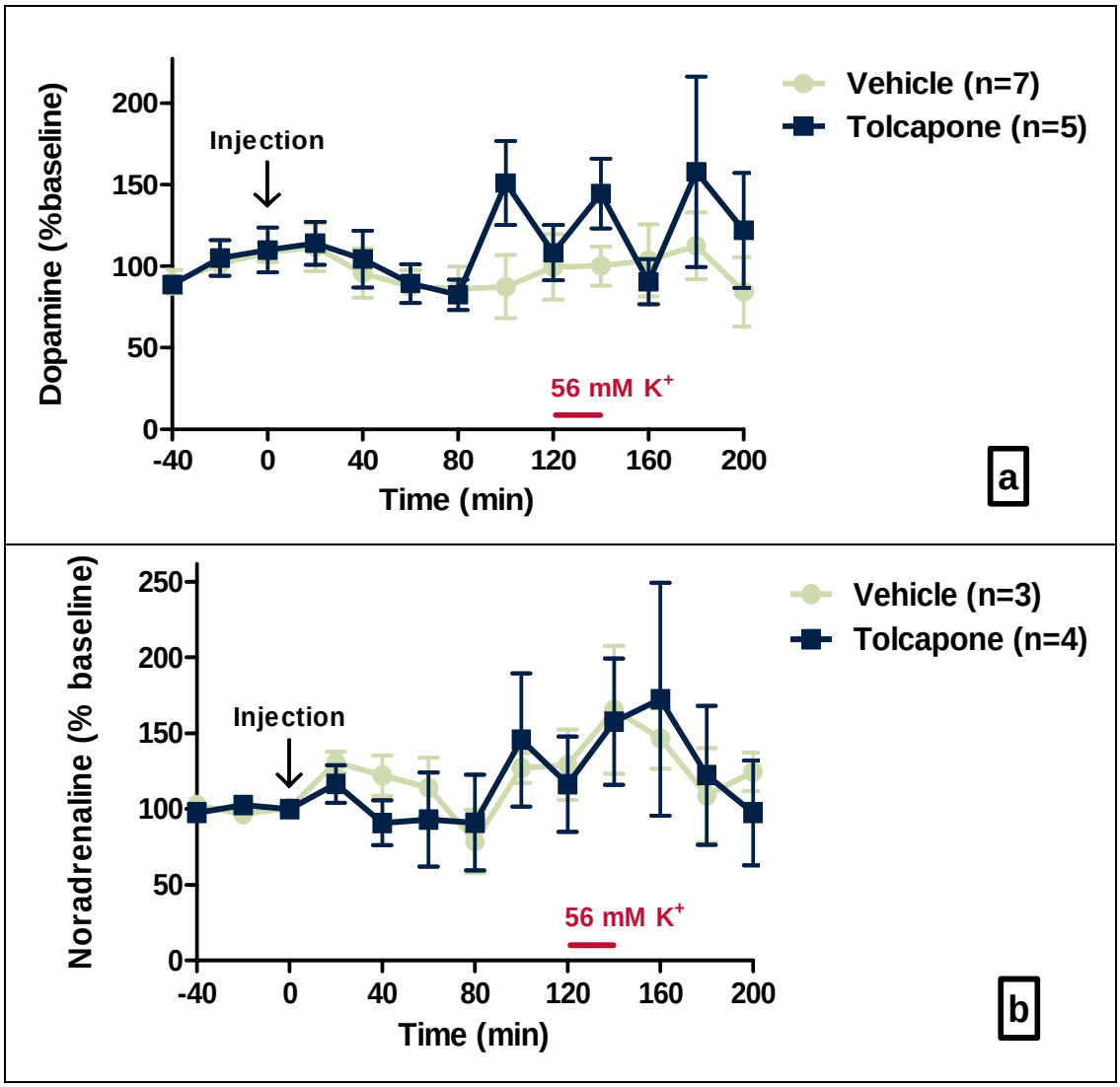


Figure 35 (a) Dopamine and (b) Noradrenaline content as a percentage relative to baseline in hippocampal dialysates from rats treated with either tolcapone or vehicle

Data for 40 µl samples collected over 20 min using a microdialysis probe with a 3mm membrane are given as percentages relative to baseline ± SEM. Vehicle or tolcapone was injected at time 0 and a high potassium challenge was administered for 20 min from 120 min post-injection.

3.3 DOPAC and HVA were detected in hippocampal perfusates

Extracellular DOPAC and HVA levels were measured in hippocampal dialysates from rats. During the 1 h baseline period period prior to treatment with tolcapone or vehicle, extracellular DOPAC and HVA levels were readily detected in the hippocampus at concentrations of 58±5 and 120±7 fmol/20 min sample, respectively.

Univariate ANOVAs investigating raw basal DOPAC and HVA levels indicated that HVA levels are fairly stable across hippocampal subregions ($F_{1,16}$=0.054; p=0.819). In contrast, extracellular DOPAC levels are approximately two-fold higher in ventral (0.07 ± 0.03 pmol / 20 min) than in dorsal subregions (0.03 ± 0.01 pmol / 20 min; $F_{1,14}$=49.003, p<0.001; Table 10). Accordingly, a repeated-measures ANOVA investigating raw DOPAC levels yielded a significant main effect of region as well as significant region*treatment, time*region and time*region*treatment interactions (p's≤0.001). Moreover, tolcapone-treated rats had 37 % higher baseline DOPAC levels ($F_{1,14}$=5.911, p=0.029), presumably due to chance. Hence, it is important to consider DOPAC (and HVA) levels as a percentage relative to the one-hour baseline prior to injection in order to avoid false positives. Following this normalisation procedure, the repeated-measures ANOVAs on pre- and/or post-treatment DOPAC no longer exhibited region-related effects (p's>0.05).

Basal DOPAC	vHC (n=8)	dHC (n=10)	Treatment
Vehicle (n=9)	70±9	32±8	P=0.029
Tolcapone (n=9)	112±9	31±8	
Region	P<0.001		P=0.022
Basal HVA	vHC (n=8)	dHC (n=12)	Treatment
Vehicle (n=10)	114±28	135±23	P=0.741
Tolcapone (n=10)	133±28	99±23	
Region	P=819		P=0.306
Basal DOPAC/HVA	vHC (n=8)	dHC (n=10)	Treatment
Vehicle (n=9)	0.7±0.2	0.3±0.1	P=0.334
Tolcapone (n=9)	1.0±0.2	0.4±0.1	
Region	P=0.005		P=0.380

Table 10 DOPAC, HVA and DOPAC/HVA data in ventral and dorsal hippocampal dialysates prior to either vehicle or tolcapone treatment

All data (in fmol/40 µl sample collected over 20 min using a microdialysis probe with a 3 mm membrane) given as mean raw data ± SEM of last three samples prior to injection; p-values from univariate ANOVAs investigating the mean baseline data are also given**.**

3.4 Effect of tolcapone on basal DOPAC and HVA levels

Extracellular DOPAC and HVA levels were measured in hippocampal dialysates from rats treated with tolcapone- or vehicle-after a baseline period of at least 1 h. The main finding of this experiment was that administration of 30 mg/kg tolcapone significantly inhibited COMT, as suggested by the fact that tolcapone increased DOPAC levels, decreased HVA levels and increased DOPAC/HVA in both the dorsal and ventral hippocampus (Figure 36 and Figure 37).

Both percentage (and raw) DOPAC and HVA data indicated a significant main effect of treatment (p's<0.05; Appendix V), when analysed using repeated-measures ANOVAs. These ANOVAs used time as a within-subjects factor, which had nine levels – corresponding to each percentage data point from one hour pre-injection until two hours post-injection. Similar ANOVAs illustrated that tolcapone increased the DOPAC/HVA ratio about 12-fold in both ventral and dorsal hippocampal perfusates (1.0 to 12.2 and 0.4 to 5.0 pmol / 20 min sample, respectively; Figure 37, p's<0.05), whilst there was no significant change in this measure in the vehicle-treated animals (Table 10).

Given that tolcapone was administered 2 h after the start of the experiment and that the effect of tolcapone developed over time, all repeated-measures ANOVAs on DOPAC, HVA and DOPAC/HVA yielded significant main effects of time and a significant treatment*time interaction (p's≤0.002; Appendix V). LSD post-hoc tests on the time-related effects indicated that tolcapone significantly increased percentage DOPAC and DOPAC/HVA relative to baseline from only 20 min post-injection onwards (p's≤0.004). In contrast, HVA levels across the whole hippocampus were significantly decreased from 60 min post-treatment (p's≤0.025). DOPAC levels in extracellular CSF were increased approximately three-fold and HVA levels were decreased nearly four-fold after tolcapone administration (see Figure 36).

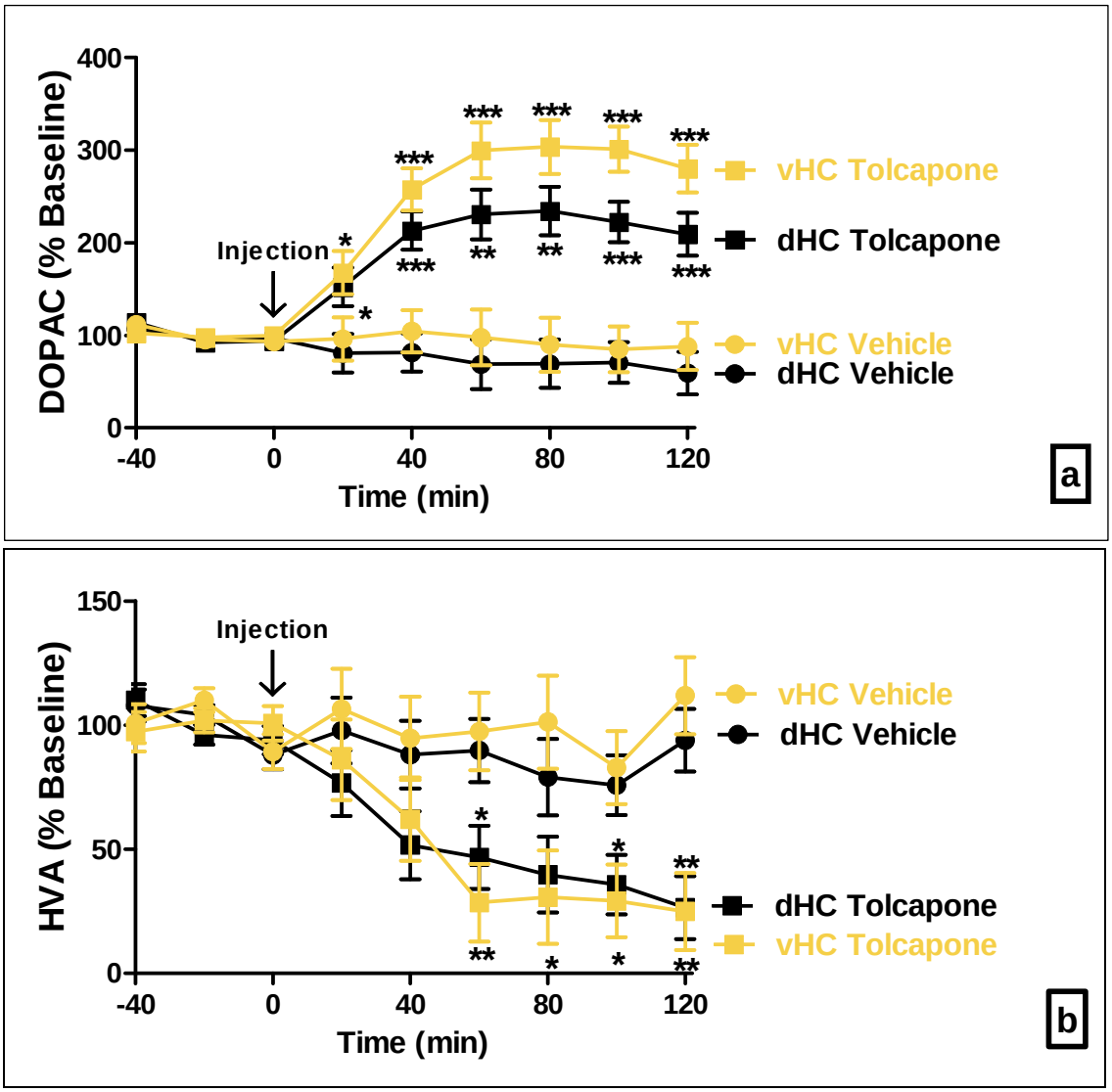


Figure 36 (a) DOPAC and (b) HVA content as a percentage relative to baseline in dorsal (dHC) and ventral (vHC) hippocampal dialysates from rats treated with either tolcapone or vehicle

Data (for 40 µl samples collected over 20 min using a microdialysis probe with a 3 mm membrane) given as mean percentages relative to baseline ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Vehicle or tolcapone was injected at time 0. DOPAC data pooled from n=9 rats and HVA data pooled from n=10 rats.

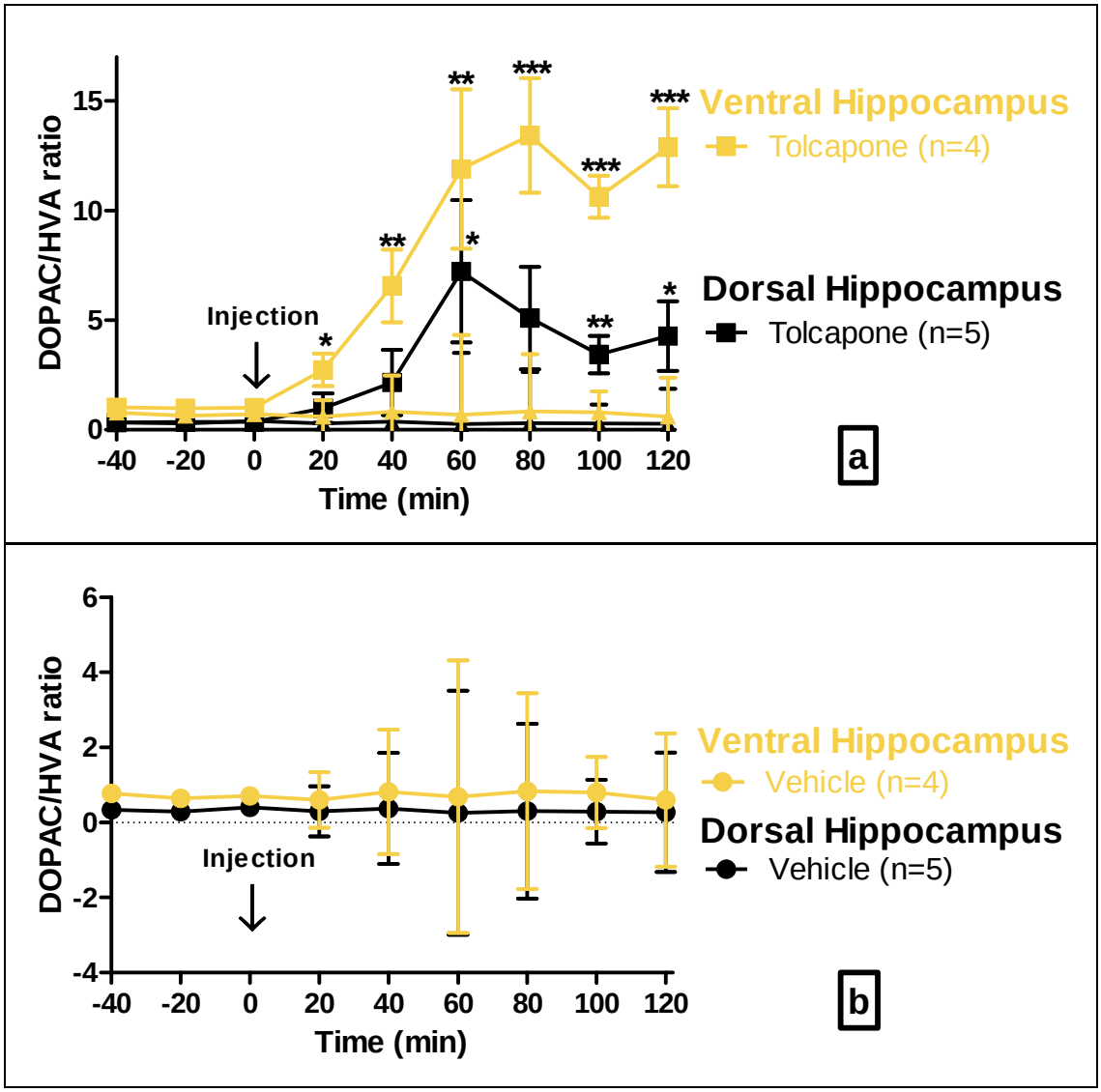


Figure 37 DOPAC/HVA ratio in dorsal and ventral hippocampal dialysates from rats treated with either vehicle or tolcapone

Data (for 40 µl samples collected over 20 min using a microdialysis probe with a 3 mm membrane) given as mean ratios ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Vehicle or tolcapone was injected at time 0. In order to see the traces corresponding to vehicle-treated rats in Figure 37a, a zoomed in Figure 37b is provided.

Discussion

This microdialysis study is the first neurochemical study to investigate the *in vivo* effects of tolcapone on hippocampal DA metabolism in the hippocampus. The main result of this experiment was that i.p. injection of tolcapone (30 mg/kg), but not vehicle, caused accumulation of extracellular DOPAC levels and depletion of extracellular HVA levels in both the dorsal and the ventral hippocampus. Tolcapone also increased DOPAC/HVA ratios relative to baseline from 20 min post-injection onwards.

4.1 Caveats of the microdialysis methodology

There are three main caveats in the use of awake microdialysis coupled with HPLC-EC for the measurement of extracellular catecholamine levels in the hippocampus.

First, the recovery surgery may have long-term effects on hippocampal neurochemistry. However, any prolonged effects of recovery surgery are not expected to differentially affect tolcapone-treated rats, compared with those that received vehicle. Also, it is encouraging to note that the acute damage to the blood-brain barrier induced by surgery is reportedly reversed within 30 minutes of probe implantation (Benveniste 1989) and that extracellular neurotransmitter levels in dialysates do not originate from damaged neurons, because these are affected by neuronal firing (Sharp 1992).

Second, even though microdialysis probes only sample an area of about 200 µm surrounding the dialysis membrane, extracellular DOPAC and HVA levels measured in the hippocampus may have been produced in distant brain structures and diffused into the hippocampus within the CSF. Nonetheless, given that both our intracellular and extracellular HPLC experiments (Chapter 3 and Chapter 4, respectively) provide very similar results, we can conclude that tolcapone affects DA metabolism in the hippocampus.

Third, and most important for the present study, basal DA (and NA) levels in hippocampal perfusates are very close to the limit of detection for HPLC-EC. This is potentially because innervations in the hippocampus are sparse, and it is challenging to insert the microdialysis probes in the most densely innervated hippocampal region (compare Figure 31 and Figure 8) – especially since it is yet unknown which part of the hippocampus receives the most dense monoaminergic innervations (see Chapter 1). The present study reliably measured DA and NA in about 60% of all animals (see Section 2 for the data selection criteria), which is a similar success rate to previous microdialysis studies in the hippocampus. The reason NA was not measured in all rats was due to its location on the chromatogram. NA peaks were located very close to the solvent front and numerous co-eluting contaminant peaks. In contrast, the measurement of hippocampal DA is genuinely challenging. Nonetheless, there are three main reasons, of increasing importance, to suggest that extracellular DA levels were accurately measured in the present study:

a) **DA measurements are not simply noise, because they appear to be altered by high potassium** (see Figure 34)**:** Peaks corresponding to extracellular DA, but not noise or contaminants, increase in height after potassium stimulation.

b) **The HPLC-EC method readily measures DA oxidisation at the same retention time in striatal perfusates:** A striatal pilot study (with the microdialysis probes inserted at coordinates AP+0.8, ML +3.5, DV -7.0; (Paxinos & Watson 1997)) showed that the retention time of DA in hippocampal samples was identical to the retention time of DA in striatal samples – hence confirming that DA was indeed the molecule being quantified in hippocampal experiments.

c) The extracellular DA levels in the hippocampus are in the range of the literature values (see Table 11), though it is important to acknowledge that previously published reports of hippocampal DA levels span from 3 to 300 fmol/20 min.

Paper	DA	NA	DOPAC	HVA	NA/DA	DOPAC/HVA
(Biggs et al 1992)	25	n/a	2332	4000	n/a	0.58
(Gariepy et al 1994)	13	n/a	n/a	n/a	n/a	n/a
(Zhang et al 1995)	320	223	187	216	0.7	0.87
(Ihalainen et al 1999)	6	18	n/a	n/a	3.0	n/a
(Kawata et al 1999)	9	n/a	n/a	n/a	n/a	n/a
(Isogawa et al 2000)	196	83	n/a	n/a	0.4	n/a
(Liu et al 2004)	9	n/a	n/a	n/a	n/a	n/a
(Kino et al 2004)	26	n/a	n/a	n/a	n/a	n/a
(Hatip-Al-Khatib et al 2005)	3	8	13	15	2.7	0.87
(Weikop et al 2007)	8	13	n/a	n/a	1.6	n/a
(Paredes et al 2007)	20	n/a	n/a	n/a	n/a	n/a
(Meurs et al 2007)	5	n/a	n/a	n/a	n/a	n/a
(Meurs et al 2008)	5	n/a	n/a	n/a	n/a	n/a
Laatikainen *et al*, present data	**6**	60	58	120	10	0.48

Table 11 Summary of published papers reporting dopamine levels in hippocampal dialysates
Papers given in chronological order; see Figure 1 for abbreviations. All concentrations are given in fmol/sample collected over 20 min using a probe with a 3 mm membrane i.e. corrections were made for differences in membrane length and aCSF/Ringer flow rate. However there were no corrections made to account for the fact that the use of loop probes (Gariepy et al 1994; Hatip-Al-Khatib et al 2005), or the use of two probes in series (Ihalainen et al 1999; Kino et al 2004), which practically doubles the membrane length. No corrections were made for the use of mice (Ihalainen et al 1999), different rat strains, or whether measurements were conducted in anaesthetised or awake rats (Biggs et al 1992; Gariepy et al 1994; Paredes et al 2007), even though these factors are known to affect neurochemical measurements (e.g. see Li et al 1998 for the effects of chloral hydrate on DA levels). Furthermore, all DOPAC and HVA data from the literature are from ventral hippocampus studies, whereas the microdialysis data given for the current study is collapsed across the dorsal and ventral hippocampus. Finally, note that there is probably publication bias in this field, because most studies with low success of hippocampal DA measurement will either not be published, or will not report their hippocampal DA data.

4.2 Tolcapone may alter hippocampal catecholamine levels

The present study measured both extracellular NA and DA levels in about 60 % of rats. However, no statistically robust effects of tolcapone, high potassium challenge, or hippocampal region were observed – potentially due to the high data variance. Nevertheless, if tolcapone had a large effect on extracellular levels of DA or NA, the present study would probably have shown this. Hence, it is possible that basal DA levels are only altered by tolcapone intracellularly (see Chapter 3), which

would mean that microdialysis studies cannot measure changes in tolcapone-induced changes in basal DA levels.

NA/DA ratios of approximately 10 were successfully calculated for extracellular levels of hippocampal NA and DA. This illustrates that the hippocampus contains more NA than DA - in line with the results of Chapter 3 (NA/DA ~20) and a denser distribution of NA than DA fibres in the hippocampus (Figure 8). However, it is worth noting that the effect of tolcapone on tissue levels of DA, but not NA (see Chapter 3), suggests that DA is a hippocampal neurotransmitter, which is released and synthesised independently of NA synthesis. This conclusion supports other neurochemical studies, which have estimated that just less than half of hippocampal DA is confined to noradrenergic neurons, presumably as a precursor to NA (Bischoff et al 1979; Westerink & de Vries 1985).

4.3 Tolcapone influences DOPAC and HVA levels

In the present study, tolcapone increased DOPAC and DOPAC/HVA by ~300 % and ~1800 %, respectively, and decreased HVA by ~60 %. This results agrees well with the effects of tolcapone on extracellular DA metabolite levels in rat striatum and prefrontal cortex (Acquas et al 1992; Brannan et al 1992; Budygin et al 1997; Janhunen et al 2005; Kaakkola & Wurtman 1993; Li et al 1998; Napolitano et al 2003; Tunbridge et al 2004b). Hence, COMT is not only important for DA metabolism in non-hippocampal regions. Instead, hippocampal COMT is also of functional significance with respect to DA signalling.

Comparing the results of Chapter 4 with those of Chapter 3 suggest that DOPAC and HVA levels were more influenced by tolcapone extracellularly than in total tissue extracts (only 20 % higher DOPAC, 6 % lower HVA and 163 % higher DOPAC/HVA were found in tissue extracts, compared with in 300 % higher DOPAC, 60 % lower HVA nd 1800 % higher DOPAC/HVA in hippocampal perfusates). This might simply be an indication of COMT's variable relative importance at different subcellular structures in the hippocampus, which in turn could be determined by

the subcellular localisation of COMT (which remains unclear to date). Another possibility is that recently produced DA metabolites are efficiently transported through the plasma membrane into the extracellular CSF, where the effects of tolcapone are thus more apparent.

Another similarity between the results of Chapter 3 and Chapter 4 is that both suggest that DOPAC levels may be more sensitive to tolcapone treatment than HVA levels. Chapter 3 shows that tolcapone-induced changes in HVA levels were limited to the prefrontal cortex, and Chapter 4 shows a delayed effect of tolcapone on HVA compared with DOPAC. These results could potentially be due to a lack of statistical power or due to a difference in sensitivity of the HPLC-EC assay for DOPAC and HVA. A more interesting explanation is that HVA levels remain relatively stable, because the amount of hippocampal HVA breakdown is counterbalanced by the activity of aldehyde dehydrogenase (AD; see Figure 2) in the absence of COMT activity. This could be through upregulated AD activity or by less efficient HVA metabolism in the hippocampus. Given that AD forms HVA from MHPA, which is downstream of COMT – it may be more likely that stable HVA levels could be due to a relative lack of HVA metabolism in the hippocampus. Alternatively, it is possible that there is a form of COMT (e.g. the recently-described COMT2 protein; Du et al 2008) that can also produce HVA from DOPAC, but is not inhibited by tolcapone.

4.4 Dorso-ventral dissociations?

The present *in vivo* study revealed dorso-ventral differences in hippocampal neurochemistry, namely that DOPAC levels are two-fold higher in ventral than dorsal hippocampal perfusated. There may also be dorso-ventral differences between the effects of COMT on DA metabolism in the hippocampus. In the present study, tolcapone stimulated DOPAC levels and DA turnover (according to the DOPAC/DA ratio) more in dorsal than ventral hippocampus extracts, as described in Chapter 3. Furthermore, the literature DOPAC/HVA ratios (0.58-0.87), measured in

ventral hippocampus perfusates (Biggs et al 1992; Hatip-Al-Khatib et al 2005; Zhang et al 1995), agree with basal DOPAC/HVA ratios in ventral (0.85), but not dorsal hippocampus perfusates (0.23).

However, these results do not directly mirror the *ex vivo* data presented in Chapter 3. The results of Chapter 3 suggested that tissue levels of DOPAC are higher in the ventral than the dorsal hippocampus, and that levels of DA were affected by tolcapone in ventral, but not dorsal hippocampal extracts. Thus, studies are needed to further investigate the potential dorso-ventral differences in hippocampal DA metabolism, exocytosis, diffusion, or active transport of DA metabolites (see Chapter 7 for further discussion).

4.5 Summary of Chapter 3 and Chapter 4

The aim of the tissue and microdialysis studies presented in Chapters 3 and Chapter 4 was to investigate the effect of COMT inhibition on hippocampal catecholamine metabolism. HPLC-EC data from hippocampal tissue extracts (Chapter 3) and microdialysis perfusates (Chapter 4) provide converging evidence for the involvement of COMT in DA metabolism not only in striatum and prefrontal cortex, but also in the hippocampus. In fact, from the tissue data presented in Chapter 3, the neurochemical changes in the hippocampus are at least as robust as in striatum and prefrontal cortex, which suggests that this novel finding is biologically relevant. This result also verifies that the high levels of COMT mRNA in the hippocampus (Matsumoto et al 2003a) result in functional COMT enzyme.

Oxford University

Chapter 5

Investigation of the effects of COMT inhibition on aspects of anxiety

Linda Maria Laatikainen

DPhil thesis on the role of COMT in hippocampal function

1 Introduction

The HPLC studies discussed in Chapter 3 and Chapter 4 established that COMT has biochemical effects in the hippocampus. The follow-up question is: "what is the role of COMT in hippocampal-dependent behaviour?" The hippocampus is critical for certain aspects of emotion and cognition (discussed in Section 1.1 below and Chapter 6). Also, both catecholamine transmission and COMT may be associated with emotionality and cognitive phenotypes (discussed in Chapter 1). Hence, the studies presented in Chapter 5 and Chapter 6 investigate the behavioural effects of COMT inhibition on specific tasks designed to test hippocampus-dependent anxiety and memory in the rat.

1.1 Role of the hippocampus in emotion

The first emotional circuit described in the brain was the "Papez circuit", in which the hippocampus was suggested to collect sensory information in order to create a conscious appreciation of one's emotional state (Papez 1937). However, the evidence for this circuit, i.e. the aberrant emotional expression resulting from temporal lobe lesions (Kluver 1937), was later attributed to the lesion of non-hippocampal structures.

In order to consider the modern theories of the hippocampal involvement in emotional control, it is important to make careful distinctions between different types of emotional states: Anxiety is the tonic emotional response to situations of conflict and uncertainty, which allows animals to avoid potentially dangerous situations (passive avoidance; see Figure 38). In contrast, fear is the phasic emotional and behavioural response to imminent or present danger, which helps an animal deal with the dangerous situation, mostly by specific behaviours such as escape (active avoidance). In other words, anxiety is the emotional response to a potential threat, whereas fear is the response to an acute threat.

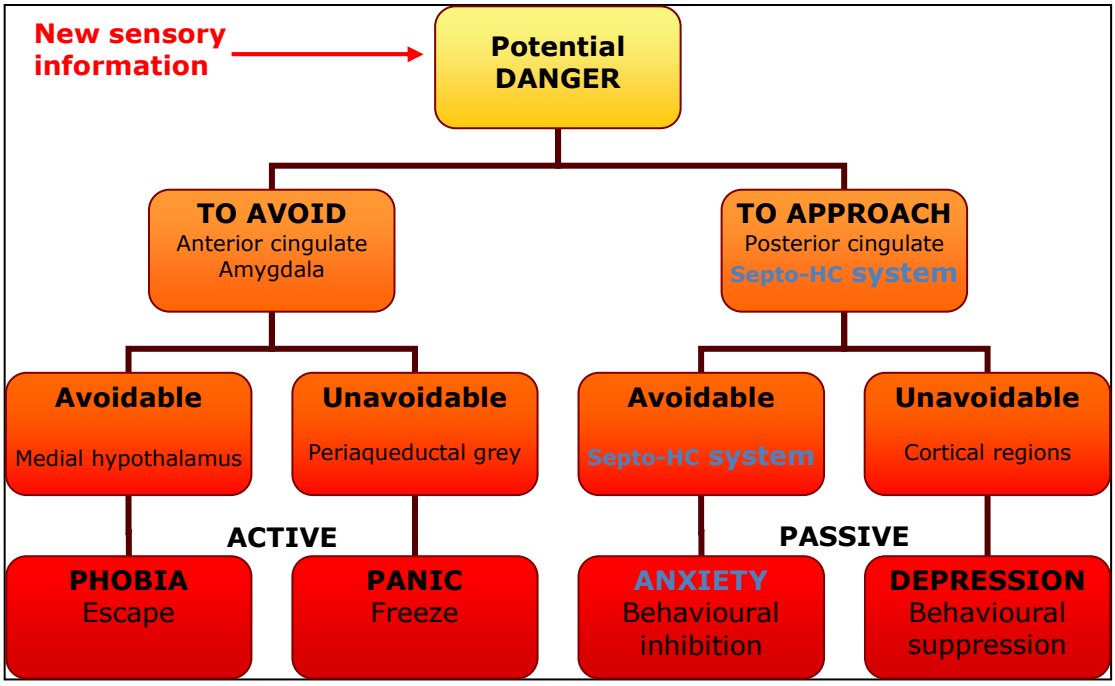


Figure 38 The concept of defensive direction

The septo-hippocampal system is involved in anxiety, but not panic or phobias (the concept of defensive direction is adapted from Gray & McNaughton 2000). Note that evidence regarding the neuroanatomical correlates of different emotional states is ambiguous. This means that the above figure is merely an interpretation of the data and as such, may undergo revisions in the future.

The reason it is important to distinguish between different emotional states is because they have different neuroanatomical correlates. Even though the ventral hippocampus is well connected to the dorsal hippocampus (which is involved in spatial memory formation), the HPA axis (which is involved in mediating the effects of stress) and the amygdala (which is associated with emotional processing, including fear and reward); all these regions control distinct aspects of emotion:

Rats with cytotoxic ventral hippocampal lesions display reduced anxiety in a hyponeophagia task, black/white two-compartment box test, light/dark exploration test, open field, successive alleys test, social interaction test, as well as the elevated plus maze and contextually conditioned freezing paradigms (Bannerman et al 2002; Bannerman et al 2003; Bannerman et al 2004a; Kjelstrup et al 2002; McHugh et al 2004; Richmond et al 1999; see subchapter 1.2.1 for details on rodent tests of anxiety). These effects are distinct from those of dorsal hippocampal or amygdala lesions (Bannerman et al 2004b; McHugh et al 2004; Phillips & LeDoux 1992; Treit & Menard 1997), which suggests that the effects of ventral

hippocampal lesions are not due to effects on the amygdala or dorsal hippocampus. Thus, this thesis considers the (ventral) hippocampus to be particularly involved in anxiety.

Additional evidence for a hippocampal involvement in anxiety comes from human functional and structural imaging studies. Ploghaus *et al* (2001) used fMRI to show increased hippocampal formation activation during unexpected situations in an aversive fear conditioning paradigm, implicating the importance of uncertainty in hippocampus-dependent anxiety. Similarly, Hasler *et al* (2007) showed increased cerebral blood flow in the anterior hippocampus of humans (which corresponds to the ventral hippocampus in rodents) during sustained contextual anxiety induced by unpredictable electric shocks. These results have been replicated using fMRI also during non-aversive paradigms (Kumaran & Maguire 2006). Moreover, Rusch *et al* (2001) used MRI-based morphometry to reveal a positive association between hippocampal volumes and trait anxiety. Thus, human neuroimaging studies are consistent with animal lesion models, which suggest a role for the hippocampus in anxiety.

One model for the involvement of the hippocampus in anxiety is the conflict resolution model of anxiety (Figure 39). According to this hypothesis, the hippocampus is involved in detection and resolution of conflict between simultaneously available, competing goals. Conflict resolution then protects an animal from danger and/or maximises the chance of rewards (i.e. protects from loss of reward). The mechanisms for this are thought to be three-fold: behavioural inhibition, increased attention and arousal, as well as a negative bias i.e. placing additional weight on information with potentially greater adverse significance, leading to a preference for avoidance rather than approach behaviour (see Figure 38).

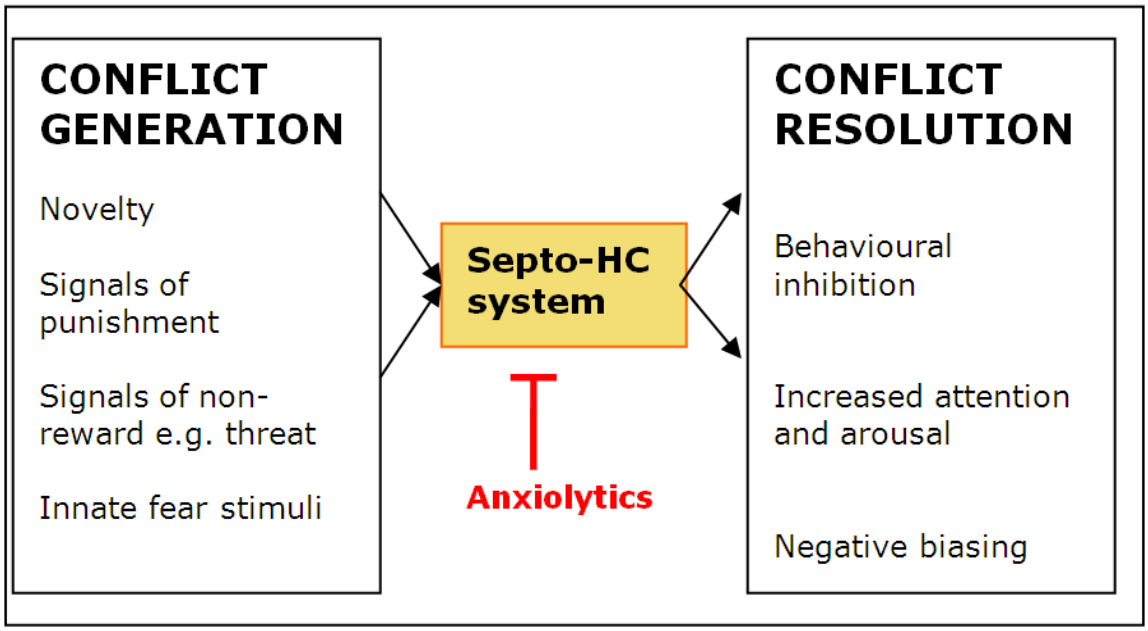


Figure 39 A schematic diagram of the septo-hippocampal involvement in conflict resolution
The role of the septo-hippocampal system in conflict resolution may underlie its role in anxiety, memory processes, attention and arousal (adapted from Gray & McNaughton 2000).

Support for the role of the hippocampus in conflict detection comes for example from rodent studies, which have shown that hippocampal lesioned rats are unable to detect associative mismatch in a non-spatial, non-aversive paradigm (Honey et al 1998). Also, rhesus monkeys with hippocampal lesions display impaired defensive reactions to fear-provoking stimuli, illustrating the role of the hippocampus in generating responses to potentially threatening stimuli (Chudasama et al 2008). Moreover, these results are in good agreement with pharmacological studies using anxiolytic drugs, notably benzodiazepines, which also increase approach behaviour and decrease passive avoidance (Gray & McNaughton 2000).

In summary, both human neuroimaging and animal lesion studies suggest a role for the ventral hippocampus in anxiety, and emphasize the importance of conflict and uncertainty in generating hippocampus-dependent anxiety.

1.2 Validation of the experimental approach

1.2.1 Assessing emotionality in rodents

Animal models of anxiety have varying degrees of face, construct and predictive validity (Uys et al 2003). Anxiety tests are validated with benzodiazepines (Gray & McNaughton 2000) and/or by investigating the effects of region-specific cytotoxic lesions (e.g. Bannerman et al 2004b). The most extensively validated and hence, most commonly used tests of anxiety are the elevated plus maze (Lister 1987; Pellow et al 1985), open field (Choleris et al 2001), light-dark box (Costall et al 1989; Crawley 1981), and hyponeophagia tasks (Britton & Britton 1981; Dulawa & Hen 2005). More recent modifications of these tasks include the black-white alley (Contet et al 2001; Deacon et al 2003; McHugh et al 2004), elevated zero maze (Deacon et al 2002b), successive alleys task (Deacon et al 2003; McHugh et al 2004), fear-potentiated startle (Tizzano et al 2002), social interaction test (McHugh et al 2004) as well as conditioned freezing (Kjelstrup et al 2002).

These rodent anxiety tasks have varying sensitivities and are usually less sensitive than cognitive tasks. Hence, data from rodent anxiety tasks are usually validated using a battery of anxiogenic tests. Using a battery of tests can also reduce the likelihood of false negative results, and help rule out nonspecific effects, such as changes in stress or activity levels (see Chapter 7; Crawley 2008).

1.2.2 Choice of tasks

There have been no publications specifically investigating the potential anxiolytic or anxiogenic properties of tolcapone in rats. Hence, the studies detailed here are designed to provide an initial behavioural screen to investigate whether tolcapone results in gross increases or decreases in anxiety-like behavioural indices.

Tolcapone-treated rats were compared with vehicle-treated rats on approach-avoidance conflict tasks because hippocampal lesions have robust effects

on unconditioned, exploratory-based approach-avoidance conflict tests (see below). Moreover, approach-avoidance tasks display both face validity by modelling human anxiety symptoms, and predictive validity by showing selectivity for known anxiolytic drugs (Crawley 2008). Approach-avoidance tasks make use of the conflicting tendencies of rats to approach food and explore novel environments, but to avoid aversive stimuli such as open areas and unfamiliar food (Cryan & Holmes 2005). In other words, because rats believe themselves to be more vulnerable to predators in open spaces, they prefer to stay in dark enclosed spaces near walls (thigmotaxis), although by doing so they miss out on any food present in the aversive environment. However, if they have low levels of anxiety, they might explore novel environments, even if they have aversive features. When rats are hungry, they are more likely to enter aversive areas in the search for food, which is why rats were food deprived prior to the food neophobia tests.

The first anxiety task was the successive alleys task. The testing apparatus consists of four alleys connected in series, designed to be increasingly anxiogenic. The latencies to enter each alley, the time spent in each alley as well as the number of crosses into each alley were recorded. Hence, the task exploits the approach/avoid conflict between a rats' innate preference for dark, enclosed spaces and their natural exploratory drive. The successive alleys task is similar to the elevated plus maze and black-white alley, without the ambiguity involved with scoring behaviour within the central square of the plus maze and with potentially increased sensitivity to test subtle anxiety differences due to the increased number of areas with varying aversive properties (Contet et al 2001; Deacon et al 2003). Notably, the successive alleys task has been shown to be sensitive to the anxiolytic effects of benzodiazapines, as well as ventral hippocampal lesions, but not amygdalar lesions in rodents (McHugh et al 2004).

The successive alleys task was followed by two food neophobia (hyponeophagia) tests. In hyponeophagia tests, prior food deprivation creates a conflict between hunger and the natural wariness of rodents to approach and

consume novel foods presented in a novel environment. The first hyponeophagia task used part of a T-maze as the novel environment and 45 mg Noyes pellets as the novel food, whereas the second hyponeophagia task involved rats being in immediate contact with the novel foodstuff (sucrose pellets at the bottom of an opaque bucket) from the start of the experiment. These hyponeophagia tests have been validated as a model of anxiety by showing that the latency to (approach and) eat novel food is decreased with anxiolytic drugs and increased with anxiogenic drugs (Britton & Britton 1981; Dulawa & Hen 2005; Shephard & Estall 1984). In agreement with these pharmacological findings; ventral hippocampus, but not amygdalar or dorsal hippocampus lesioned rats demonstrate behaviour consistent with reduced anxiety (Bannerman et al 2002; Bannerman et al 2003; McHugh et al 2004).

1.3 Aims and hypotheses

The aim of the present study was to assess the behavioural consequences of acute, systemic tolcapone injections on anxiety in male rats. This is the first study designed to specifically investigate the impact of COMT on ventral hippocampus-dependent function.

As discussed in Chapter 1, there is some human evidence for a link between low COMT activity and emotion-related phenotypes. More importantly, Smolka *et al* (2005) and Drabant *et al* (2006) have implicated the hippocampus in the links between COMT and emotional processing (discussed in Chapter 1). However, very few studies have investigated the anxiety-related behavioural effects of tolcapone in rodents or genetic COMT knockout in mice; and the results are inconsistent. Gogos *et al* (1998) showed that homozygous COMT knockout female (but not male) mice demonstrate increased anxiety in a light/dark box. In contrast, tolcapone administration in rats and mice increased exploratory activity in the open field test (Maj et al 1990). Hence, it is not possible to formulate accurate *a priori* hypotheses about the behavioural phenotype of tolcapone-treated rats.

2 Methods

The present chapter describes the findings from three different anxiogenic behavioural tasks. Accordingly, the methods section is divided into three parts.

All anxiety tasks were conducted in the same cohort of rats and performed in the order in which the tasks are described below. The time intervals between the three anxiogenic behavioural studies were 6 days between tasks 1 and 2, and 3 days between tasks 2 and 3. Treatment allocations were kept the same throughout the study. Accordingly, the same rats always received tolcapone or vehicle one hour prior to testing. Furthermore, the order of injection and testing of the rats was also kept constant across the three tasks.

All three anxiety tests were performed in different testing rooms. All thirty animals were run on the same day and injected in the holding room in batches of six animals. At the start of testing, non-experimental rats were put on the apparatus to give it a rat odour. The apparatus was cleaned with a damp cloth between each rat, and rats were kept separate from their cage mates until the end of their testing to prevent social transmission of food preferences, which is a very potent form of non-spatial learning (Burne et al 2010; Strupp & Levitsky 1984).

All behavioural testing procedures were carried out in the laboratory of Dr David Bannerman in the Oxford University Department of Experimental Psychology in accordance with the Animals (Scientific Procedures) Act 1986 and associated Home Office guidelines.

2.1 Animals

Thirty male Lister-Hooded rats (Harlan, OLAC, Bicester, UK) weighing 222 – 249 g during the successive alleys task, 225 – 256 g during the first hyponeophagia task and 231-262 g during the second hyponeophagia task, were used. All rats were housed in groups of three in standard open caging (N.K.P. Cages Ltd, Kent, UK). The home cages contained wood chips as bedding, a white

cardboard refuge, two cuboid wood blocks for gnawing, as well as copious amounts of shredded brown paper as nesting material. The rats were maintained on a 12 h light/dark cycle (lights on 07.00 – 19.00) in a temperature- and humidity-controlled room, with all testing conducted during the light cycle. Water was available ad libitum. Due to the hyponeophagia tasks being appetitively motivated, food was removed the evening before behavioural testing for approximately 20 h.

2.2 Drugs

Tolcapone (Roche Products Ltd, Welwyn, UK) does not dissolve well, so it was made up fresh daily by suspending it in 0.9 % saline with a few drops of Tween-80. To improve suspension of tolcapone, the tolcapone-containing solution was vortexed and sonicated briefly prior to injections to ensure as accurate dosing as possible. Tolcapone was administered intraperitoneally at a dose of 30 mg/kg. The vehicle was 0.9 % saline with a few drops of Tween-80. All animals were injected by the same person.

The experimenter was blind to the treatment allocations throughout behavioural testing. All testing was started 1 h post-injection, since tolcapone significantly altered all measures of COMT inhibition in the hippocampus by 60 min post-treatment (see Chapter 4).

2.3 Successive alleys task

2.3.1 Apparatus

The wooden apparatus consisted of four alleys connected in series, designed to be increasingly anxiogenic (Figure 39; Deacon et al 2003). Each alley was 45 cm long. Alley 1 had 29 cm high walls, was 9 cm wide and was painted black. Alley 1 lead to alley 2, which was 9 cm wide, but had 2.5 cm high walls and was grey. A 2 cm step down lead to alley 3, which was 6.7 cm wide, had 0.5 cm high walls and was white. A 0.5 cm step down lead to alley 4, which was also white, 3.5 cm wide with 0.3 cm high walls. The apparatus was elevated 80 cm above the floor.

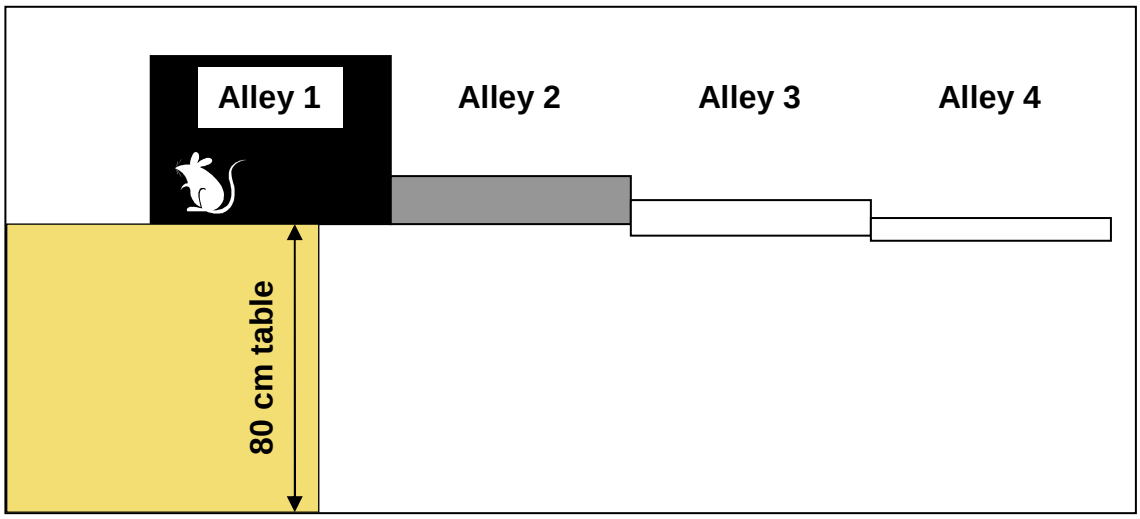


Figure 40 Successive alleys apparatus

Four linearly connected alleys of increasingly anxiogenic character are shown from the side. The diagram is not drawn to scale. The position and direction of the rat is illustrated at the start of a test.

On the day of testing, rats were moved from the holding room to a room other than the experimental room 10 min prior to testing to equilibrate arousal levels. Then, one hour after injection, each rat was individually placed in the apparatus for 5 min by placing it at the closed end of alley 1 facing the back wall. The latency to enter each of the alleys for the first time as well as the time spent in each alley were recorded. A rat was considered to have entered the next alley when it placed all four feet on the next alley. The number of faecal boli and whether or not any urination occurred were also recorded during the test. The apparatus was cleaned with a damp cloth between each rat, and rats were kept separate from their cage mates until the end of their testing.

2.4 Hyponeophagia tests

On the day of testing, rats were removed from the holding room approximately 15 min before testing (which occurred 1 h after injection) to standardize arousal levels. Each trial lasted a maximum of 3 min. If a rat did not eat by the 3 min cut-off time, the rat was placed alone in a separate 'waiting cage' for about 5 min before retesting for another 3 min. The test parameter was the cumulative latency to start eating. Cumulative latency is the total time taken from placing a rat onto the apparatus to the time of eating, including time spent on retesting trials. The number of faecal boli and whether or not any urination occurred was also recorded.

2.4.1 First hyponeophagia test

The test was conducted on a 30 cm long section of an open wooden maze, painted uniform grey (30 x 10 x 2 cm at 43 cm off the floor). The apparatus contained a metal food well (2 x 2.5 x 2.5 cm, located 5 cm from the end of the section), containing eight 45 mg Noyes food pellets (Sandown Scientific, Hampton, UK). Noyes pellets were a novel food for the rats, in order to increase the potential danger of eating, as perceived by the rats.

Each rat was placed on the apparatus facing away from the food well and removed once they had finished eating most of the 8 pellets provided. In addition to recording cumulative latency to eat, the first hyponeophagia test also involved recording the cumulative latency to first contact (determined by sniffing or licking of pellets).

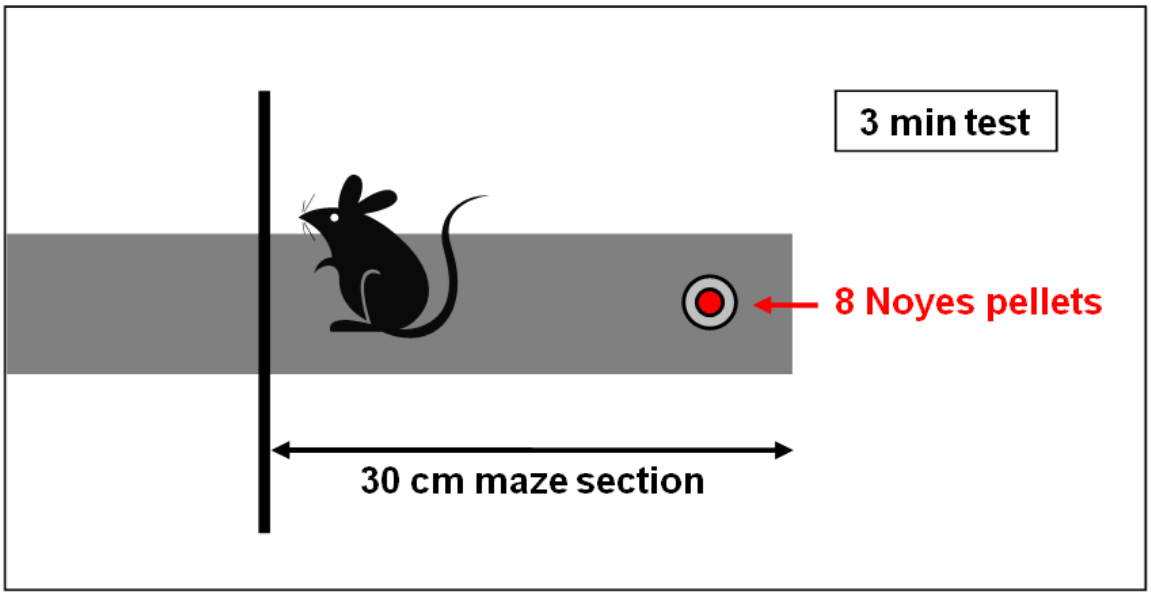


Figure 41 Apparatus for the first hyponeophagia test

The position and direction of the rat is shown from above at the start of an experiment. The black line illustrates the 20 cm x 30 cm black wooden wall separating the rest of the T-maze from the 30 cm section used for the present experiment. The diagram is not drawn to scale.

2.4.2 Second hyponeophagia test

To remove the potential confound of latency to contact in the first hyponeophagia task, the second hyponeophagia task was conducted in a standard white 10 litre bucket, containing a layer of about 1 cm of Noyes sucrose reward pellets on the floor. Sucrose pellets were a novel food for the rats, in order to increase the potential danger of eating.

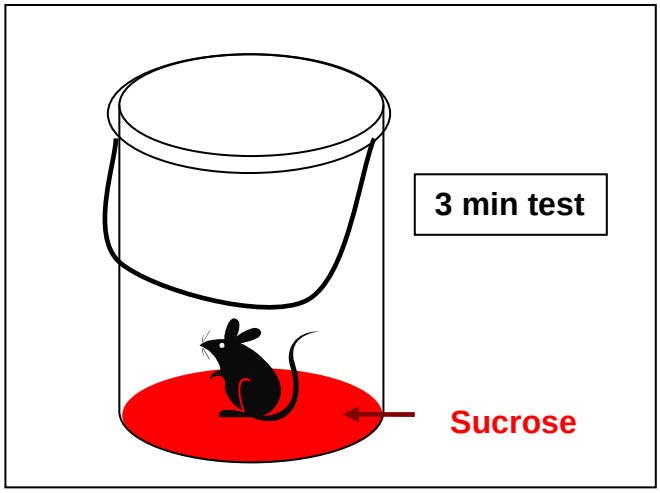


Figure 42 Apparatus for the second hyponeophagia test

White opague bucket containing about 1 cm of Noyes sucrose reward pellets (red) is shown from the side. The diagram is not drawn to scale.

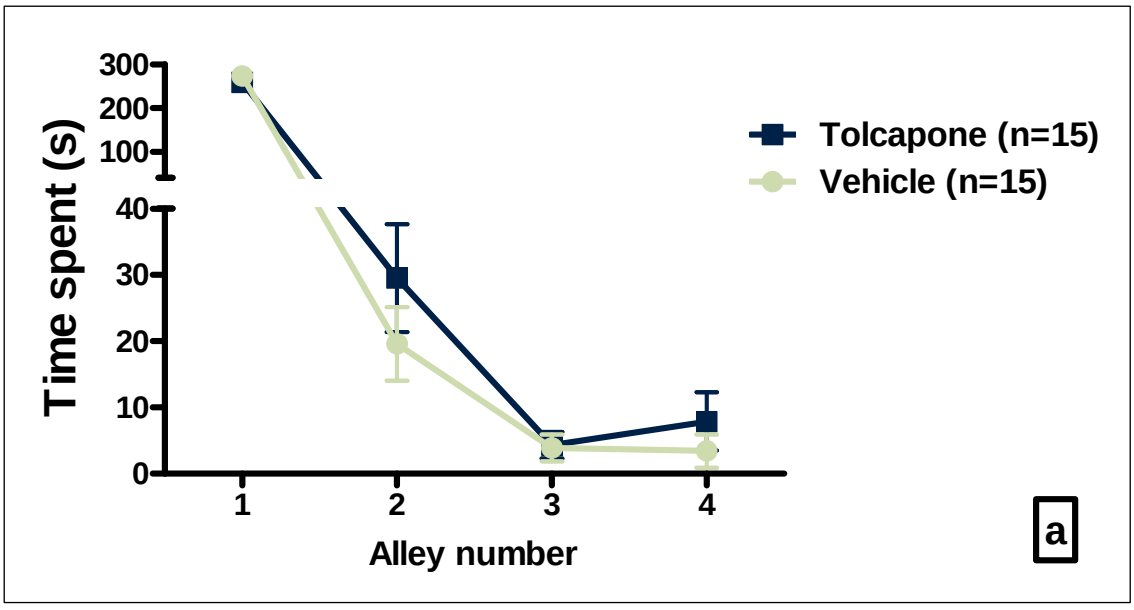
2.5 Data analysis

Data were analysed using SPSS 15.0 statistics software (SPSS Inc, Chicago, IL, USA) and plotted using GraphPad Prism 5.01 (GraphPad Software Inc, La Jolla, CA, USA). The successive alleys data for time spent in each alley and number of entries into each alley were analysed using repeated-measures ANOVAs with LSD post-hoc tests, using treatment as a between-subjects factor and alley number as a within-subjects factor. Given that the data for time spent in each successive alley are not independent, the numerator degrees of freedom were adjusted and the significance of the ANOVA was checked using ANOVA distribution tables. The latencies to cross into each alley were treated as independent variables and analysed using independent samples t-tests. In contrast, the hyponeophagia test data on the latency to first contact and eat food pellets were investigated using independent samples t-tests. Defecation data were analysed using non-parametric Mann-Whitney U-tests, whereas the binomial urination data were analysed using Chi-squared tests.

3 Results

3.1 Tolcapone did not influence behaviour during the successive alleys task

Tolcapone did not affect any of the test parameters studied in the successive alleys task (Figure 43). The latencies to move into alley 2 ($t(28)=0.562$, $p=0.579$), alley 3 ($t(28)=0.404$, $p=0.689$) and alley 4 ($t(28)=0.027$, $p=0.978$) were unaffected by tolcapone treatment. Similarly, tolcapone did not alter the time spent in each alley (treatment*alley interaction, $F_{2,84}=0.814$, $p=0.393$; Figure 43a). Additionally, tolcapone had no influence on the number of times rats crossed into the alleys (no main effect of treatment, $F_{1,28}=0.369$, $p=0.549$; or treatment*alley interaction, $F_{3,84}=0.088$, $p=0.914$). Finally, a Mann-Whitney U test on defecation ($U=88$, $p=0.170$), and a Chi-squared test on urination ($X^2(1, N=30)=0.37$, $p>0.5$) did not reveal any effect of treatment.



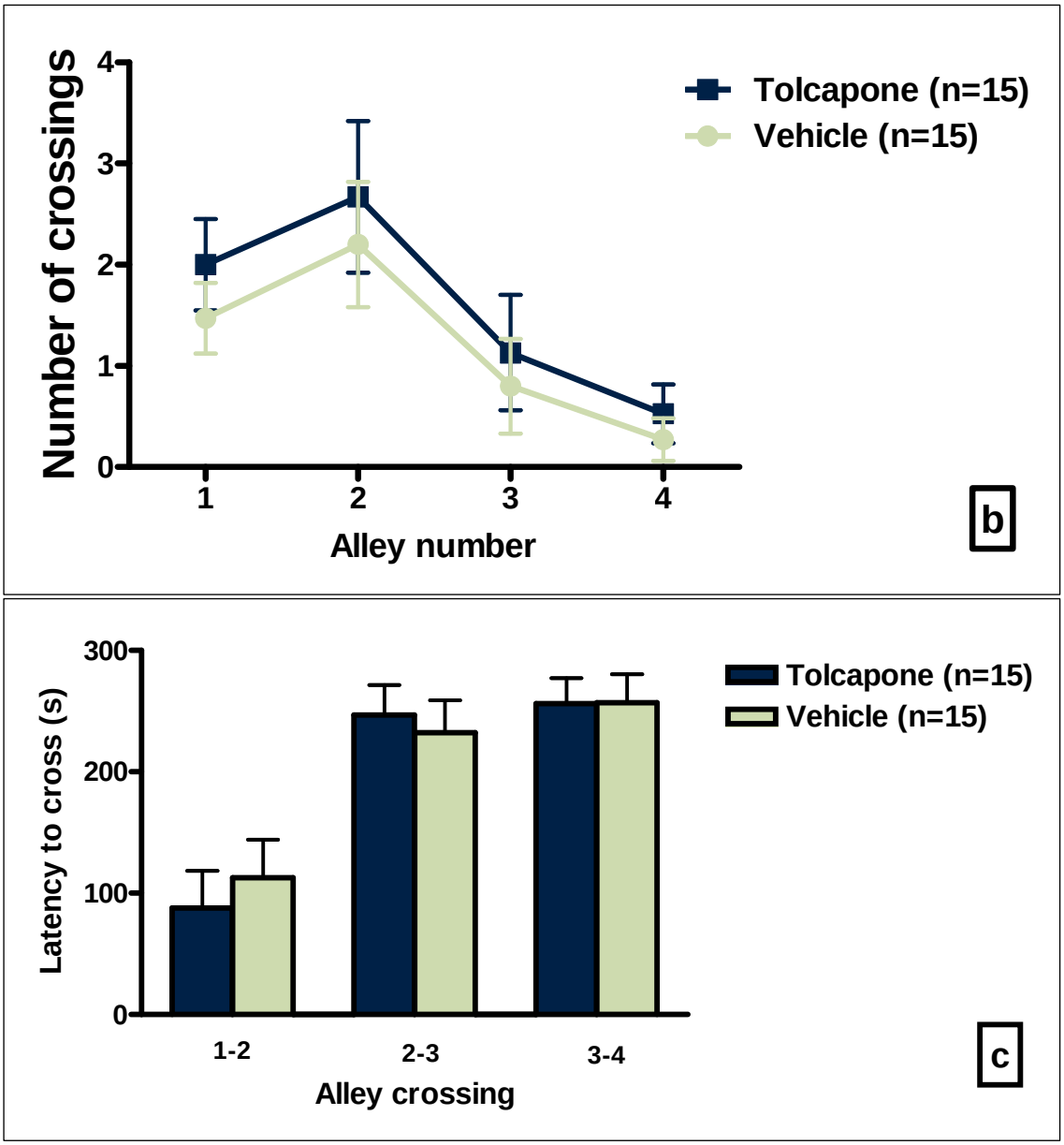


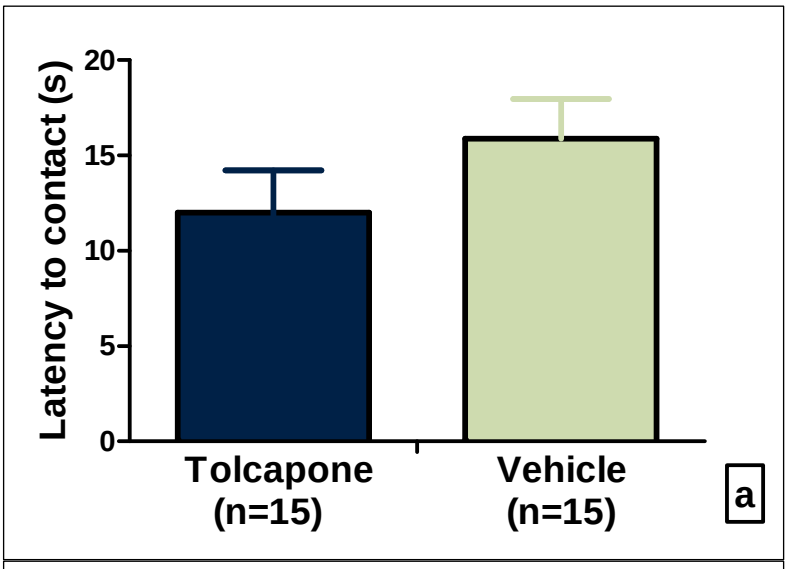
Figure 43 Effect of tolcapone on (a) total time spent in, (b) number of entries into, and (c) latency to cross into, the four successive alleys 1-4

Mean data ± SEM are shown (units given in Figure).

3.2 Tolcapone did not influence the results of the hyponeophagia tests

Tolcapone had no effect on rodent behaviour during the hyponeophagia tasks. During the first hyponeophagia test, latencies to first contact ($t(28)=1.264$, $p=0.217$), latencies to eat ($t(28)=1.007$, $p=0.323$) and the differential of latency to contact minus latency to eat the novel food ($t(28)=1.091$, $p=0.285$) were unaltered by treatment (Figure 44). Also, tolcapone did not influence defecation ($U=81.5$, $p=0.184$) or urination ($X^2(1, N=30)=0.14$, $p>0.5$) during the first hyponeophagia task.

Similar to the first hyponeophagia test, tolcapone treatment did not affect latency to eat ($t(28)=0.874$, $p=0.389$; see Figure 45) in the second hyponeophagia test. Similarly, tolcapone did not influence defecation ($U=112.5$, $p=1.0$) or urination ($X^2(1, N=30)=0.16$, $p>0.5$). Moreover, a repeated-measures ANOVA combining the latency to eat data from both hyponeophagia tasks found no main effect of treatment ($F_{1,28}=1.407$, $p=0.245$).



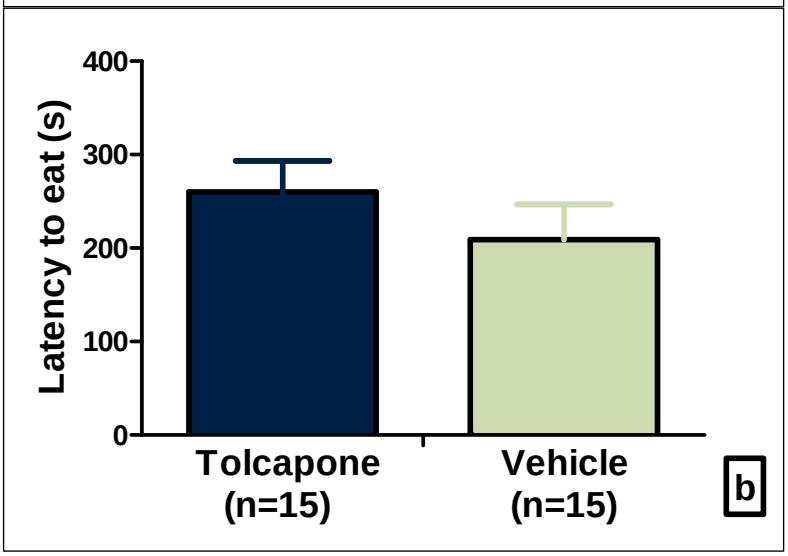


Figure 44 Effect of tolcapone on latency to (a) first contact and (b) eat novel food rewards on the first hyponeophagia task

Data (in seconds) are given as (a) mean latency to first contact ± SEM and (b) mean latency to eat ± SEM.

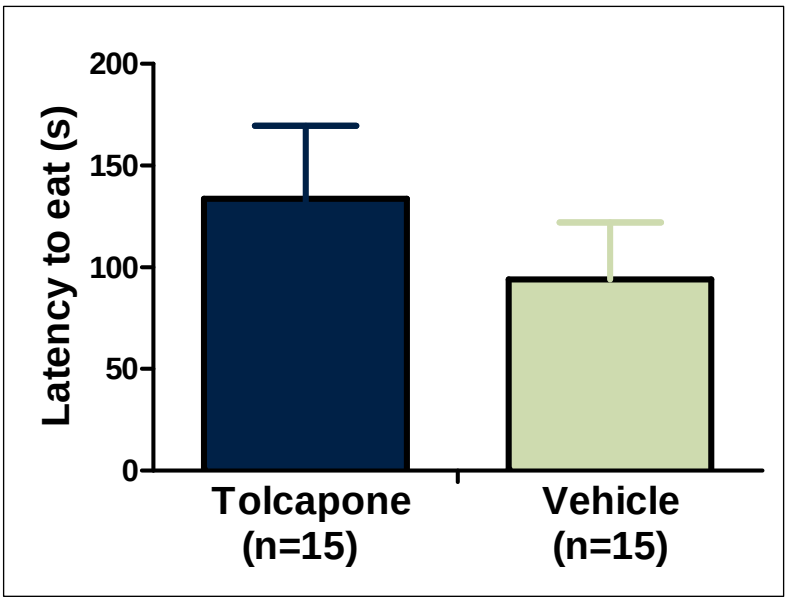


Figure 45 Effect of tolcapone on latency to eat novel food during the second hyponeophagia task

Data (in seconds) is given as mean latency to eat ± SEM.

4 Discussion

The present study found no effect of COMT inhibition on anxiety-related behaviours in male rats. The time spent in the four successive alleys 1–4 did not reveal any differences between tolcapone and vehicle-treated rats. Similarly, the number of entries into alleys 1–4 and the latencies to cross into alleys 2–4 were unaffected by treatment. Additionally, urination and defecation were not different between treatment groups.

However, the time-spent data showed that rats spent most of the time in the first alley of the maze (data not shown), irrespective of drug treatment. Hence, it appears that the open alleys 2–4 (and 3–4 in particular, see Figure 43) may be too aversive. Because these rodents as a cohort may be too anxious to venture out into the open alleys, this task may not be sensitive enough to measure tolcapone-induced changes in anxiety.

Even though the successive alleys data may have been confounded by a floor or ceiling effect, it is more likely that tolcapone has no effect on anxiety in male rats – given that neither of the hyponeophagia tasks yielded any significant effects of treatment.

These negative data may suggest that COMT is not involved in the molecular mechanisms of anxiety. This is perhaps slightly surprising since tolcapone impacts upon hippocampal neurochemistry (chapters 3–4) and the hippocampus is involved in anxiety (see subchapter 1.1). An alternative explanation is that acute administration of tolcapone cannot reproduce the developmental and adaptive aspects of chronically low COMT activity, which was studied by both Gogos *et al* (1998) and the human genetic association studies (discussed in Chapter 1). Moreover, it is possible that, because hippocampus-dependent anxiety tasks also involve non-hippocampal brain regions (e.g. the PFC; Deacon et al 2003), the systemic effects of tolcapone in non-hippocampal regions dampened the

behavioural phenotypes observed as a result of COMT inhibition in the hippocampus alone.

The lack of positive results could potentially also be explained by sex differences, since Gogos *et al* (1998) only demonstrated increased anxiety in female homozygous COMT knockout mice. Studying anxiety in both females and males could test this hypothesis. However, this would at least double the number of rats tested, because the menstrual cycle phase may further increase the variability of the data.

4.1 Summary of Chapter 5

The present study found no gross effect of tolcapone on anxiety-related behaviours in male rats. The implications of this result for a possible involvement of COMT in ventral hippocampal function are discussed in Chapter 7.

Future COMT-related studies on anxiety should investigate different types of emotionality and a wider range of quantifiable anxiety measures. The links between COMT and cognition are better established than the involvement of COMT in emotionality (see Chapter 1). Hence, Chapter 6 investigates the effects of COMT inhibition on hippocampus-dependent cognitive tasks.

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Chapter 6

Investigation of the effects of COMT inhibition on hippocampus-dependent cognition

Linda Maria Laatikainen

DPhil thesis on the role of COMT in hippocampal function

1 Introduction

The present study investigated the influence of COMT inhibition on hippocampus-dependent memory tasks. As discussed previously, the basis of this research is that the hippocampus expresses functional COMT (Chapters 2-4; Matsumoto et al 2003a) and has a crucial role in certain types of memory (see Section 1.1).

1.1 The hippocampus and memory

There is universal consensus that the mammalian hippocampus is involved in certain aspects of learning and memory. Nevertheless, the precise role the hippocampus plays in cognition is commonly debated, because it is unclear which aspect of learning and memory requires hippocampal function (Andersen P 2006).

Research on the role of the hippocampus in cognition began with the finding that removal of large parts of the medial temporal lobe, including the hippocampal formation resulted in amnesia in patient H.M. (Scoville & Milner 1957). It has been suggested that the hippocampus is crucial for declarative (explicit) memory, which includes memories of episodes and semantic items (Eichenbaum 2000a; Tulving 2002; Tulving & Markowitsch 1998). In contrast, the hippocampus is not necessary for procedural (implicit) memory, which is important for encoding skills and habits (Squire 1986).

In rodents the hippocampus seems to be especially important for spatial memory. The earliest evidence for a hippocampal involvement in spatial memory came from electrical recordings in the hippocampi of behaving rats. O'Keefe and Dostrovsky (1971) found hippocampal cells that selectively increased their firing rate only when the rat occupied a well-defined region of the environment, called the 'place field', and rarely fired outside the place field. Logically, these cells were named 'place cells'. These data suggested that firing of hippocampal cells creates a "cognitive map" of the environment (O'Keefe 1990; O'Keefe & Dostrovsky 1971;

O'Keefe et al 1979). Hence, this cognitive map hypothesis suggests the involvement of the hippocampus in spatial memory. Spatial memory is considered as a subtype of declarative memory, and is involved in recording information about the environment and its spatial orientation ("spatial cognitive mapping"). The hippocampus is required in order to recognize places and to orient oneself in space. Moreover, it has been proposed that the role of the hippocampus includes binding aspects of an event to its spatiotemporal context, so-called "contextual binding" (Boyer et al 2007; Eichenbaum 2000a; b).

In addition to *in vivo* electrophysiological experiments; molecular studies, animal lesion models and human neuroimaging have also provided support for a hippocampal role in spatial memory processing: First, *c-fos* staining experiments suggest that the rat dorsal hippocampus is activated during tests of spatial working memory (Vann et al 2000). Second, dorsal (but not ventral) hippocampal lesions result in spatial memory impairments in rats, mice and non-human primates (Aggleton et al 1986; Bannerman et al 2002; Bannerman et al 1999; Deacon et al 2002a; Deacon & Rawlins 2002; Morris et al 1986; Moser et al 1995; Olton & Papas 1979; Sanderson et al 2006). Third, fMRI and PET studies in humans have found that individuals with good navigational accuracy show increased activation of the posterior hippocampus (dorsal hippocampus in rodents) (Hartley et al 2003; Maguire 1997; Maguire et al 1997).

Finally, structural neuroimaging studies suggest that humans with extensive spatial navigation experience exhibit larger posterior hippocampus volumes than age-matched individuals from the general population (Maguire et al 2000; Maguire et al 2003).

1.2 Methods for studying hippocampus-dependent cognition in rodents

The hippocampus is crucially involved in mediating the spatial aspects of declarative memory processing (see Section 1.1). Hence, most studies on hippocampus-dependent cognition involve testing spatial memory functions.

Studies of spatial memory (storage of allocentric spatial information) in rodents have proposed an important distinction between spatial working memory and spatial reference memory (Olton 1979; Olton & Papas 1979). Working memory in rodents refers to a short-term, rapidly-encoded memory process, where the information necessary to make a correct response varies from one trial to the next. One hypothesis is that this type of flexible working memory is driven by short-term habituation to recently-visited, familiar spatial locations (Sanderson et al 2007). In contrast, reference memory in rodents is a form of associative memory, which involves gradual acquisition of associations between an outcome (e.g. reward) and its spatial location across a series of trials (Olton & Papas 1979).

Traditionally, spatial memory was investigated using the radial maze task, which can include both working and reference memory components (Olton 1979; Olton & Papas 1979). In the interest of investigating spatial memory more quickly and easily, spatial working memory and spatial reference memory are now usually explored using separate tests. The classic tests of spatial working memory are spontaneous and rewarded alternation (spatial non-matching to place), performed either on T- or Y-shaped mazes (see below). In contrast, typical tests of spatial reference memory are the hidden platform Morris watermaze task (Morris 1984; Morris et al 1986), as well as multi-trial passive avoidance and elevated Y-maze tests (Deacon et al 2002a).

1.3 Validation of methodology

There were two main criteria for selecting tasks to investigate the role of COMT in the hippocampus. The most important criterion was that the tests were robustly hippocampus-dependent – as shown by cytotoxic lesion studies. The second criterion was that tasks could be completed within 1 – 8 h hours of acute tolcapone administration. The test duration criteria of 1 – 8 h was based on microdialysis experiments, which show significant inhibition of COMT from 60min post-treatment (see Chapter 4), and based on human and rodent pharmacokinetic data, which suggest that a time frame of up to 8 h is viable since tolcapone has a relatively slow elimination rate (see Chapter 3).

1.3.1 Delayed rewarded alternation task

Rewarded alternation (also called non-matching-to-place or forced-choice alternation) is a spatial working memory task, which makes use of rodents' instinctive tendency to explore novel or less familiar environments. Each rewarded alternation trial consists of two distinct phases: the sample run, during which the rat has access to one of the goal arms; and the choice run, when the rat has access to both goal arms. The rat only receives a food reward if it correctly alternates between two available goal arms during each trial (Figure 45; Deacon & Rawlins 2006; Rawlins & Olton 1982).

Rewarded alternation was the rewarded spatial working memory task of choice because it is commonly used, and is robustly hippocampus-dependent in both rat and mouse lesion studies (Aggleton et al 1986; Deacon et al 2002a; Rawlins & Olton 1982). Also, since the cognitive effects of tolcapone are unknown, the rewarded alternation task design can incorporate a variable inter-run delay so as to increase the sensitivity of the task to allow for the detection of cognitive enhancement (hence, the task is named "delayed" rewarded alternation).

1.3.2 Spatial novelty preference task

The single trial spatial novelty preference task is a spatial short-term memory task, which also involves two phases: the exploration period, during which a rat is restricted to exploring only two of the three available arms of a Y-maze; and the test period, when the rat can freely explore the Y-maze. Previous work has shown that rodents display a natural preference for the novel arm during the test period of this task. However, if a rat does not recognise which arm of the maze is novel, it would be expected to spend equal time in all three arms of the Y-maze during the test period – indicating a spatial short-term memory deficit.

This task was chosen, because it has been shown to be hippocampus-dependent in rodents (Sanderson et al 2007), and because it is neither appetitively nor aversively motivated. Also, it appears that rats may use similar familiarity-based strategies to solve both the rewarded alternation and spatial novelty preference task (Sanderson et al 2009). Thus, the spatial novelty preference task can be considered as an unrewarded version of the T-maze alternation task, whilst offering a means of validating any alternation task results on an independent spatial memory paradigm.

1.4 Aims and hypotheses

The aim of the present chapter was to investigate the contribution made by COMT to aspects of learning and memory. In this study tolcapone-treated rats were assessed on two well-validated, hippocampus-dependent spatial working memory tasks (Rawlins & Olton 1982; Sanderson et al 2007).

Given that DA agonists in the hippocampus improve cognition (Bach et al 1999; Packard & White 1991), whereas decreased DA and NA signalling impairs memory (Gasbarri et al 1996; Korz & Frey 2007; O'Carroll et al 2006), tolcapone was expected to improve spatial working memory. Further support for this hypothesis comes from the cognitive enhancing effects of tolcapone in humans (Apud et al 2007; Gasparini et al 1997; Giakoumaki et al 2008) and rats

(Khromova et al 1997; Lapish et al 2009; Liljequist et al 1997; Tunbridge et al 2004b), as well as the cognitive phenotypes of genetically manipulated COMT mice (Babovic et al 2008; Papaleo et al 2008). However, it is important to note that these cognitive phenotypes are not strictly hippocampus-dependent.

2 Methods

2.1 Animals

The delayed, rewarded alternation task was conducted in one cohort of twenty-four experimentally-naïve male Lister-Hooded rats (n=24 at 209 – 263 g during rewarded alternation testing; Harlan, OLAC, Bicester, UK). In contrast, the spatial novelty preference task was performed on two separate cohorts of experimentally naïve male Lister-Hooded rats (n=61 at 230 – 317 g during novelty preference testing; Harlan) in order to avoid caveats related to multiple injections. The cohorts were tested one month apart in separate testing rooms.

All rats were housed in groups of three in standard open caging (N.K.P. Cages Ltd, Kent, UK) at standard conditions (lights on 7.00 – 19.00, 21 ± 1 °C temperature, 50 % humidity, ad libitum water). The home cages contained wood chips as bedding, a white cardboard refuge, two cuboid wooden blocks for gnawing, as well as copious amounts of shredded brown paper as nesting material. Because the delayed rewarded alternation task is appetitively motivated, rats used for this task were not on *ad libitum* food like the rats used for spontaneous novelty seeking. Instead, rats used for the rewarded alternation task were maintained on a restricted feeding schedule at no less than 85 % of their free-feeding body weight for 27 – 30 days prior to testing.

All animal procedures were carried out in the laboratory of Dr David Bannerman in the Oxford University Department of Experimental Psychology in accordance with the Animals (Scientific Procedures) Act 1986 and associated Home Office guidelines.

2.2 Delayed Rewarded Alternation Task

2.2.1 Apparatus

Testing was conducted in an open wooden T-maze, raised 43 cm off the floor (arms 61 x 10 x 2 cm). The start arm led to two goal arms, all of which were painted a uniform grey colour. Each goal arm contained a metal food well (2 cm in height and 2.5 cm in diameter), located 5 cm from the end of the arm. A moveable block of wood (natural wood colour, 20 x 10 x 10 cm) could be placed on the maze to deny access to one of the goal arms (making the maze L shaped).

All habituation, training and experiments were conducted by the same person in the same experimental testing room. The experimenter was located ~1 m from the maze, the holding cages were ~2 m from the maze and the door was ~3 m from the maze.

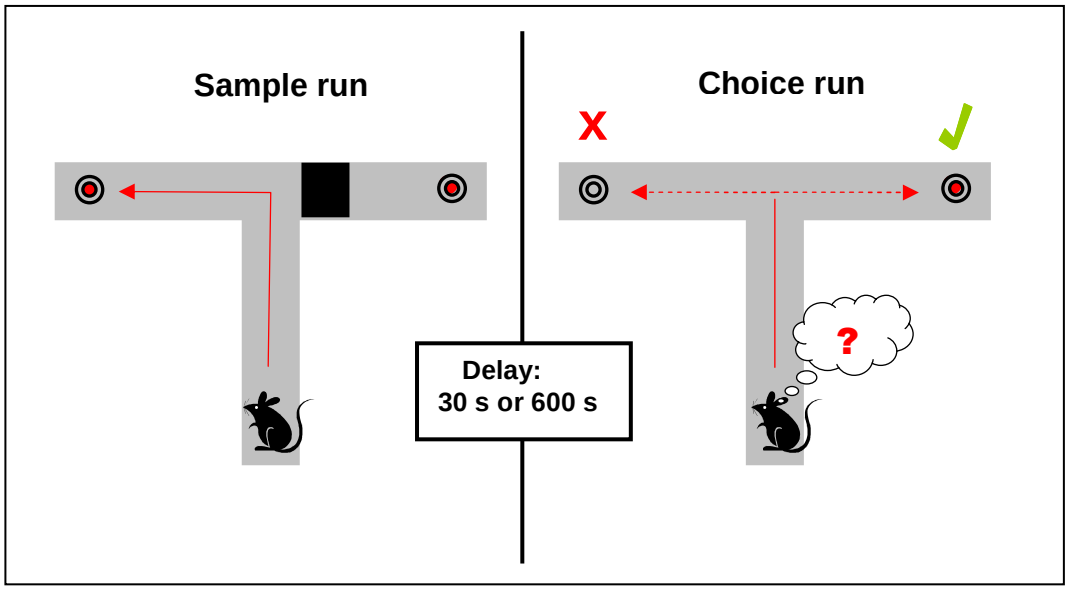


Figure 46 Diagram of delayed rewarded alternation task design and apparatus
The diagram above presents a view of the testing apparatus from above. The wooden T-maze, painted uniform grey, is shown with the two food wells located 5 cm from the end of each goal arm (either filled [shown in red] or empty) as well as the removable wooden block (illustrated as a black square), which was used to block access to one goal arm during the sample run. Solid red lines represent run paths for the rat, whereby dotted red lines represent two alternative run paths rats can choose between on the choice run. The picture on the left shows a rat at the start of a sample run, with the wooden block in place. In contrast, the left-hand picture shows a rat at the start of a choice run, whereby both goal arms of the T-maze are accessible.

2.2.2 Habituation

During the first 12 days of food deprivation animals were fully habituated to running on the maze and to eating the sucrose pellets (45-mg Noyes sucrose pellets; Sandown Scientific, UK) that were used as a reward. Initially, rats were habituated for 5 min in groups of three, with food freely available in both goal arms. Then rats were habituated individually, with access only to food in one of the goal arms (using the wooden block to prevent access into the other goal arm). Once all the animals were running freely and consuming the food rewards, training on the reward alternation task was started from day 13 (of food deprivation) onwards.

2.2.3 Training

Training and testing were performed in batches of six rats run in a circulating order through the squad. Rats were first trained to run from the start arm to the only accessible goal arm, the sample arm, which was baited with one sucrose pellet. After eating the sucrose pellet during this sample run, the rats were again placed on the start arm of the maze. During this choice run, rats were rewarded for going into the novel goal arm by the presence of two sucrose pellets in this arm. The previously visited sample arm now had no food available. There were no delays present during this stage of training. The location of the rewarded goal arm was varied in a pseudorandom fashion between runs, with no more than two consecutive choice runs with the same configuration. Each rat had an equal number of left and right rewarded goal arms within each block of 10 choice runs. This zero delay training was continued until all animals were choosing the rewarded goal arm on more than 80 % of trials.

Twenty days after the start of the restrictive food schedule, 30 s and 600 s delays were introduced between the sample run and the choice run (see Figure 46). During delays, rats were placed in 30 x 12.5 x 15 cm wooden holding cages with a wire-mesh floor and a transparent Perspex lid. The order of delays was varied in a

pseudorandom fashion, with no more than two consecutive trials with the same delay. The number of 30 s and 600 s delay trials, as well as the number of times each goal arm contained the reward was balanced across each block of 20 choice runs. Choice data from the four consecutive training days were combined into a single block of 40 trials for analysis.

2.2.4 Behavioural testing

The performance of the rats on the rewarded alternation task was measured on four consecutive days of testing. Each day six animals were tested, of which two were drug-treated, two were vehicle-treated and two were non-injected controls (see Chapter 5 Section 2.2 for the tolcapone and vehicle preparation methods). Each day the same person injected rats intraperitoneally one hour prior to the start of behavioural testing in a room separate to the testing or holding rooms. Each rat received four blocks of 10 trials on the testing day. The latency to eat on the sample run was also recorded. The total time of testing each day was approximately 6 – 7 h.

2.3 Spatial novelty preference task

2.3.1 Apparatus

The task was conducted on a Y-maze constructed from transparent Perspex, which was mounted on a transparent Perspex board (47.5 cm x 52.5 cm; see Figure 47). The walls of the maze were 11.5 cm high and 0.3 cm thick. Each arm was 20 cm long and 8 cm wide. During the 5 min exploration period, the entrance to one of the arms (named the novel arm) was blocked with a sheet of opaque Perspex. The maze was placed in a testing room containing a variety of extra-maze cues.

A thin layer (<0.5 cm) of wood chips, as used for the home cage bedding, covered the floor. This was not changed between rats, but was redistributed between the exploration period and the test period for individual rats, as well as

between rats. Before the start of the experiment, two non-experimental rats were placed in the maze for a few minutes to make the environment more similar for the first versus subsequent rats. Also, when wood chips were replaced with clean ones after each batch of 6 rats, some old wood chips were left in the Y-maze.

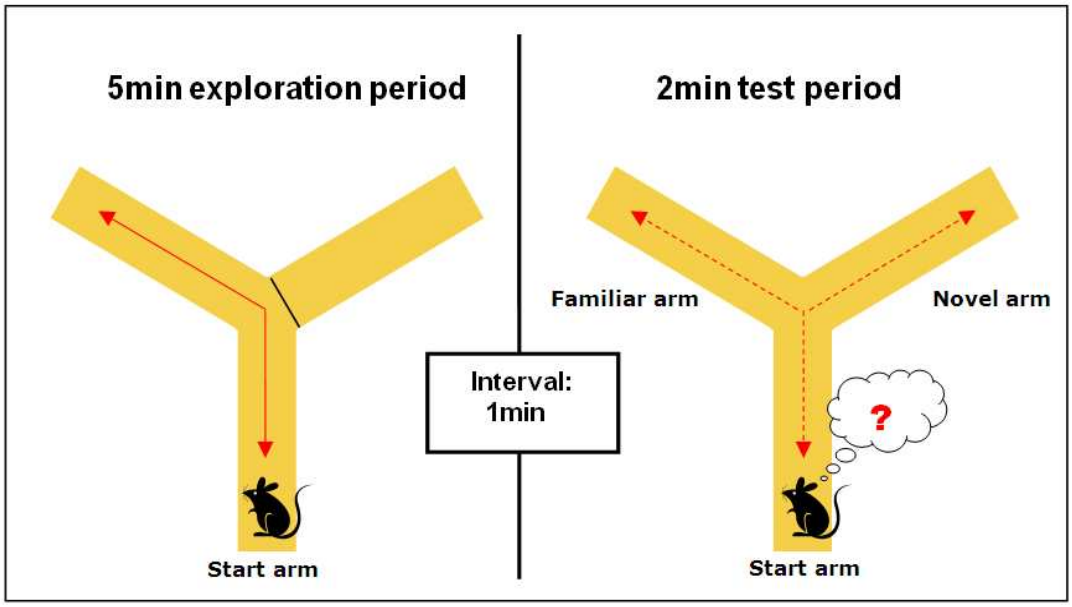


Figure 47 Diagram of the spatial novelty preference task design and apparatus

The diagram above represents a view of the testing apparatus from above. The transparent Perspex Y-maze is shown with the removable opaque Perspex wall (illustrated as black line in the left-hand picture), which is used to block access to the novel arm during the 5 min exploration period. Solid red lines represent run paths for the rat, and dotted red lines represent two run paths rats can choose between during the 2 min test period. The picture on the left shows a rat at the start of a 5 min exploration period, with the opaque Perspex barrier in place. In contrast, the right-hand picture shows a rat during the 2 min test period, with both the familiar and novel arms accessible.

2.3.2 Behavioural testing

The Spatial Novelty Preference task does not involve any training. Testing was performed in batches of six individually running rats, of which two were drug-treated, two were vehicle-treated (see Chapter 5 Section 2.2 for details regarding the preparation of the drug and vehicle) and two were simply handled (non-injection controls) in order to investigate the effects of injection stress on cognition. Each day the same person injected rats intraperitoneally one hour prior to the start of behavioural testing in a room separate from either the testing or the holding room. Three squads of six were run each day at similar times of the day.

Each rat was assigned two arms (start and "to be familiar" arms) to which it was exposed during the first part of the task (exploration period). The remaining third arm represented the novel arm during the second part of the task (test period). Allocation of arms (start, to be familiar and novel) to specific spatial locations and to the arms of the Y-maze was counterbalanced within each experimental group. The experimenter always sat still behind the start arm.

During the exploration period, rats were placed at the end of the start arm and given 5 min to spontaneously explore both the start arm and the "to be familiar" arm. Access to the novel arm of the Y-maze was prevented using a removable opaque Perspex wall (see Figure 47). During this exploration period, time spent in each arm, as well as the number of entries into each arm, were recorded using a manually operated multi-channel event timer/counter. An entry into an arm was defined by a rat placing all four paws inside an arm. Similarly, a rat was considered to have left an arm if all four paws were placed outside the arm. 5 min after a rat left the start arm it was removed from the Y-maze and returned to its home cage.

The test period began 1 min after the rats were returned to their home cage. During the test period, rats were placed back at the end of the start arm and were allowed to explore freely all three arms of the Y-maze for 2 min after they left the start arm. The number of entries into each arm and the time that rats spent in each arm were recorded during the test period.

2.4 Data analysis

Data were analysed using PASW statistics 18.0 software (SPSS Inc, Chicago, IL, USA) and plotted using GraphPad Prism 5.01 (GraphPad Software Inc, La Jolla, CA, USA).

For most delayed rewarded alternation data analyses, the choice data from a given delay were combined into one block of 20 trials at each delay for each rat. Repeated-measures ANOVAs with LSD post-hoc tests were performed (details

described in the results section). In case of statistically significant Mauchly's test of sphericity, a Huynh-Feldt correction was used to alter the degrees of freedom and thereby alter the F-ratio significance level.

Novelty preference was quantified by calculating a discrimination ratio [time spent in novel arm divided by total time spent in novel and familiar arms during the 2min test period]. In order to statistically analyse the novelty preference data, two different methods of data analysis were utilised: First, one-way ANOVAs with LSD post-hoc tests were used to compare tolcapone-, vehicle- and non-injected rats. Second, planned comparisons were used to compare tolcapone and vehicle-treated rats, where appropriate, based on a priori prediction. Notably, including rat cohort as a between-subjects factor had no statistically significant effect on novelty preference and was hence not included as part of the final analysis. Raw data regarding the number of entries into, and the time spent in the arms of the maze were analysed using repeated-measures ANOVAs with treatment as a between-subjects factor and arm type as a within-subjects factor.

3 Results

3.1 Delayed rewarded alternation task

3.1.1 Performance during pre-training

During the training phase, all rats performed significantly better on the 30 s delay condition than on the 600 s delay condition ($t(23)=8.094$, $p<0.001$). Nonetheless, according to a one-sample t-test, performance on the 600 s delay trials was still clearly above chance ($t(23)=8.789$, $p<0.001$). Baseline performance for the 30 s delay condition was 87.5 ± 1.8 % and for the 600 s delay condition 68.8 ± 2.1 %.

Importantly, there was no evidence of inherent differences in performance between the rats randomly allocated to be in the three different treatment groups, as indicated by an ANOVA showing no overall effect of treatment ($F_{2,21}=0.464$, $p=0.635$) or delay*treatment interaction ($F_{2,21}=0.889$, $p=0.426$) in baseline performance during training (i.e. prior to injection).

3.1.2 Performance during behavioural testing

The main finding of the delayed rewarded alternation task was that tolcapone-treated rats performed better than vehicle-treated rats on trials using the 30 s delay condition. Moreover, non-injected rats exhibited intermediate levels of performance.

Choice data were included in a repeated-measures ANOVA, with a between-subjects factor of treatment (tolcapone, vehicle, non-injection) and a within-subjects factor of delay (30 s vs 600 s). This analysis revealed a significant treatment*delay interaction ($F_{2,21}=4.772$, $p=0.020$) and a significant main effect of delay ($F_{1,21}=102.054$, $p<0.001$). There was no main effect of treatment ($F_{2,21}=1.977$, $p=0.163$). Subsequent investigation of the treatment*delay interaction using LSD post-hoc tests showed that there was a significant difference between tolcapone- and vehicle-treated rats in the 30 s delay condition ($p=0.001$),

but not in the 600 s delay condition (p=0.701; Figure 48). Non-injected rats also tended to perform better than vehicle-treated rats on the 30 s delay condition (p=0.060).

As illustrated in Figure 48, task performance after a 600 s delay is similar across the three treatment groups (62.9 ± 1.9 %). This level of performance was significantly worse than during the training days (68.8 ± 2.1 %, t(23)=2.322, p=0.029), however it was still significantly above chance (50 %, t(23)= 6.793, p<0.001).

Analysis of the latencies to run and/or eat on the sample trial across different trial delays during testing did not show any effect of treatment or delay*treatment interaction (p's>0.05 during training and testing; Appendix VI "latencies to eat"). However, during testing, rats entering the sample phase of a 600 s trial were slower to run/eat than rats on 30 s trials ($F_{1,21}$=11.278, p=0.003).

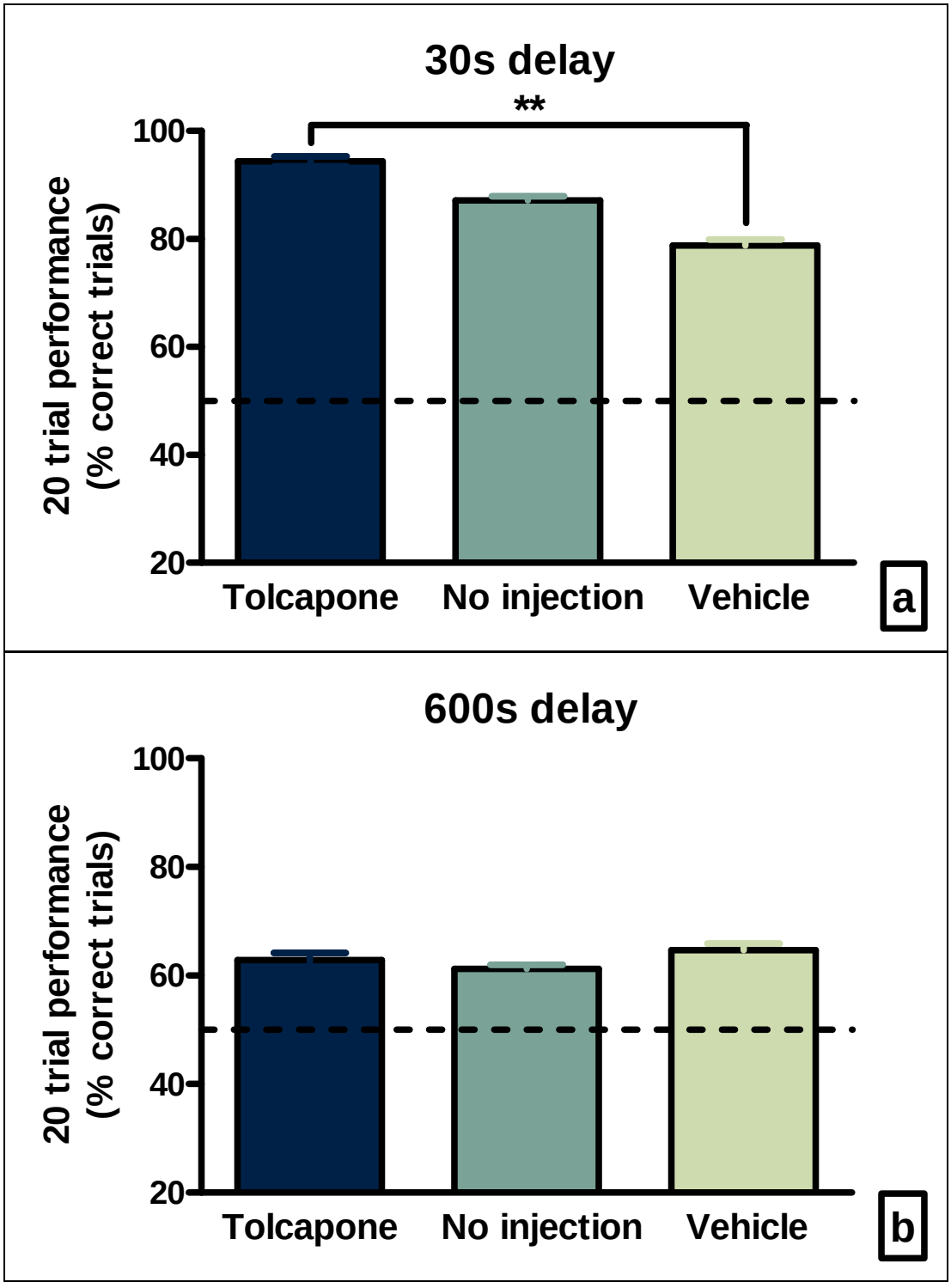


Figure 48 Effect of tolcapone and vehicle treatment on delayed rewarded alternation

The figure above illustrates the performance of tolcapone-treated rats, vehicle-treated rats or non-injected controls at (a) 30 s delay or (b) 600 s delay as a percentage of correct trials out of 20 total trials. Post-hoc tests show that tolcapone-treated rats perform significantly better than vehicle-treated rats on the 30 s delay task ($p=0.001$), as shown by the double asterix. There is also a trend for vehicle-injected rats to perform worse than "no injection" controls on 30 s delay tasks ($p=0.06$).

3.1.3 Analysis of proactive interference

Proactive interference over the course of repeated rewarded alternation trials is a possible explanation for poorer performance of vehicle-treated rats on behavioural memory tasks. According to this theory, memory traces from previous trials may interfere with performance on the current trial. Hence, one might expect that performance would get worse over the course of the day as the number of performed trials increases. However, a crude analysis comparing the first block of ten versus the second block of ten trials at each delay did not yield any statistically significant effect of block ($F_{1,21}$=0.388, p=0.540), treatment*block ($F_{2,21}$=1.195, p=0.322) or treatment*block*delay ($F_{2,21}$=0.307, p=0.739; Figure 49 and Appendix VI "Block" section).

An alternative method of analysing proactive interference involves analysing whether memories from previous trials interfere with more recent memories: ANOVAs were performed with treatment as a between-subjects factor (tolcapone, vehicle, non-injection) and delay condition on the current trial (30 s, 600 s) as well as delay condition on the preceding trial (30 s, 600 s) as within-subjects factors. This method of analysis did not reveal a statistically significant effect of previous delay condition ($F_{1,21}$=0.004, p=0.953) or previous delay*treatment ($F_{2,21}$=0.761, p=0.480). However, there was a trend-level three-way interaction between previous and current delay condition as well as treatment type ($F_{2,21}$=2.985, p=0.072; see Figure 50 and Appendix VI "previous delay" section). Namely, vehicle-treated rats were only different from tolcapone- (p=0.001) and non-injected rats (p=0.002) on 30 s delay conditions that were preceded by 600 s delay conditions.

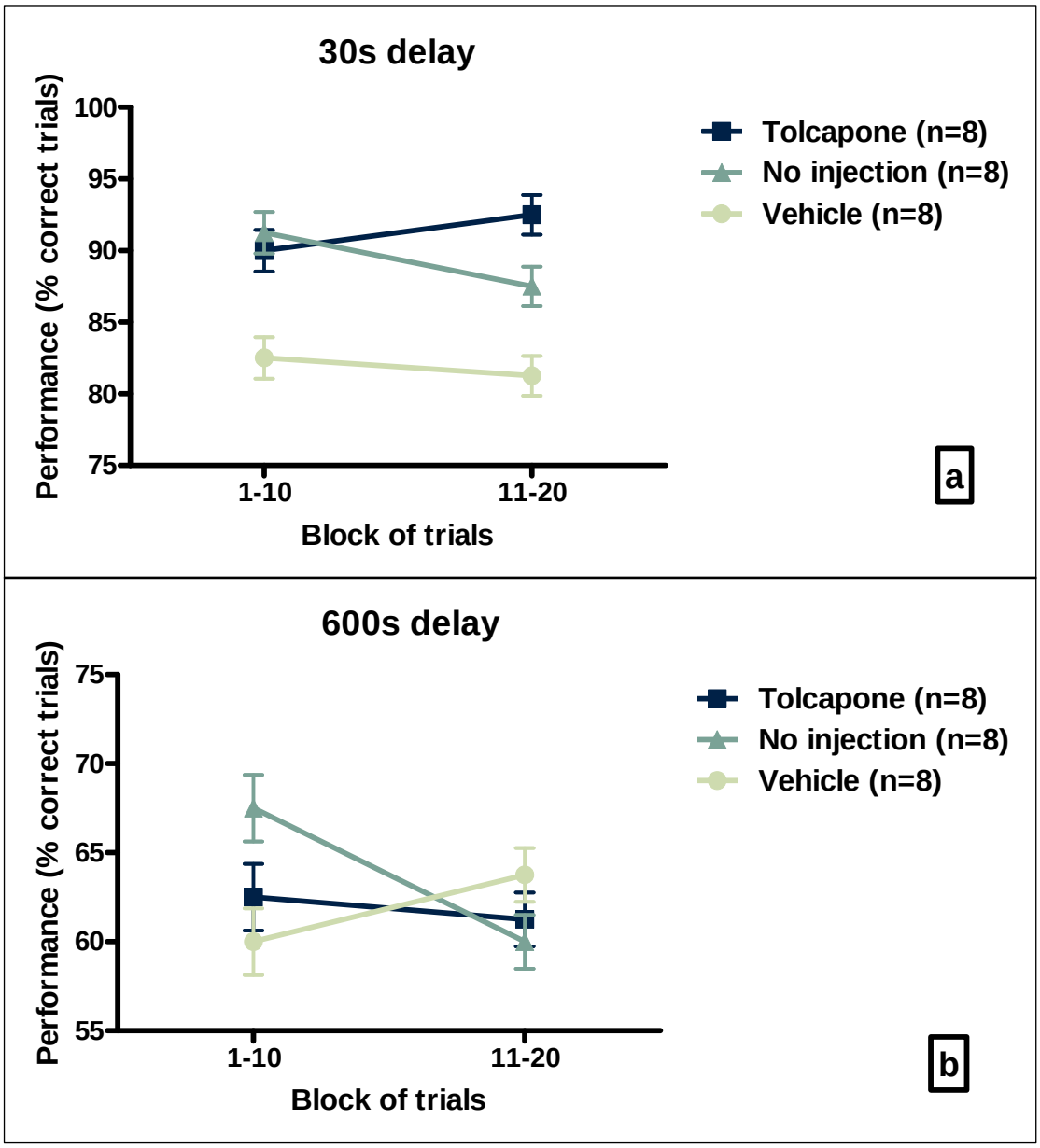


Figure 49 Performance on the delayed rewarded alternation task by block of trials

Performance of tolcapone-, vehicle- and non-injected rats during the trial blocks 1-10 and 11-20 (corresponding to approximately 1 – 4 and 4 – 7 h post-injection) during (a) 30 s or (b) 600 s delay trials (data given as number of correct choices out of 10 possible correct choices ± SEM).

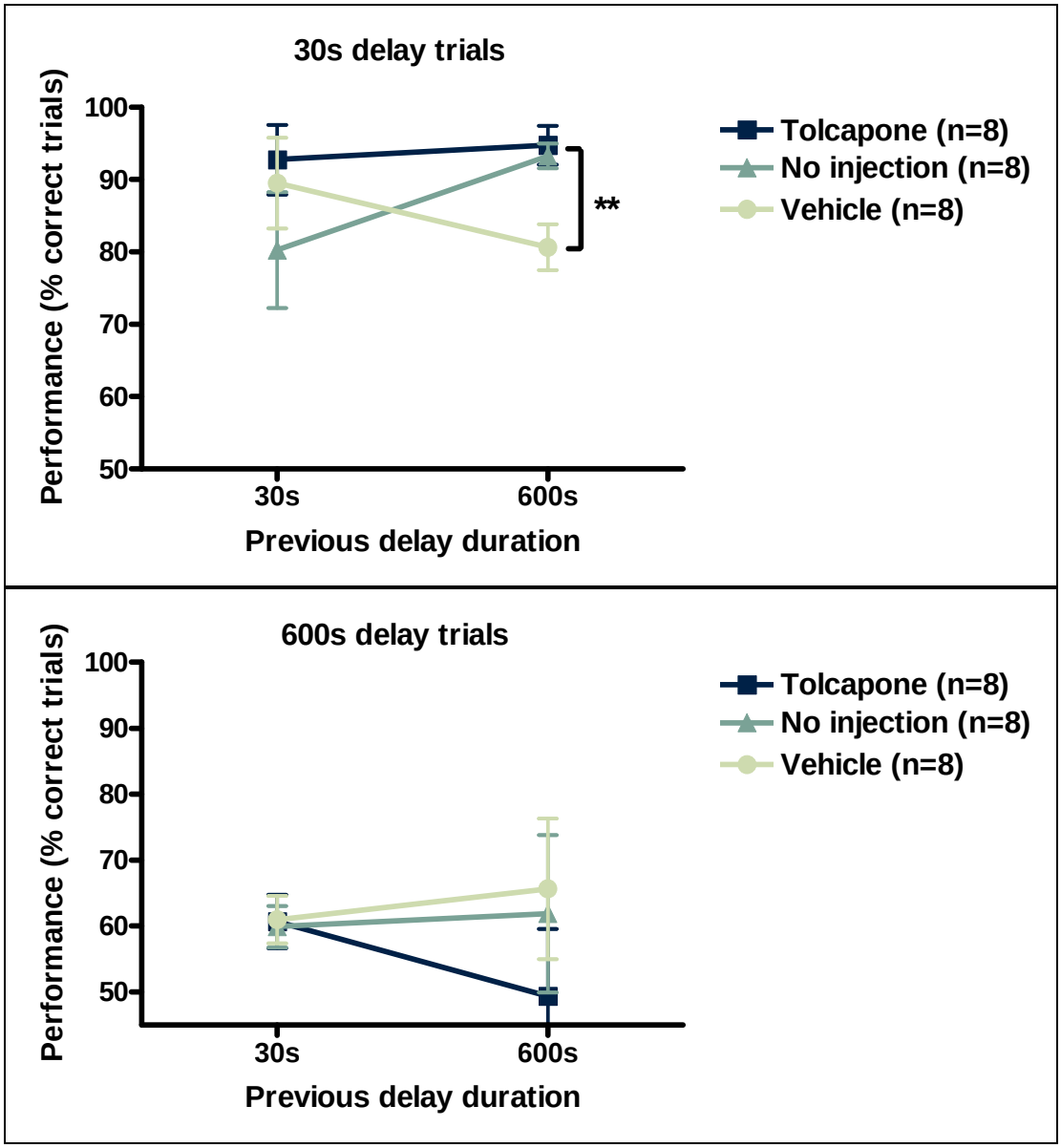


Figure 50 Performance on the delayed rewarded alternation task by previous delay duration

The figure illustrates performance 1–7 h post-injection on (a) 30 s and (b) 600 s delay trials, when the previous delay duration was either 30 s or 600 s (data given as % correct trials ± SEM; ** $p<0.01$).

3.2 Spatial Novelty Preference Task

3.2.1 Effect of tolcapone on spatial novelty preference

The main result of the spatial novelty preference task was that tolcapone significantly improved spatial novelty preference performance relative to vehicle-injected controls, whereas non-injected controls again exhibited intermediate levels of novelty preference.

As stated in section 2.4, novelty preference was quantified by calculating time spent in the novel arm divided by total time spent in the novel and familiar arms during the 2 min test period. A one-way ANOVA with treatment as a between-subjects factor did not yield an overall main effect of treatment ($F_{2,58}$=1.85, p=0.166). However, given the results of the rewarded alternation experiment, a planned comparison comparing tolcapone- and vehicle-treated rats indicated that tolcapone-treated rats prefer the novel over the familiar arm more than vehicle-treated rats (Figure 51; $F_{1,38}$=4.67, p=0.037).

Notably, all treatment groups spent equal amounts of time in the familiar arm during the 5 min exploration period, as analysed using univariate ANOVAs ($F_{2,58}$=0.057, p=0.945 including all three treatment groups; or $F_{1,38}$=0.004, p=0.947 using planned comparisons). This confirms that the preference of tolcapone-treated rats for the familiar arm is not simply because the familiar arm is more familiar to tolcapone- than vehicle-treated rats.

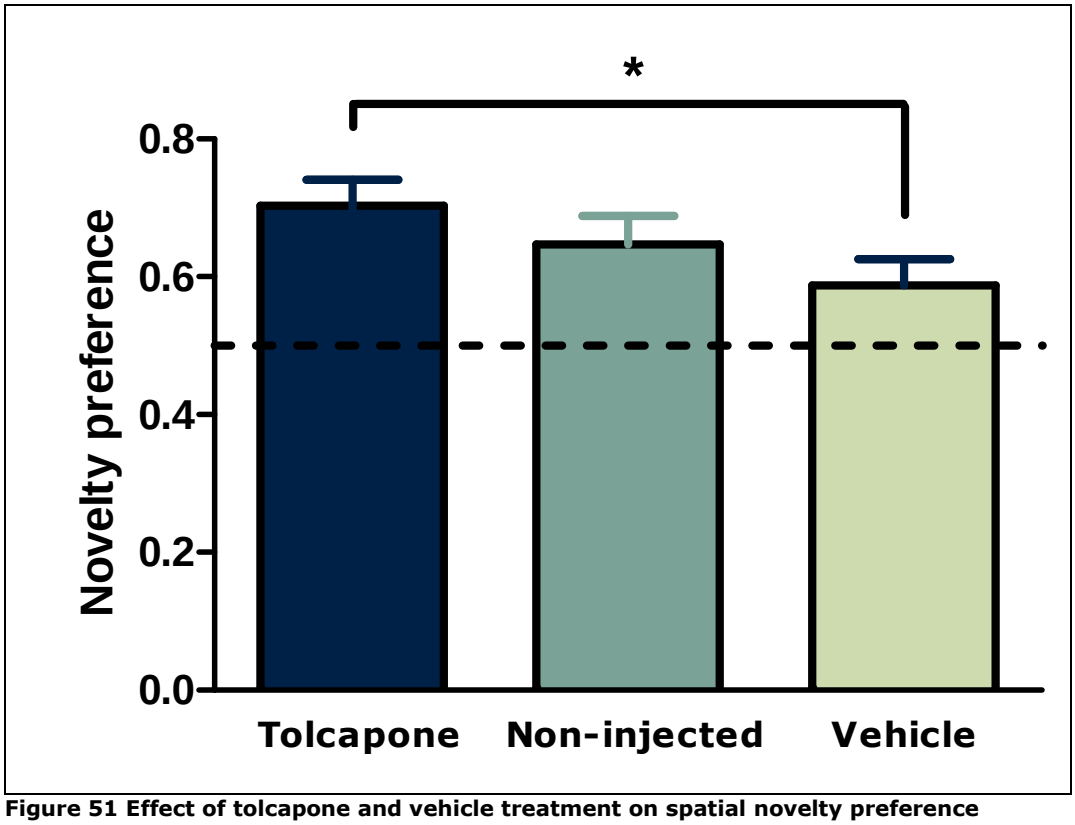


Figure 51 Effect of tolcapone and vehicle treatment on spatial novelty preference
Novelty preference was quantified by calculating the ratio of total time spent in novel arm divided by total time spent in novel and familiar arms during the 2 min test period (tolcapone-treated rats n=20, non-injected rats n=21, vehicle-treated rats n=20).

3.2.2 Effect of tolcapone on the number of times rats enter each arm of the Y-maze

Studying the effect of tolcapone on the number of times rats enter each arm of the Y-maze showed that arm entries were not affected by treatment. During the exploration period, raw "arm entry" data did not differ between the start or familiar arms ($F_{1,58}$=128.685, p<0.001) and were not affected by treatment ($F_{2,58}$=0.973, p=0.384; as studied using a repeated-measures ANOVA with a two-level within-subjects factor (start, familiar). Also, there was no treatment*arm type interaction ($F_{2,58}$=0.399, p=0.672).

In contrast, during the 2 min test period, rats preferred to enter the novel arm over the start or familiar arm. However, treatment did not influence the arm entry data. A repeated-measures ANOVA, including treatment as a between-subjects factor (tolcapone, vehicle, non-injected) and arm type as a within-subjects

factor (novel, familiar), revealed a main effect of arm type ($F_{1,58}$=128.685, p<0.001), but not treatment ($F_{2,58}$=0.476, p=0.623) or treatment*arm type interaction ($F_{2,58}$=0.126, p=0.882). LSD post-hoc tests indicated that all rats spend more time in the novel arm than the familiar arm. A similar repeated-measures ANOVA with arm type as a three-level within-subjects factor (novel, familiar, start) also suggested a main effect of arm type ($F_{2,116}$=78.125, p<0.001), but no effect of treatment ($F_{2,58}$=0.462, p=632) or treatment*arm type interaction ($F_{4,116}$=0.654, p=0.625). LSD post-hoc tests on the raw entry data suggested that rats entered the novel arm more often than either the start (p<0.001) or the familiar arm (p<0.001). The arm entry data during the test period did not show a preference for the familiar arm over the start arm (p=0.884).

The lack of a treatment effect on novelty preference implied by the raw entry data was also supported by the results of an one-way ANOVA analysing novelty preference [as calculated using number of entries into the novel arm divided by total number of entries into the novel and familiar arms during the 2 min test period]. Hence, tolcapone-treatment had no effect on the number of entries into each arm of the maze ($F_{2,58}$=0.866, p=0.426; or $F_{1,38}$=1.337, p=0.255 using a planned comparison, tolcapone vs. vehicle).

Moreover, the arm which rats entered first during the test period was not affected by treatment, irrespective of data analysis method (p's>0.6). Almost all rats entered the novel arm first.

4 Discussion

4.1 Delayed rewarded alternation

Tolcapone improved spatial working memory performance on the delayed rewarded alternation task. This conclusion is in agreement with the current human (Apud et al 2007; Gasparini et al 1997; Giakoumaki et al 2008) and rodent (Babovic et al 2008; Khromova et al 1997; Lapish et al 2009; Liljequist et al 1997; Papaleo et al 2008; Tunbridge et al 2004b) literature which implies that COMT inhibition may have beneficial effects on aspects of memory performance.

The present study conducted proactive interference analyses to attempt to understand the processes underlying the tolcapone-induced cognitive enhancement. However, memory of the reward location in a previous trial did not appear to interfere with performance of the current trial, as observed in previous studies (Aggleton et al 1986). Hence, tolcapone-induced changes in cognitive performance did not appear to be due to an altered susceptibility to proactive interference (see Sanderson et al 2007 for a similar result). Furthermore, tolcapone-treated rats were also improved at the single trial spatial novelty preference task (see below), which cannot be explained by proactive interference.

The mechanisms underlying delayed rewarded alternation performance are not fully understood. It is often assumed that rewarded alternation requires learning of a win-shift rule, whereby animals gradually learn not to return to a location in which they have already consumed food within a trial. However, given that rats performed well above chance at the start of testing (data not shown), it appears that rats can alternate without having to learn a win-shift rule. Alternative hypotheses include that rewarded alternation provides a measure of how well rodents detect and perceive relative novelty/familiarity, and that spatial alternation requires stimulus-specific, short-term habituation to recently experienced stimuli (Sanderson et al 2010). If no novelty detection and/or habituation occur, then rats

are equally as likely to enter the two goal arms, when given a choice – indicating poor working memory performance.

Given that tolcapone had no effect on proactive interference, tolcapone-induced spatial working memory enhancement may be due to improved short-term memory for the familiar goal arm (Sanderson et al 2007). This memory enhancement may reflect a drug-induced change in the attentional state or a change in processing the salience of the reward (sucrose pellets in the present study). Hence, tolcapone-treated rats may go into the novel goal arm because they are more motivated by the two sucrose pellets, or they attend more to the rewarding or arousing properties of the sucrose pellets than vehicle-treated rats. Hence, the spatial novelty preference task was performed to distinguish between these two options.

4.1.1 Task-specific caveats of the rewarded alternation experiment

The alternation data from drug-naïve rats treated with a single tolcapone injection suggest a fairly robust memory enhancement. However, the data raise two task-specific issues, which are discussed below:

First, tolcapone improved alternation on the 30 s delay trials but not 600 s delay trials. One explanation is that the 600s delay task is too difficult for all rats. However, this hypothesis is not entirely straight forward, because all rats performed significantly above 50 %, even at the 600 s delay condition. Alternative explanations may reflect the nature of the delay condition or that tolcapone only enhances cognitive performance when performance is good.

Second, rats perform significantly worse after the 600 s delay on the testing day than on the training days. This may reflect the fact that when rats are tested, they are in the same physical environment as during training, but may not be in the same physiological state due to the stress of injection. However, it is worth noting that the non-injected control group was also worse on the testing day than the training. This could potentially be due to transmission of the effect of stress

between cage mates. Also, given that during testing rats were run 40 times daily, but only 10 times during training, worse performance during testing may reflect increased arousal due to having a higher information load and hence higher task difficulty. However, Figure 49 suggests that performance did not worsen with increased information load. Hence, this result may even be due to task design-specific effects.

4.2 Spatial novelty preference task

Tolcapone-treated animals showed a greater novelty preference, compared with vehicle-treated rats. Hence, the spatial novelty preference task confirmed the memory enhancing effects of the COMT inhibitor tolcapone on hippocampus-dependent short-term memory. As stated above, this result is in accordance with studies suggesting a role for COMT, dopamine and the hippocampus in certain forms of learning and memory. Also, the habituation theory discussed above can be applied to the spatial novelty preference task. Increased habituation of tolcapone-treated rats to the familiar arm could not be explained by increased exposure to the familiar arm during the exploration period. Instead, rats spent similar amounts of time in the familiar arm, irrespective of treatment. Thus, preference for novelty during the test period is proposed to be due to short-term habituation to the recently-visited locations during the exploration period. The fact that rats explored less during the test period than the exploratory period (data not shown) suggests that some habituation to the familiar environment had occurred over time, which supports the habituation theory. Hence, it appears that tolcapone improves short-term habituation, which may underlie the spatial working memory performance on win-shift tasks in rodents.

A potential caveat of the spatial novelty preference data is that it was pooled from two separate cohorts of rats, which were tested in different testing rooms that have different spatial cues. However, including experimental cohort as a factor had no effect on the results. Hence, hippocampus-dependent cognitive tasks

appear to be sensitive to the cognitive enhancing effects of tolcapone - even over the background noise of using different rat cohorts and experimental conditions.

It is also encouraging that comparison of the performance of tolcapone-, vehicle- and non-injected rats on the spatial novelty preference task mirrors the results seen in the delayed rewarded alternation task. This suggests that the results are reliable and robust even across different types of spatial working memory tasks. Since tolcapone improves performance on the novelty preference task, which does not incorporate any overt rewards, tolcapone appears to improve spatial working memory irrespective of changes in reward processing. This means that tolcapone-induced cognitive enhancement cannot be solely driven by an increased interest in rewards, an enhanced motivation by rewards or an increased association between a spatial location and a reward. This suggests that the effects of tolcapone on catecholamine metabolism in the hippocampus (and elsewhere, see discussion below) could potentially influence a wider range of cognitive tasks than traditionally expected (mesolimbic dopamine is traditionally thought to be involved in learning the association between a reward and its spatial location; Berridge & Robinson 2003). However, this hypothesis requires further validation, especially since the mechanisms underlying hippocampus-dependent short-term memory are a matter of controversy (Sanderson & Bannerman 2007).

4.3 Effects of injection stress on hippocampus-dependent behaviour

Non-injected rats performed at intermediate levels between tolcapone- and vehicle-treated rats on both short-term hippocampus-dependent spatial working memory tasks. This suggests that the changes in stress and arousal due to injection can influence hippocampus-dependent rat behaviour.

One interpretation of the result is that injection of tolcapone is beneficial to spatial working memory (as predicted by the literature reviewed in Chapter 1), whilst injection of vehicle is detrimental to performance. An alternative explanation is that the stress caused by any injection is detrimental to spatial working memory

and that tolcapone rescues this deficiency – though in this case one might expect tolcapone to bring performance only to the level of the non-injected controls and not above it. The most likely interpretation is that the effects of tolcapone are superimposed on a background of stress-induced impairment in cognitive performance. The molecular mechanisms underlying this explanation are discussed below.

The effect of injection stress probably follows the well-established inverted U-shaped function between acute stress and performance on cognitive tasks initially formulated by Yerkes and Dodson (1908). According to this function, stress up to a certain threshold level enhances cognitive performance by increasing levels of attention and arousal relative to the "non-stressed" state. However, higher levels of stress become deleterious. Interestingly, the effect of COMT inhibition on cognitive performance is likely to also follow an inverted U-shaped function. Since COMT affects DA and NA metabolism (see Chapter 3 and Chapter 4), it is likely that intermediate levels of COMT activity are needed to produce intermediate levels of brain catecholamine levels, which result in maximal cognitive performance (see Chapter 1).

Since the effects of both stress and COMT inhibition on cognitive performance may follow inverted U-shaped functions, it is tempting speculate both effects can be integrated into a single inverted U-shaped function. Since COMT affects DA, which is directly involved in both cognition and arousal, it is tempting to base this integrative U-shaped function on the relationship between DA levels and cognitive performance (Figure 5). However, as illustrated by Figure 52, such an inverted U-shaped cannot explain why tolcapone-treated animals performed better than non-injected animals, which in turn performed better than vehicle-treated rats.

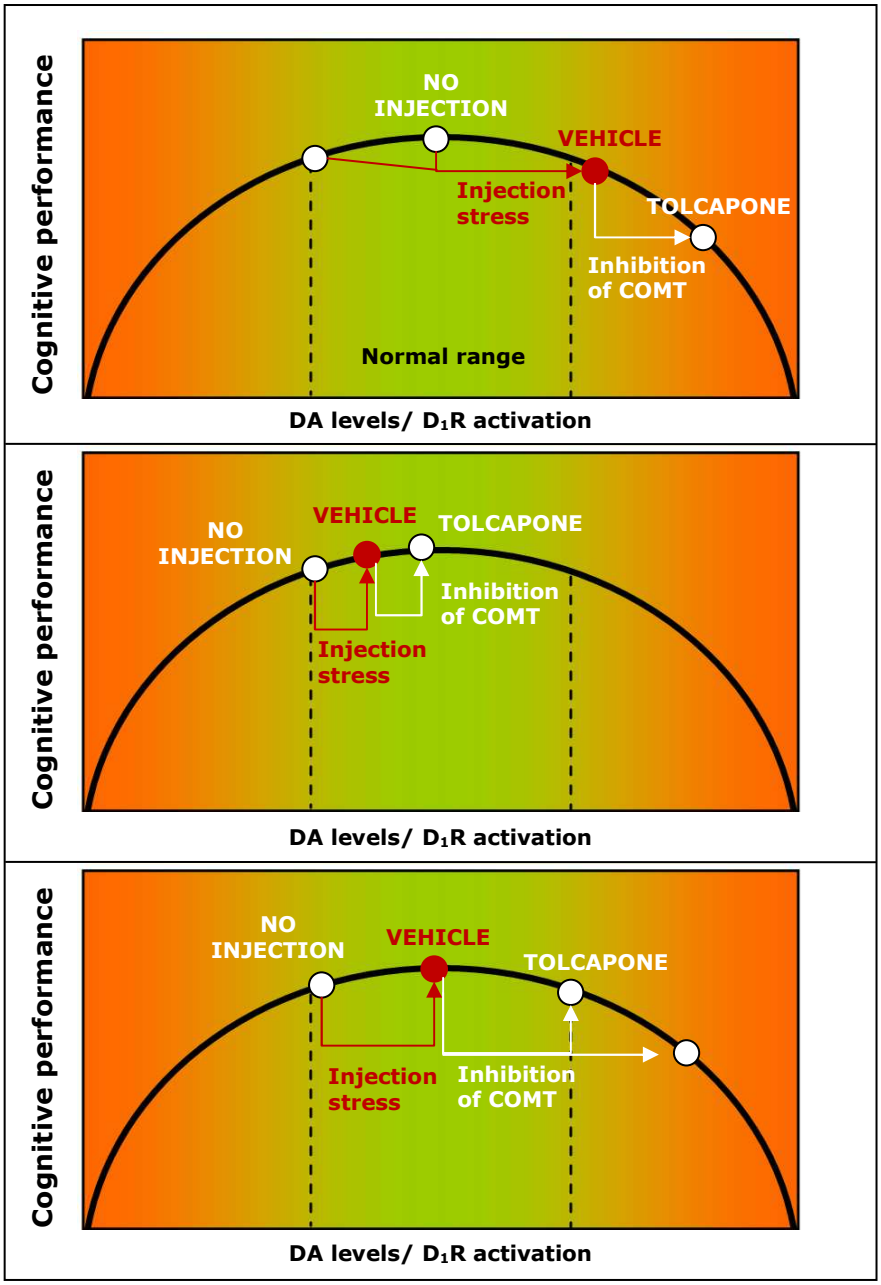


Figure 52 Three attempts at explaining the effects of injection stress and tolcapone administration on cognitive performance using the "U-shaped curve" presented in Figure 5

The top figure suggests that tolcapone-treated rats perform worse than vehicle-treated rats, which may or may not perform worse than non-injected rats – depending on the baseline level of DA. The middle figure proposes that tolcapone-treated rats perform better than vehicle-treated rats, which perform better than non-injected rats. The bottom hypothesis proposes that vehicle-treated rats perform better than tolcapone-treated rats, which may or may not perform worse than non-injected rats.

Hence, it appears that the results of the present study cannot be explained by a single, symmetrically shaped inverted U-shaped relationship between cognitive performance and central DA signalling. Instead, the molecular mechanisms underlying the effects of stress and tolcapone on hippocampus-dependent behaviour are likely to be distinct and involve complex interactions between different molecular mediators.

4.4 Summary of Chapter 6

The data presented in this chapter suggest a memory-enhancing effect of the COMT inhibitor tolcapone on hippocampus-dependent short-term memory. Hence, this data supports previous studies that show that tolcapone is a cognitive enhancer in humans (Apud et al 2007; Gasparini et al 1997; Giakoumaki et al 2008) and rodents (Babovic et al 2008; Khromova et al 1997; Lapish et al 2009; Liljequist et al 1997; Papaleo et al 2008; Tunbridge et al 2004b).

The present study also suggests that tolcapone can improve spatial working memory irrespective of changes in reward processing. This means that tolcapone-induced cognitive enhancement does not necessarily require the formation of associative memory traces between rewards and spatial locations. This implies that tolcapone may affect non-associative short-term working memory – though this hypothesis remains to be tested.

Future research should investigate the effects of COMT activity on aversively motivated cognitive tasks, associative memory tasks, spatial reference memory tasks and non-hippocampal cognitive tasks. Also, the combined effects of injection stress and COMT inhibition on cognitive performance warrant further study.

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Chapter 7

General discussion

Linda Maria Laatikainen

DPhil thesis on the role of COMT in hippocampal function

1 Summary of main findings

Matsumoto *et al* (2003a) have shown that COMT mRNA is abundant in the rat hippocampus. Nevertheless, very little is known about the expression and function of COMT in either the rodent or human hippocampus. Hence, the aim of this thesis was to elucidate the role of COMT in hippocampal function. Table 12 summarises the main findings of this thesis.

Chapter	Subject	Sample	Technique	COMT activity manipulation	Main finding
2	**Human:** control, schizophrenia, bipolar disorder	Post-mortem hippocampal homogenates	qRT-PCR	COMT genotype	**COMT mRNA unaffected by COMT genotype or psychiatric diagnosis**
3	**Rat**: female and male	Tissue homogenates	HPLC-EC	Tolcapone	**Tolcapone alters hippocampal dopamine metabolism**
4	**Rat:** male	Hippocampal dialysates	HPLC-EC	Tolcapone	
5	**Rat:** male	n/a	Successive alleys task	Tolcapone	**Tolcapone has no impact on anxiety**
			Neophagia	Tolcapone	
6	**Rat:** male	n/a	T-maze alternation	Tolcapone	**Tolcapone enhances spatial working memory performance**
			Novelty preference	Tolcapone	

Table 12 Summary of the main findings of this thesis

Chapter 2 investigated the expression levels of COMT mRNA in the human hippocampus, particularly with respect to COMT genotype and psychiatric diagnosis. Hippocampal COMT mRNA levels were not altered in schizophrenia or bipolar disorder, nor with respect to COMT genotype. Nonetheless, this study was able to robustly detect COMT mRNA in the human hippocampus, consistent with the data in rat hippocampus. Chapter 3 and Chapter 4 elucidated that COMT activity affects catecholamine metabolism in the hippocampus. This confirmed that

hippocampal COMT is active. Finally, Chapter 5 and Chapter 6 showed for the first time that COMT activity affects performance on some tasks known to depend on hippocampal function. These findings and the questions arising from these results are discussed in more detail below.

1.1 Hippocampal COMT mRNA expression is not altered in schizophrenia or bipolar disorder, nor affected by COMT genotype

Chapter 2 concluded that overall hippocampal COMT mRNA is not altered in schizophrenia or bipolar disorder, nor affected by COMT genotype. These findings are consistent with earlier studies in prefrontal cortex (Chen et al 2004a; Matsumoto et al 2003b), and support the idea that functional SNPs within the COMT gene operate by altering COMT activity rather than COMT mRNA expression (although see Nackley et al 2006).

The observation that overall hippocampal COMT mRNA is not affected by COMT genotype supports data suggesting that functional SNPs within the COMT gene do not operate by altering mRNA expression (Scanlon et al., 1979; Boudikova et al., 1990; Lotta et al., 1995; Matsumoto et al., 2003b; Chen et al., 2004). Hence, it appears that the effects of the $Val^{158}Met$ SNP on COMT function and psychopathogenesis are not mediated by changes in COMT mRNA expression. This is an interesting observation, since it suggests that there is no compensatory increase in mRNA expression in Met^{158} allele carriers, who have low COMT activity due to the increased thermolability of the Met^{158} form compared with the Val^{158} form of the COMT protein. Hence, research is required to evaluate the relationship between functional COMT SNPs and COMT protein synthesis, folding and metabolism.

Future research is also required to investigate the possibility that COMT mRNA expression changes are specific to certain COMT splice variants, or certain schizophrenia and bipolar disease subtypes. Also, qRT-PCR has poor spatial resolution, since it requires the post-mortem hippocampus samples to be

homogenised. Hence, given previous reports of lamina-specific alterations in neocortical COMT expression (Matsumoto et al 2003b), there could be hippocampal subfield-specific alterations in COMT mRNA expression.

1.2 COMT inhibition alters hippocampal dopamine metabolism

HPLC-EC data from hippocampal tissue extracts (Chapter 3) and microdialysis perfusates (Chapter 4) provided converging evidence for the involvement of COMT in DA metabolism not only in striatum, prefrontal cortex and cerebellum, but also in the hippocampus. In the rat hippocampus, 30 mg/kg systemic tolcapone significantly inhibited COMT, as illustrated by an accumulation of DOPAC and a depletion of HVA. In fact, the neurochemical changes in the hippocampus were at least as robust as in other rat brain regions studied.

The results of Chapter 3 and Chapter 4 also suggested gender- and hippocampal subregion-specific effects of COMT on DA metabolism throughout the brain – as discussed below. Together, the data presented in Chapter 3 and Chapter 4 confirmed the hypothesis that COMT inhibition affects hippocampal DA metabolism, and hence proved that COMT mRNA in the hippocampus is indeed translated into a functional enzyme that is relevant for the regulation of DA in this region. Future research into the hippocampal role of COMT should perhaps include studies on absolute levels of COMT activity in the hippocampus using radiochemical COMT assays.

1.3 COMT activity affects some aspects of hippocampus-dependent rat behaviour

The role of COMT in cognition is better established than the involvement of COMT in emotionality. Notably, there are several studies suggesting that tolcapone improves cognitive performance in humans and rodents, as discussed in Chapter 6. In agreement with this literature, Chapter 6 found that tolcapone-treated rats performed better than vehicle-treated rats on hippocampus-dependent cognitive

tasks. In contrast, the behavioural screen of anxiety-related phenotypes in Chapter 5 did not suggest a role for COMT in ventral hippocampus-dependent emotionality. Hence, hippocampal COMT may play a greater role in hippocampus-dependent memory than anxiety-related phenotypes. However, it is important to acknowledge that the data in Chapter 5 cannot exclude the possibilities that COMT may only have a role in certain types of anxiety, or only in females, and that the effects of tolcapone may be masked by the effects of injection on arousal and stress (see below).

Chapter 6 extended the current literature by showing tolcapone-induced cognitive enhancement on a spatial short-term memory task, which does not involve an overt reward (spatial novelty preference) and by showing that non-injected rats had intermediate levels of cognitive performance. These results imply that injection stress affects cognitive performance and that COMT inhibition appears to improve hippocampus-dependent short-term memory irrespective of changes in reward processing.

The mechanisms involved in the effects of stress on tolcapone-dependent cognitive enhancement are unknown. One possible mechanism for the overlapping effects of stress and tolcapone on cognition could be related to catecholamine signalling. However, Figure 52 illustrates that the effects of stress and tolcapone on cognitive performance cannot be explained by a single inverted U-shaped curve relating DA transmission to cognition. Instead, the effects of stress and tolcapone on cognition are probably mediated by interactions between catecholamines and mediators of stress released by the HPA axis (see e.g. Alexander et al 2010; Korz & Frey 2003; Pacak et al 1995), though this hypothesis remains to be tested.

Planned future experiments include investigation of the hippocampus-dependent behavioural effects of decreased COMT activity in mice expressing the human Met^{158} form of COMT. Comparing Met^{158} mice with wildtype mice expressing Leu^{148} (similar to human Val; Lotta et al 1995) should complement the tolcapone data of this thesis well by "mimicking" the human variation in COMT activity. Also,

the use of Met^{158} mice avoids confounds relating to injection stress. Furthermore, these mice should be better than acute drug administration at modelling the potential compensatory effects of decreased COMT activity throughout development due to the presence of the Met^{158} allele.

2 Broader perspectives

There are many unanswered questions regarding COMT and its role in hippocampal function. The sections below discuss some of these fundamental questions, which affect the interpretation of most of the results presented in this thesis.

2.1 Cellular and subcellular localisation of COMT

The most popular theories regarding the subcellular and cellular localisation of COMT are summarised in Chapter 1. However, the COMT localisation data are markedly inconsistent. For example, there is no consensus on whether COMT is primarily a pre-synaptic, post-synaptic or extra-synaptic protein. Also, it remains unknown whether the COMT active site is located outside or inside cells. This is partly due to methodological problems such as the lack of S- and MB-COMT specific antibodies.

The initial suggestion, that MB-COMT is localised on the plasma membrane and protrudes into the synapse (Aprille & Malamud 1975; Karhunen et al 1995) would explain how COMT can sense catecholamine levels in the synapse and hence, effectively regulate them. However, as discussed in Chapter 1, COMT is unlikely to be functional in the extracellular space due to high extracellular Ca^{2+} levels (Jeffery & Roth 1984). The alternative theory, that MB-COMT is localised on intracellular membranes in the post-synaptic cell, does not highlight any obvious means for COMT to sense and metabolise catecholamine levels in the extracellular space – especially given that COMT is particularly important for DA metabolism in brain regions that exhibit low DAT immunoreactivity (e.g. the PFC; Karoum et al 1994; Sesack et al 1998).

Not only is the localisation of COMT an interesting topic of controversy, it also has important implications for the interpretation of the results of this thesis. For example, Chapter 3 found that tolcapone stimulated DA levels more in ventral

than dorsal hippocampal extracts. This dorso-ventral difference may be due to biologically important processes, such as differences in catecholamine metabolism, exocytosis, diffusion, active transport or negative feedback mechanisms (see below). However, without knowing the localisation of COMT, it is impossible to distinguish, which one of these processes is differentially affected by COMT inhibition in the dorsal and the ventral hippocampus. Also, dorso-ventral differences in the effects of tolcapone on hippocampal metabolite levels could reflect pharmacodynamic and pharmacokinetic differences, or anatomical differences between hippocampal regions not only with respect to the localisation of COMT but also other dopaminergic markers. For example, DA terminals are more densely distributed in the ventral than the dorsal hippocampus (see Chapter 1; e.g. Verney et al 1985) and DA levels are higher in ventral than dorsal hippocampal extracts (Hortnagl et al 1991), which could explain the increased potential for tolcapone to stimulate DA levels in ventral compared to dorsal hippocampal extracts.

In summary, there are many unanswered questions with respect to the localisation of dopaminergic markers, most notably COMT. More research is required in order to identify the subfields, subregions, cells and intracellular compartments in which COMT is most important for controlling hippocampal function.

2.2 Sexually dimorphic effects of COMT

The tissue extract study in Chapter 3 showed sexually dimorphic effects of tolcapone on DOPAC, HVA and/or DOPAC/HVA in striatum, prefrontal cortex and cerebellum. In contrast, hippocampal DOPAC/HVA ratios appeared unaffected by sex. Gogos et al (1998) also observed that sexually dimorphic effects of COMT are brain region-specific, since the sexually dimorphic effects of genetic COMT knockout on DA levels were present in the frontal cortex, but not in the striatum or hypothalamus.

The data from this study and Gogos et al (1998) not only suggest that COMT-related sex differences are region-specific. Gogos (1998) found that COMT knockout homozygous male, but not female, mice had two- to three-fold higher DA in cortical extracts. Similarly, Käenmäki et al (2009) found that cortical neurochemistry was affected in male, but not female S-COMT knockout mice. These results are in contrast to the more pronounced effect of tolcapone on DA metabolite levels in female than male rats in Chapter 3. This discrepancy may be due to species differences, and/or sex differences in the metabolism of tolcapone or the vulnerability to stress within rats. For example, steroids may interact with stress response mediators, potentially making females more vulnerable than males to the effects of stress and arousal on COMT activity (Dalla et al 2005; Frye 2007; Garcia-Caceres et al 2010; Jabbi et al 2007). Thus, sexually dimorphic effects of COMT may be brain region-, species-, drug- and even stress-specific.

In addition to these questions regarding the specificity of sex differences in COMT function, there are many unanswered questions regarding the mechanisms underlying sexually dimorphic effects of COMT. The most popular hypothesis is that free oestrogen activates oestrogen receptors, which get phosphorylated, dimerise and bind to oestrogen response elements (EREs) in the promoter regions of the COMT gene in order to epigenetically down-regulate COMT (Salama et al 2006; Xie et al 1999). Thus, an oestrogen-dependent decrease in female COMT activity (see Chapter 3; e.g. Philippu et al 1981) is believed to underlie sexually dimorphic neurochemical effects of COMT (Gogos et al 1998; Huotari et al 2002; Kaenmaki et al 2009; Tammimaki et al 2010). Moreover, these neurochemical changes potentially underlie sexually dimorphic effects of COMT on behaviour (Babovic et al 2008; Gogos et al 1998; Tammimaki et al 2008), perhaps via downstream effects on NMDA-dependent LTP maintenance (e.g. Foy et al 1999; Shang et al 2010).

However, this hypothesis may not be able to explain sexually dimorphic effects of COMT in the CNS, since sex differences in COMT activity have only been demonstrated in the periphery. Also, Jiang et al (2003) found that 17β-estradiol

exposure of 72 h reduced COMT activity in human breast carcinoma cells, but not in a glial cell line. Moreover, Chapter 2 illustrated that hippocampal COMT mRNA levels are not affected by sex, consistent with previous post-mortem studies of mRNA levels in the CNS (e.g. Matsumoto et al 2003b). Only one previous study by Dempster et al (2006) has found sex differences in central COMT mRNA levels. However, this study showed that females exhibited greater cerebellar COMT mRNA levels than males, which does not agree with the hypothesis described above.

In summary, oestrogen-based theories cannot explain the full range of COMT-related sex differences. Thus, future research is needed to develop alternative hypotheses to explain the sexually dimorphic effects of COMT in the CNS. New theories may involve the effects of other systemic steroids such as progesterone on COMT (Frye 2007; Frye et al 2007; Salama et al 2006). Given that sex differences in COMT function occur on the background of sex differences in DA function and psychopathology (e.g. schizophrenia: Aleman et al 2003; Gur et al 1996; Leung & Chue 2000; Szymanski et al 1995), new hypotheses may also incorporate the effects of sex hormones on downstream targets related to catecholamine transmission (e.g. OCT3; Wu et al 1998) and on genes known to be involved in neurodevelopment or psychopathogenesis. Moreover, dynamic changes in hormonal levels may have functional, structural and psychopathological consequences through life and during the oestrous cycle (e.g. Prange-Kiel et al 2009). Thus, future experiments on sex differences in COMT function should perhaps span the entire course of sexual and reproductive maturation and degeneration. Finally, differences in sex hormones are not the only pathway resulting in sexually dimorphic effects in the brain. For example, genes residing on sex chromosomes can directly influence sexually dimorphic traits in a sex hormone-independent manner (Davies & Wilkinson 2006). Thus, investigating interactions between genetic and hormonal pathways resulting in sexually dimorphic neurobiology may lead to improved theories on sexually dimorphic effects of COMT in the CNS.

2.3 Dorso-ventral dissociations in the hippocampus

The *ex vivo* neurochemical study presented in Chapter 3 found dorso-ventral differences in rat hippocampal neurochemistry. Consistent with an *ex vivo* study by Ishikawa et al (1982), dorsal hippocampal tissue extracts had higher DOPAC and HVA content than ventral hippocampal tissue extracts of vehicle-treated rats. The results of Chapter 3 also suggested that tolcapone increases DOPAC levels and DA turnover to a greater extent in dorsal than ventral hippocampal extracts. Chapter 3 also found that tolcapone increased DA levels to a greater extent in ventral than dorsal hippocampal extracts.

One way of explaining these results is that there are dorso-ventral differences in metabolism, transport, diffusion and negative feedback mechanisms within the hippocampal catecholamine systems. For example, tolcapone may cause DOPAC to increase in ventral, but not dorsal hippocampal extracts, because it amplifies the naturally high DOPAC levels in ventral hippocampus and hence, saturates DOPAC metabolism and/or transport mechanisms. Such saturation would perhaps not occur with respect to HVA levels, because the effect of tolcapone is to decrease the naturally high HVA levels in the dorsal hippocampus. An explanation based on dorso-ventral differences in DA metabolism could also explain the effects of tolcapone on tissue DA. Namely, perhaps DA levels continue to accumulate intracellularly after tolcapone, because tolcapone did not cause changes in DA turnover in ventral hippocampal extracts.

However, these suggested dorso-ventral differences cannot explain the differences between the *ex vivo* and *in vivo* neurochemical data of this thesis. Whilst tissue levels of DOPAC are higher in the dorsal than the ventral hippocampus, the *in vivo* microdialysis study found that extracellular levels of DOPAC are higher in ventral than dorsal hippocampus. Also, whilst tolcapone only increases tissue levels of DOPAC in the dorsal hippocampus, tolcapone alters extracellular levels of both DOPAC and HVA in both the dorsal and ventral hippocampus. These differences illustrate that further research is required to

investigate the compartment-specificity of dorso-ventral differences within the hippocampus.

In addition to the questions regarding anatomical and neurochemical differences between the dorsal and ventral hippocampus, the behavioural data presented in Chapter 5 and Chapter 6 highlight an intriguing question: Are the dorso-ventral differences in hippocampal neurochemistry related to the differential effects of COMT inhibition on dorsal and ventral hippocampus-dependent behaviours? Given that dorsal, but not ventral hippocampal lesions in rats impair performance on hippocampus-dependent cognitive tasks (see Chapter 6), the results of this thesis imply that COMT could be more crucial for dorsal than ventral hippocampal function. Similarly, the effect of tolcapone on tissue levels of DA metabolites in the dorsal, but not the ventral hippocampus may also imply that COMT is more relevant for dorsal than ventral hippocampal function. Thus, the neurochemical and behavioural results of this thesis could be linked i.e. effects of tolcapone on dorsal hippocampus-dependent short-term working memory could be at least partly mediated by tolcapone-induced changes in dorsal hippocampal DA metabolism. This is an important hypothesis to consider in the future, as this research could provide information on the mechanisms by which the COMT Val^{158}Met SNP can affect cognitive performance (see Chapter 1) by not only affecting COMT activity in the prefrontal cortex (Egan et al 2001), but also the hippocampus. Possible ways of testing this hypothesis are by evaluating hippocampus-dependent behaviour during hippocampal microdialysis experiments, potentially in the presence of DA-specific antagonists, and/or hippocampal subregion-specific lesions.

2.4 What molecular mechanisms mediate the effects of COMT?

The present thesis established that COMT is expressed and is enzymatically functional in the hippocampus. Also, COMT impacts hippocampus-dependent memory functions. However, the molecular mechanisms that mediate the effects of

COMT in the hippocampus (and elsewhere) are unknown. This raises two major questions.

The first question is which neurotransmitters mediate the effects of COMT on hippocampal neurochemistry. The neurochemical studies in Chapter 3 and Chapter 4 found that NA/DA ratios were about 10-20, consistent with the fact that NA innervation of the hippocampus is much more prominent than DA innervation of the hippocampus (see Figure 8). However, despite the higher levels of NA in the hippocampus, it does not appear that the effects of COMT are necessarily mediated by hippocampal NA. Instead, Chapter 3 showed that COMT inhibition affected tissue levels of DA, but not NA, in the hippocampus – consistent with other neurochemical studies on the effects of reduced COMT activity (e.g. Gogos et al 1998). This suggests that there exists a hippocampal pool of DA, which is independent of NA. However, the present thesis cannot fully distinguish between the role of hippocampal DA as a precursor to NA or as a neurotransmitter independent of NA.

The second question (already highlighted in subchapter 2.7) is whether the tolcapone-induced changes in hippocampal catecholamine metabolism could underlie tolcapone-induced changes in hippocampus-dependent behaviour. Although the data presented in this thesis cannot show that the effects of tolcapone on DA (or NA) metabolism mediate the behavioural effects of tolcapone, this would make a lot of sense on theoretical grounds: NA modulates LTP in the DG, whereas DA is crucial for LTP in the CA1 (see Chapter 1; though DA also affects synaptic plasticity in the DG; Hamilton et al 2010). Hence, it appears that both NA and DA are needed for LTP modulation across the transverse axis of the hippocampus. Since LTP is a form of synaptic plasticity thought to encode hippocampus-dependent spatial learning, NA and DA are probably both needed for maximal performance on hippocampus-dependent spatial learning tasks. Indeed, this appears to be the case – as discussed in Chapter 1. Whilst optimal NA transmission probably improves cognitive performance by enhancement of cortical signal, DA improves cognition by attenuating noise (Arnsten 2007). Thus, optimal NA and DA

levels may act synergistically and/or reciprocally to improve cognition by affecting the signal/noise ratio of neuronal information transfer.

In summary, NA and DA may act together to mediate the cognitive-enhancing effects of COMT inhibition, though neurochemical data suggest that DA plays a greater role than NA in the hippocampal effects of COMT.

2.5 Relative involvement of COMT in hippocampal versus non-hippocampal function

The present study focused almost exclusively on the role of COMT in the hippocampus. Hence, microdialysis probes were only inserted in the hippocampus and only hippocampus-dependent behavioural tasks were utilised in Chapter 5 and Chapter 6.

However, most brain regions are interconnected and function as circuits. Thus, it is important to acknowledge that different hippocampal subregions (e.g. Bannerman et al 2004b), as well as various non-hippocampal brain regions, influence specific aspects of hippocampal function. For example, cortical areas aid cognitive processing (e.g. Dias & Aggleton 2000; Yoon et al 2008), the striatum modulates reward processing, whereas the HPA axis and amygdala mediate the effects of stress, emotional arousal and attention on cognitive performance (Korz & Frey 2005; McGaugh 2000). The potential influence of cortical regions on hippocampus-dependent behaviour and extracellular levels of DA metabolites in the hippocampus is particularly pertinent, given the known role for COMT in regulating cortical DA and modulating cortical-dependent behaviours, and given the known cross-talk between the hippocampus and the PFC (see Chapter 1).

A possible experiment to investigate the relative involvement of COMT in different brain regions would be to compare the behavioural and neurochemical consequences of different region-specific COMT knockout mice, or region-specific intracerebral infusions of tolcapone (e.g. Huotari et al 2001). This type of experiment could identify if tolcapone-related behavioural effects are mediated by the local effects of tolcapone on hippocampal neurochemistry rather than being a downstream consequence of COMT acting in other brain regions.

2.6 Tolcapone as a cognitive enhancer

Even though the results of Chapter 6 are very preliminary, they agree well with previous reports of tolcapone-induced cognitive enhancement in humans and

rodents (e.g. Apud et al 2007; see Chapter 1 subchapter 2.7.2). Thus, it would be worth testing if COMT inhibitors could be used clinically in the treatment of cognitive deficits.

The proposition of COMT inhibitors as cognitive enhancers is not novel. The use of tolcapone as an adjunct to antipsychotic treatments for improving cognitive dysfunction and the poor side-effect profiles of antipsychotics is already under investigation. Tolcapone is an attractive cognitive enhancer given that it is used clinically as an adjunct to Parkinson's disease treatment, which means that toxicological trials and practice have shown that COMT inhibition is relatively safe in humans. Also, the fact that anxiety symptoms are not likely to be a major side-effect of tolcapone-like drugs (see Chapter 5) enhances their therapeutic candidacy still further.

The research presented in Chapter 6 make tolcapone a particularly attractive therapeutic substrate for the treatment of cognitive deficits in schizophrenia, because it now appears that COMT influences hippocampal and cortical function - both of which are dysregulated in schizophrenia. Also, the tolcapone-induced enhancement of cognitive performance on hippocampus-dependent tasks is important for schizophrenia research since DA dysfunction and the hippocampus are both crucial in its pathogenesis (see Chapter 1 and Chapter 2). Moreover, there is a shortage of treatment strategies for cognitive deficits in schizophrenia (Arnsten 2004; Davidson & Keefe 1995; Gold 2004; Marder 2006a; b; McGurk et al 2007; McKenna 2006; Mishara & Goldberg 2004; Sharma & Antonova 2003), even though cognitive deficits in schizophrenia are a good predictor for poor prognosis (Green 1996; Green et al 2004; Kopelowicz et al 2005). Hence, the results of Chapter 6 are of particular interest to research on cognitive deficits in schizophrenia, though other disorders involving cognitive deficits may also benefit from future research in this field.

3 Final conclusions

This thesis has described the first multifaceted characterisation of the function of hippocampal COMT. The characterisation of COMT in the hippocampus yielded interesting data from a gene expression, neurochemical and behavioural point-of-view:

The results of Chapter 2 showed that COMT mRNA is expressed in the human hippocampus, complementing earlier rodent studies. However, hippocampal COMT mRNA expression was not altered in schizophrenia or bipolar disease, nor affected by COMT genotype.

The neurochemical studies presented in Chapter 3 and Chapter 4 indicated that COMT is enzymatically active and capable of regulating DA and its metabolism in the hippocampus both *ex vivo* and *in vivo*. Furthermore, it appears that COMT may be more relevant for dorsal than ventral hippocampal function. Moreover, in non-hippocampal regions COMT appears to have gender-specific effects on catecholamine metabolism.

Finally, Chapters 5 and 6 showed that systemic COMT inhibition affects hippocampus-dependent cognitive phenotypes, but not hippocampus-dependent emotionality. Notably, COMT inhibition improved spatial working memory even in the absence of overt rewards, which suggests a potential role for catecholamine metabolism in more general aspects of cognitive processing than simpy reward processing. Moreover, there may be a link between the fact that COMT inhibition appeared to affect DOPAC levels more in dorsal than ventral hippocampus (see Chapter 3) and that tolcapone affected dorsal hippocampus-dependent spatial working memory performance more than ventral hippocampus-dependent emotionality (Chapter 6 and Chapter 5, respectively).

In conclusion, the studies presented in this thesis demonstrate that COMT has a role in hippocampus-dependent behaviour and that the mechanisms for this

may involve the role of COMT in hippocampal catecholamine metabolism. Additionally, COMT inhibitors are suggested to have potential therapeutic use as an adjunct to antipsychotic treatment in schizophrenia. However, further research is required to fully understand the physiological, pathophysiological and therapeutic importance of COMT in the hippocampus.

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Appendices

Linda Maria Laatikainen

DPhil thesis on the role of COMT in hippocampal function

Appendix I COMT expression data: ANOVAs

Univariate ANOVAs conducted on raw COMT, GAPDH, TFRC and TBP mRNA expression data as well as on normalised expression data

		df	F	p
NORMALISED COMT mRNA DATA				
1 between-subjects factor (diagnosis/SNP/haplotype/demographics) with brain weight as a covariate				
	Diagnosis	2,97	0.115	0.891
	rs4680	2,98	0.917	0.403
	rs4818	2,98	0.622	0.539
	rs4633	2,98	0.525	0.593
	A-287G	2,98	0.982	0.378
	rs6269	2,96	0.319	0.728
	Haplotype	2,31	0.019	0.981
	Alcohol	5,94	1.212	0.309
	Drug use	5,93	1.911	0.100
2 between-subjects factors (diagnosis, SNP/haplotype) with brain weight as a covariate				
rs4680	Diagnosis	2,91	0.158	0.854
	rs4680	2,91	0.850	0.431
	Diagnosis*rs4680	4,91	1.225	0.306
rs4818	Diagnosis	2,91	1.558	0.216
	rs4818	2,91	0.846	0.433
	Diagnosis*rs4818	4,91	2.258	0.069
rs4633	Diagnosis	2,91	0.105	0.900
	rs4633	2,91	0.619	0.541
	Diagnosis*rs4633	4,91	1.743	0.147
A-287G	Diagnosis	2,91	1.110	0.334
	A-287G	2,91	0.339	0.713
	Diagnosis*A-287G	4,91	0.929	0.451
rs6269	Diagnosis	2,89	1.622	0.203
	rs6269	2,89	0.576	0.564
	Diagnosis*rs6269	4,89	2.215	0.074
Haplotype	Diagnosis	2,26	1.210	0.314
	Haplotype	2,26	0.841	0.443
	Diagnosis*Haplotype	2,26	3.538	0.044
RAW COMT mRNA DATA				
1 between-subjects factor (diagnosis/SNP/haplotype/demographics) with brain weight as a covariate				
	Diagnosis	2,97	1.170	0.315
	rs4680	2,98	0.162	0.850
	Haplotype	2,31	0.745	0.483
	rs6269	2,96	0.424	0.655
	rs4633	2,98	0.103	0.902
	rs4818	2,98	0.367	0.694
	A-287G	2,98	0.111	0.895
	Alcohol	5,94	0.809	0.546
	Drug use	5,93	1.702	0.142
2 between-subjects factors (diagnosis, rs4680) with brain weight as a covariate				
	Diagnosis	2,91	1.523	0.224
	rs4680	2,91	0.171	0.843
	Diagnosis*rs4680	4,91	0.801	0.528

GEOMETRIC MEAN OF HOUSEKEEPER GENES mRNA DATA			
2 between-subjects factors (diagnosis, rs4680) with brain weight as a covariate			
Diagnosis	2,91	0.158	0.854
rs4680	2,91	0.850	0.431
Diagnosis*rs4680	4,91	1.225	0.306
1 between-subjects factor (diagnosis/SNP/haplotype/demographics) with brain weight as a covariate			
Rs4680	2,98	0.917	0.403
Haplotype	2,31	0.019	0.981
rs6269	2,96	0.319	0.728
rs4633	2,98	0.525	0.593
rs4818	2,98	0.622	0.539
A-287G	2,98	0.982	0.378
Alcohol	5,94	1.369	0.243
Drug use	5,93	2.033	0.081
2 between-subjects factors (diagnosis, rs4680) with brain weight as a covariate			
Diagnosis	2,91	0.158	0.854
rs4680	2,91	0.850	0.431
Diagnosis*rs4680	4,91	1.225	0.306

Appendix II COMT expression data: Independent samples T-tests

Univariate ANOVAs conducted on raw COMT, GAPDH, TFRC and TBP mRNA expression data as well as on normalised expression data

	df	t	p
NORMALISED COMT mRNA DATA			
Sex	99	-0.383	0.702
Smoking	61	0.845	0.402
Side of brain	99	0.846	0.400
Suicide	99	-0.770	0.443
RAW COMT mRNA DATA			
Sex	99	0.890	0.376
Smoking	61	-0.545	0.588
Side of brain	99	0.253	0.801
Suicide	99	0.041	0.967
GEOMETRIC MEAN OF HOUSEKEEPER GENES mRNA DATA			
Sex	99	0.946	0.347
Smoking	61	-0.891	0.377
Side of brain	99	-0.556	0.580
Suicide	99	0.538	0.592

Appendix III Tissue extract study data

Repeated-measures ANOVAs conducted using treatment and sex as between-subjects factors and region as a within-subjects factor. (Note that the appropriate p-values are highlighted, depending on the value obtained from Mauchly's test of sphericity.)

		df	F	p	p Huynh-Feldt	Mauchly's Test of Sphericity
ALL BRAIN REGIONS INCLUDED						
DA	region	4,72	298.215	0.000	0.000	0.000
	region*sex	4,72	0.274	0.894	0.644	
	region*treatment	4,72	0.797	0.531	0.402	
	region*sex*treatment	4,72	0.237	0.917	0.670	
	sex	1,18	0.255	0.620		
	treatment	1,18	0.737	0.402		
	sex*treatment	1,18	0.232	0.636		
NA	region	4,72	17.321	0.000	0.000	0.206
	region*sex	4,72	1.661	0.168	0.168	
	region*treatment	4,72	0.382	0.821	0.821	
	region*sex*treatment	4,72	0.614	0.654	0.654	
	sex	1,18	0.000	0.989		
	treatment	1,18	0.094	0.762		
	sex*treatment	1,18	0.017	0.898		
NA/DA	region	4,72	54.815	0.000	0.000	0.206
	region*sex	4,72	0.157	0.959	0.932	
	region*treatment	4,72	1.754	0.148	0.164	
	region*sex*treatment	4,72	0.414	0.798	0.754	
	sex	1,18	1.488	0.238		
	treatment	1,18	3.264	0.088		
	sex*treatment	1,18	0.027	0.875		
DOPAC	region	4,72	432.076	0.000	0.000	0.000
	region*sex	4,72	3.370	0.014	0.075	
	region*treatment	4,72	2.518	0.049	0.123	
	region*sex*treatment	4,72	0.214	0.930	0.688	
	sex	1,18	3.811	0.067		
	treatment	1,18	4.512	0.048		
	sex*treatment	1,18	0.347	0.563		
HVA	region	4,72	111.569	0.000	0.000	0.000
	region*sex	4,72	4.263	0.004	0.045	
	region*treatment	4,72	23.293	0.000	0.000	
	region*sex*treatment	4,72	3.256	0.016	0.079	
	sex	1,18	3.058	0.097		
	treatment	1,18	23.049	0.000		
	sex*treatment	1,18	3.649	0.072		
DOPAC/ HVA	region	4,72	38.956	0.000	0.000	0.000
	region*sex	4,72	5.208	0.001	0.026	
	region*treatment	4,72	20.833	0.000	0.000	
	region*sex*treatment	4,72	4.476	0.003	0.039	
	sex	1,18	4.619	0.045		
	treatment	1,18	25.281	0.000		
	sex*treatment	1,18	4.458	0.049		
DOPAC/ DA	region	4,72	20.753	0.000	0.000	0.000
	region*sex	4,72	0.265	0.900	0.829	
	region*treatment	4,72	5.602	0.001	0.003	
	region*sex*treatment	4,72	0.105	0.980	0.944	
	sex	1,18	0.263	0.614		

	treatment	1,18	17.917	0.001		
	sex*treatment	1,18	0.051	0.823		
ONLY DORSAL AND VENTRAL HIPPOCAMPUS INCLUDED						
DA	region	1,19	0.778	0.389	0.389	0.000
	region*sex	1,19	0.771	0.391	0.391	
	region*treatment	1,19	1.860	0.189	0.189	
	region*sex*treatment	1,19	0.005	0.943	0.943	
	sex	1,19	0.222	0.643		
	treatment	1,19	4.826	0.041		
	sex*treatment	1,19	0.195	0.664		
NA	region	1,19	0.652	0.429	0.429	0.000
	region*sex	1,19	0.833	0.373	0.373	
	region*treatment	1,19	0.182	0.674	0.674	
	region*sex*treatment	1,19	0.058	0.812	0.812	
	sex	1,19	2.744	0.114		
	treatment	1,19	0.424	0.523		
	sex*treatment	1,19	0.632	0.437		
NA/DA	region	1,19	0.030	0.864	0.864	0.000
	region*sex	1,19	0.054	0.819	0.819	
	region*treatment	1,19	0.185	0.672	0.672	
	region*sex*treatment	1,19	0.001	0.970	0.970	
	sex	1,19	0.244	0.627		
	treatment	1,19	3.778	0.067		
	sex*treatment	1,19	0.160	0.694		
DOPAC	region	1,19	16.291	0.001	0.001	0.000
	region*sex	1,19	0.253	0.621	0.621	
	region*treatment	1,19	10.543	0.004	0.004	
	region*sex*treatment	1,19	0.275	0.606	0.606	
	sex	1,19	0.099	0.757		
	treatment	1,19	17.578	0.000		
	sex*treatment	1,19	0.061	0.807		
HVA	region	1,19	28.565	0.000	0.000	0.000
	region*sex	1,19	0.729	0.404	0.404	
	region*treatment	1,19	0.329	0.573	0.573	
	region*sex*treatment	1,19	0.424	0.523	0.523	
	sex	1,18	2.339	0.143		
	treatment	1,18	0.396	0.537		
	sex*treatment	1,18	1.223	0.283		
DOPAC/ HVA	region	1,19	0.122	0.731	0.731	0.000
	region*sex	1,19	0.203	0.657	0.657	
	region*treatment	1,19	3.212	0.089	0.089	
	region*sex*treatment	1,19	0.669	0.424	0.424	
	sex	1,19	1.588	0.223		
	treatment	1,19	6.968	0.016		
	sex*treatment	1,19	0.610	0.444		
DOPAC/ DA	region	1,19	13.050	0.148	0.002	0.000
	region*sex	1,19	0.364	0.162	0.553	
	region*treatment	1,19	13.269	0.912	0.002	
	region*sex*treatment	1,19	0.122	0.864	0.731	
	sex	1,19	0.278	0.604		
	treatment	1,19	8.335	0.009		
	sex*treatment	1,19	0.280	0.603		

Appendix IV Optimisation of the microdialysis protocol

Table of problems encountered in optimising the microdialysis coupled with HPLC-EC protocol and the ways in which these were solved.

Optimising	Problem	Solution	Date
Surgery	Achieving stable chloral hydrate anaesthesia takes longer than the surgery itself	Isoflurane anaesthesia	Jun-08
	Rats pull guide cannula out after surgery	Stronger cement, larger scull screws and moving from 2 to 6 scull screws per rat	Sep-08
	Smaller brain sizes in smaller rats means that rat brain atlas co-ordinates cannot be used.	Optimizing anaesthesia and probe coordinates	Jan-09
	Mash from regular food pellets is too dry	Readybrek-porridge (Weetabix Food Company) used as post-surgery food	Jan-09
	Commercial cannulae are inserted in the brain	Surgery made more sterile using glass bead steriliser	Mar-09
Probes	No DA detected with 2 mm membrane probes	Moved to 4 mm membrane	May-08
	4mm membrane bends easily	Moved to 3 mm membrane	Jun-08
	Probe moves in cannula	Probe attached to cannula using cement	Jun-08
	Rats detach tubing from the probe	Glue tube onto probe	Jun-08
	Not all probes are long enough to reach vHC	Increased shaft length (22 mm)	Aug-08
	Cannula moves when the plug is pulled out	Scoring cannulae for increased grip on cement Making plugs that fit less tightly in cannulae	Jan-09
	Self-made probes are laborious to make and break or leak easily. Also, the DA recovery and amount of tissue damage cannot be standardised.	CMA/11 commercial probes used (these are more labour-efficient, robust and show minimum tissue damage, which may be crucial given that COMT can get inhibited in inflammatory pain states (Tchivileva et al 2009))	Feb-09
	CMA/11 commercial probes are expensive	**MAB6.14.3 commercial probes used**	Apr-09
	MAB6.14.3 commercial probes are expensive	Probes are reused up to three times	Jun-09
Microdialysis	Hyperactivity of rats in testing bowl causes probes to break	Habituation of rats to the bowl, rat collar and to a plastic "hat" resembling the probe	Jul-08
	I.p. injections may cause stress-induced increase in hippocampal DA (Ihalainen et al 1999; Isogawa et al 2000; Zhang et al 1995).	Rats habituated to rougher handling associated with injections	Nov-08
	Habituation not found to have major improvement on rat behaviour when first placed in test bowl or during injections	Habituation discontinued as it is laborious and ultimately, behaviour seems to be more strongly determined by rat cohort and individual rat behaviour	Jan-09

	Locomotor activity measures unreliable	Activity not measured in test bowl	Jan-09
	Rats remove collection tubes	Low internal diameter FAP tubing used to allow sample collection at a distance from the rat without increasing the dead volume	Feb-09
	Varying volume in collection tubes	Cleaning swivel and replacing tubing daily to avoid dirt and air	Feb-09
Sample storage	DA oxidation to produce orthoquinone derivates (Marsden & Joseph 1986). Also DA is degradated by light and at room temperature	-Sample storage at 4 °C, in the dark (by covering collection tubes with aluminium foil) and for minimum time possible - Coloured FEP tubing and collection tubes - No protease inhibitor, antioxidants or EDTA are added in either the collection tubes or aCSF, because they increase the likelihood of contamination and do not significantly improve DA recovery in pilot experiments over the course of our storage time (Kern & Bernards 1997)	May-08 until May-09
HPLC	Several NA and DA metabolite standards (DOMA, DHPG, normetanephrine, 3-MT and VMA) show more than one peak due to poor purity of chemicals, also sample collection and preparation methods for certain measures of NA turnover and release (e.g. Karoum et al 1977) are too time-consuming for the present study	Only NA, DA, DOPAC, HVA, 5HIAA and HMPG studied	Jul-08
	High noise levels despite replacing old parts, adding earth connections etc.	**Purchasing new Decade I and Decade II HPLC systems**	Oct-08
	Broad, co-eluting peaks	Shorter (4.6x100 mm, 3 µm particle) Microsorb C18 column used	Sep-08
	Setting up new Decade I	Optimising Signal/Noise by manipulating voltage, range, filter and temperature settings as well as mobile phase composition	Jan-09
	Setting up new Decade II	Optimising Signal/Noise by manipulating voltage, range, filter and temperature settings as well as mobile phase composition	Jan-09
	Moderate noise levels	Clean electrode as well as purchase new tubing, a pulse dampener	Mar-09
	Autozero not functional	Rewiring of decade II necessary	Apr-09
	Contaminants coincide with peaks of interest, which are very small in size	Altering the mobile phase composition and making fresh solutions to minimise contamination and to optimize peak position and separation	Apr-08 until Oct-09
	Basal dopamine levels at the detection limit of the HPLC machine	Narrow-bore column (1-2.1 mm internal diameter), 25 µm spacer, lower flow-rate and smaller injection volumes tried; but not used in final protocol due to high risk of column blockage	Apr-09

Appendix V Microdialysis data *Repeated-measures ANOVAs conducted using treatment as a between-subjects factors and region as a within-subjects factor. (Note that the appropriate p-values are highlighted in yellow, depending on the value obtained from Mauchly's test of sphericity.)*

		df	F	P	p Huynh-Feldt	Mauchly's Test of Sphericity
RAW DATA						
DOPAC	time	8,112	22.289	0.000	0.000	0.000
	time*region	8,112	14.245	0.000	0.000	
	time*treatment	8,112	29.197	0.000	0.000	
	time*region*treatment	8,112	13.771	0.000	0.000	
	region	1,14	47.780	0.000		
	treatment	1,14	36.806	0.000		
	region*treatment	1,14	20.750	0.000		
HVA	time	8,128	10.992	0.000	0.000	0.000
	time*region	8,128	0.484	0.865	0.786	
	time*treatment	8,128	4.322	0.000	0.002	
	time*region*treatment	8,128	0.843	0.567	0.523	
	region	1,16	0.000	0.986		
	treatment	1,16	4.916	0.041		
	region*treatment	1,16	0.776	0.392		
DOPAC/HVA	time	8,112	9.225	0.000	0.000	0.000
	time*region	8,112	1.750	0.095	0.192	
	time*treatment	8,112	9.282	0.000	0.001	
	time*region*treatment	8,112	1.662	0.116	0.208	
	region	1,14	3.961	0.066		
	treatment	1,14	13.934	0.002		
	region*treatment	1,14	2.659	0.125		
PERCENTAGE DATA						
DOPAC	time	8,112	17.013	0.000	0.000	0.000
	time*region	8,112	1.873	0.071	0.112	
	time*treatment	8,112	27.018	0.000	0.000	
	time*region*treatment	8,112	0.631	0.751	0.674	
	region	1,14	4.054	0.064		
	treatment	1,14	62.350	0.000		
	region*treatment	1,14	0.828	0.378		
HVA	time	8,128	13.320	0.000	0.000	0.028
	time*region	8,128	0.434	0.899	0.872	
	time*treatment	8,128	8.039	0.000	0.000	
	time*region*treatment	8,128	0.441	0.894	0.867	
	region	1,16	0.137	0.717		
	treatment	1,16	15.421	0.001		
	region*treatment	1,16	0.337	0.570		

Appendix VI Delayed Rewarded Alternation Data *: Repeated-measures ANOVAs conducted using treatment and gender as between-subjects factors, and region as a within-subjects factor.*

		df	F	P	p Huynh-Feldt	Mauchly's Test of Sphericity
Performance (during testing)	Delay	1,21	102.054	0.000	0.000	0.000
	Delay*treatment	2,21	4.772	0.020	0.020	
	Treatment	2,21	1.977	0.163		
Performance (during training)	Delay	1,21	64.874	0.000	0.000	0.000
	Delay*treatment	2,21	0.889	0.426	0.426	
	Treatment	2,21	0.464	0.635		
Latency on sample trials (during testing)	Delay	1,21	11.278	0.003	0.003	0.000
	Delay*treatment	2,21	0.648	0.533	0.533	
	Treatment	2,21	0.474	0.629		
Latency on sample trials (during training)	Delay	1,21	0.019	0.892	0.892	0.000
	Delay*treatment	2,21	1.046	0.369	0.369	
	Treatment	2,21	0.067	0.935		
Proactive interference analysis						
Block	Delay	1,21	128.900	0.000	0.000	0.000
	Delay*treatment	2,21	1.531	0.240	0.240	
	Block	1,21	0.388	0.540	0.540	0.000
	Block*treatment	2,21	1.195	0.322	0.322	
	Delay*Block	1,21	0.025	0.876	0.876	0.000
	Delay*Block*treatment	2,21	0.307	0.739	0.739	
	Treatment	2,21	0.941	0.406		
Previous delay	Delay	1,21	51.184	0.000	0.000	0.000
	Delay*treatment	2,21	1.607	0.224	0.224	
	PreviousDelay	1,21	0.004	0.953	0.953	0.000
	PreviousDelay*treatment	2,21	0.761	0.480	0.480	
	Delay*PreviousDelay	1,21	0.532	0.474	0.474	0.000
	Delay*Prev*treatment	2,21	2.985	0.072	0.072	
	Treatment	2,21	0.108	0.898		

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DPhil thesis on the role of COMT in hippocampal function

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