D-AMINO ACID OXIDASE, D-SERINE AND THE DOPAMINE SYSTEM: THEIR INTERACTIONS AND IMPLICATIONS FOR SCHIZOPHRENIA

JILL F. BETTS

WOLFSON COLLEGE, UNIVERSITY OF OXFORD

SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN PSYCHIATRY

HILARY TERM 2012

‘As the creeper that girdles the tree-trunk,

the Law runneth forward and back;

For the strength of the Pack is the Wolf,

and the strength of the Wolf is the Pack.’

-‘The Jungle Book’, by Rudyard Kipling

Abstract

D-amino acid oxidase (DAO) is a flavin-dependent enzyme that is expressed in the mammalian brain. It is the metabolising enzyme of several D-amino acids, including D-serine, which is an endogenous agonist at the glycine co-agonist site of the glutamatergic NMDA receptor. As such, regulation of D-serine levels in the brain by DAO may indirectly modulate the activity of NMDA receptors. The expression and activity of DAO have been reported to be increased in schizophrenia. It has been identified as a putative susceptibility gene for the disorder, and as a potential therapeutic target.

This thesis explored three aspects of the interface between DAO and the DA system. First, the expression of DA was investigated in the ventral tegmental area (VTA), the source of the dopaminergic mesocortical pathway. Traditionally, DAO was considered to be an enzyme confined to the hindbrain and to glia, but more recent studies have reported its expression in additional brain regions, and also in neurons. DAO mRNA and protein was found to be expressed in the VTA, and was present in both neurons and glia in this region, whereas in the cerebellum, DAO expression appeared solely glial.

DA output from the VTA is regulated by NMDA receptors, and hence expression of DAO in the VTA suggests that it may serve a role in modulating cortical DA via regulation of D-serine levels and NMDA receptor function. The second part of this thesis investigated the effects of DAO inhibition and D-serine administration on DA levels in the prefrontal cortex (PFC) using *in vivo* microdialysis. Systemic DAO inhibition and D-serine administration resulted in increases in extracellular levels of DA metabolites in the PFC, despite no detectable change in DA. Similarly, DA metabolites in the PFC increased after local application of D-serine to the VTA, but no change was detected in DA. However, local DAO inhibition in the VTA resulted in increased levels of both DA and its metabolites, and DAO inhibition combined with D-serine administration also produced increases in DA. This suggested that DAO and its regulation of D-serine levels may serve

to indirectly modulate mesocortical DA function, and this may be mediated via the VTA. This notion was supported in the final section of this thesis, in which the expression of three DA genes was measured in the PFC of a novel line of DAO knockout mice. In this pilot study, there was evidence for an increase in Comt and Drd2 mRNAs in the knockout mice. As such, constitutive abolition of DAO activity may also alter mesocortical DA function.

These studies provide new insights into the presence and role of DAO beyond the hindbrain, and point to a potentially important physiological function in modulating the activity of the mesocortical DA system via the VTA. This could be therapeutically relevant in the context of elevating cortical DA in the treatment of schizophrenia, and may provide supporting evidence for the clinical use of DAO inhibitors.

Acknowledgements

Firstly I would like to extend my thanks to my supervisor Professor Paul Harrison, who has provided unlimited support, patience and guidance throughout my studies, and without whom this thesis would not exist. I would also like to thank Professor Trevor Sharp as my co-supervisor for providing pharmacological expertise and guidance throughout my microdialysis work. Thank you to the MRC for providing me with a studentship and allowing all of this to happen in the first place.

Many thanks go to all those who have helped me in the lab over the years: Dr Kate Groves for teaching me the joys of microdialysis; Drs Linda Laatikainen, Sonny Tan and Mikko Käenmäki for their HPLC expertise; Dr Tracy Lane for her qRT-PCR guidance; and Mary Walker and Li Chen for living up to their reputation as 'Lab Angels'. I would also like to give special thanks to Dr (Uncle) Phil Burnet for all his help with molecular biology and for fielding many silly questions, and Dr Judith Schweimer for her constant support and advice, and of course her help in learning to wrestle.

Thank you to all my friends in the Harrison and Sharp labs for making my DPhil such an enjoyable experience - to Yvonne Couch for her extensive knowledge of online distractions, to Dr Chris Barkus for giving up his time to teach me stats and for being a health and safety hazard, to Katharina Stumpenhorst for putting up with my singing and to Sarah Farrell for providing endless amusement.

My time in Oxford would have been much less entertaining were it not for the Wolfpack, who have been my second family – especially Aurélie, Bel, Christian, Duncan and Iana. Thanks also go to WCBC and OUWLRC for providing so many good times on and off the water, and for making the last 3 years so much fun.

Finally I would like to thank my family – Mum, Dad, Edd and Helen – for their love, support and encouragement, and for being wonderfully bonkers. How many beans make five?

Contents

This thesis contains approximately 35,000 words.

Figures

Chapter 1:

Introduction

D-amino acid oxidase (DAO, DAAO) is a flavin-dependent oxidase first described in the pig kidney in 1935 that catalyses the oxidation of D-amino acids (Krebs, 1935). The D-amino acids are first oxidised to the corresponding imino acids by DAO, and these are then non-enzymatically hydrolysed to form the corresponding α-keto acid. Hydrogen peroxide (H_2O_2) is also generated as part of the first stage of the reaction, and ammonia (NH_3) produced in the final step (Figure 1).

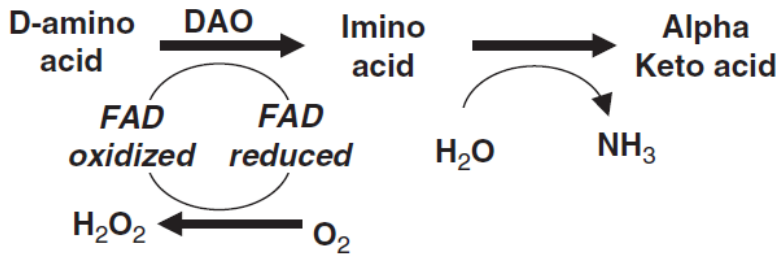


Figure 1. The oxidation of D-amino acids by DAO. *FAD: flavin adenine dinucleotide, the prosthetic group of DAO. Figure adapted from Verrall et al., 2010.*

DAO is stereospecific for D-amino acids (Krebs, 1935), and preferentially acts on those that are neutral or basic, such as D-alanine, D-serine and D-proline, for which DAO shows affinities (Km) of 1.3mM, 7.5mM and 8.5mM, respectively (Molla *et al*., 2006). Despite traditionally being studied in the kidney and liver, DAO has long been established to be present in the mammalian brain (see Neims *et al*., 1966). However, its function therein remained unknown until D-amino acids were also found in brain tissue (e.g. Dunlop *et al*., 1986; Hashimoto *et al*., 1992; Hashimoto and Oka, 1997; Fisher *et al*., 1991; Table 1). In 1995, Schell and colleagues demonstrated that the concentration of D-serine in the brain was reciprocally distributed with the expression of DAO (Schell *et al*., 1995), suggesting that DAO may be a functional brain enzyme. D-alanine and D-proline levels were shown to be considerably lower than D-serine levels in the brain (Table 1), and as such D-serine was considered to be potentially the primary important DAO substrate in the brain.

D-amino acid	Brain region	Human	Rat	Mouse
D-serine	**Cerebral cortex**	130 ± 10 (pfc)[a]	350 ± 10[b]	310 ± 10[c]
		108 ± 6 (pfc gm)[d]	359 ± 74[e]	353 ± 83[f]
		128 ± 12 (pfc wm)[d]	210 ± 8[g]	520 ± 98[e]
		66 ± 41 (fc)[h]	3202 ± 40[i]	387 ± 78[j]
		157 ± 49 (fc, m)[k]		423 ± 21[l]
		192 ± 68 (fc, f)[k]		332 ± 17[m]
		117 ± 30 (pc)[n]		420 ± 25[k]
				285 ± 20[p]
				300 ± 10[q]
	Hippocampus		280 ± 20[b]	227 ± 19[j]
			208[e]	341 ± 29[l]
			231 ± 9.7[g]	340 ± 30[m]
			1470 ± 50[r]	228 ± 20[j]
				275 ± 20[q]
	Cerebellum	ND[d]	20 ± 1[b]	33 ± 21[s]
			ND[g]	ND[c]
				32 ± 20[f]
				28 ± 16[e]
				97 ± 29[j]
				12 ± 2[l]
				7.5 ± 0.2[t]
				15 ± 1[m]
				19 ± 1[i]
				3.7[q]
D-alanine	**Cerebral cortex**	9.5 ± 2.9 (gm)[u]	ND[g]	ND[c]
		12.3 ± 10.2 (wm)[u]	~4[t]	3.5 ± 0.8[f]
				12 ± 2[l]
				12 ± 2[k]
				~5 ± 0.5[q]
	Hippocampus		ND[g]	11 ± 3.5[l]
				10 ± 3[l]
				~4[q]
	Cerebellum		ND[g,t]	ND[c]
				11 ± 2[l]
				~1[q]
				2.2 ± 1.1[f]
				9 ± 2[k]
D-proline	**Cerebral cortex**			0.31 ± 0.15[v]
	Hippocampus			0.11 ± 0.06[v]
	Cerebellum			0.29 ± 0.12[v]

Table 1. Concentrations of D-amino acids in the adult mammalian brain. *Abbreviations: f, female; fc, frontal cortex; gm, grey matter; m, male; ND, not detectable or trace levels; pc, parietal cortex; pfc, prefrontal cortex; wm, white matter. Units are nmolg^{-1} tissue, converted where necessary from alternative units used in the original publication. Some values are estimates from graphical data, and so are approximate. ([a]Hashimoto et al., 1993c; [b]Hashimoto et al., 1993a; [c]Hashimoto et al., 1993b; [d]Kumashiro et al., 1995; [e]Nagata et al., 1994a; [f]Nagata et al., 1994b; [g]Hamase et al., 1997; [h]Nagata et al., 1995; [i]Song et al., 2008; [j]Nagata et al., 1998; [k]Hamase et al., 2005; [l]Morikawa et al., 2001; [m]Wang and Zhu, 2003; [n]Bendikov et al., 2007; [p]Labrie et al., 2009; [q]Miyoshi et al., 2009; [r]Hashimoto et al., 2007; [s]Nagata et al., 1992; [t]Morikawa et al., 2003; [u]Fisher et al., 1991; [v]Hamase et al., 2001).*

1.1 DAO: genetics

The human DAO gene comprises 11 known exons and is located at chromosome 12q24 (see Verrall *et al*., 2010). Analysis of cDNA encoding human kidney DAO has revealed that the human protein consists of 347 amino acids, and has a molecular mass of approximately 39kDa (Momoi *et al*., 1988). Like human DAO, pig DAO also consists of 347 amino acid residues (Fukui *et al*., 1987), whereas mouse only has 345 (Tada *et al*., 1990), and rat is intermediate, with 346 (Konno, 1998). Amino acid sequence homology is high between human and pig DAO (84.4%; Momoi *et al*., 1988), and slightly lower between human and mouse (78%; Tada *et al*., 1990) and human and rat (80%; Konno, 1998).

1.2 DAO: function in the mammalian brain

The discovery that concentrations of D-serine in the brain are reciprocally distributed to the expression of DAO (Schell *et al*., 1995) provided evidence that DAO has a functional role in the mammalian brain. Several further pieces of evidence support this theory, including the characterisation of DAO mutant mice. The ddY/DAO- mutant mouse (Konno and Yasumura, 1983; Sasaki *et al*., 1992) lacks DAO activity due to a single glycine-arginine substitution at position 181 (Gly181Arg; Sasaki *et al*., 1992). These mice show elevated levels of DAO substrates in most brain regions (Morikawa *et al*., 2001), although interestingly show different elevation patterns between the different amino acids (Table 2). D-alanine, for example, is elevated in all brain regions of ddY/DAO- mice when compared with wild-type, whereas D-serine shows a marked increase in the cerebellum (~10 times more), but no difference in the forebrain (Morikawa *et al*., 2001). This suggests that the neurobiology of DAO in the mammalian brain is complex, and that there is regional variation in its activity and physiological relevance. Nevertheless, it provides clear evidence that abolishing DAO activity has an effect on the levels of D-amino acids present in the brain, and points to a functional role of DAO in regulating their levels. However,

whether the elevations in brain levels of the D-amino acids in the ddY/DAO- mice occurs specifically as a result of a lack of central DAO levels remains difficult to determine, since DAO is also highly expressed in the periphery, and so elevated systemic D-amino acid levels may have a contributing effect (see Burnet *et al*., 2011). However, a recent study used lentivirus-delivered RNA-interference to knock down DAO specifically in the mouse cerebellum, and reported a 77% increase in cerebellar D-serine levels as a result (Burnet *et al*., 2011).

D-amino acid	Brain region	Fold change in ddY/DAO- mice
D-serine	**Cerebral cortex**	NC[a,b,c,d] 1.13[e] 1.15[f]
	Hippocampus	NC[b,c,d] 1.29[f]
	Cerebellum	5.2[a] 6[g] 14[b] 11[c] 36[d]
D-alanine	**Cerebral cortex**	3.9[a] 5.1[b] 5.2[h] 10[d]
	Hippocampus	5.4[b] 6.1[h] 10[d]
	Cerebellum	8.2[a] 7.3[b] 8.9[h] ~50[d]
D-proline	**Cerebral cortex**	NC[i]
	Hippocampus	NC[i]
	Cerebellum	2.4[i]

Table 2. Changes in concentrations of D-amino acids in the brains of DAO knockout mice. *Values show the significant fold increases of the D-amino acid in the ddY/DAO- mouse compared with wild-type mice. NC; no significant change.([a]Nagata et al., 1994b; [b]Morikawa et al., 2001; [c]Wang and Zhu, 2003; [d]Miyoshi et al., 2009; [e]Hashimoto et al., 1993b; [f]Song et al., 2008; [g]Kumashiro et al., 1995; [h]Hamase et al., 2005; [i]Hamase et al., 2001).*

1.3 DAO: regulation of NMDA receptor function

Glutamate is the principal excitatory neurotransmitter in the central nervous system, and is ubiquitously expressed throughout the brain. There are four main subtypes of glutamate receptor: the N-methyl-D-aspartate receptor (NMDA receptor), the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA receptor), the kainate receptor, and the metabotropic receptors. The first three of these, including the NMDA receptor, are ionotropic receptors; that is, they are ligand-gated ion channels.

The NMDA receptor has a heterotetrameric structure and consists of two NR1 subunits and two regionally localised NR2 subunits (Laube *et al*., 1998). The major endogenous agonist at the receptor, glutamate, has a binding site on the NR2 subunit (Laube *et al*., 1997), whereas the binding site for the co-agonist (the 'glycine site') is located on the NR1 subunit (Kuryatov *et al*., 1994; Wafford *et al*., 1995). The receptor requires both the occupation of the glutamate binding site and the glycine binding site in order for receptor activation to occur (Kleckner and Dingledine, 1988). Furthermore, the receptor is subject to a voltage-dependent magnesium block (Ascher and Nowak, 1988); membrane depolarisation is required to remove magnesium ions from the receptor pore before the receptor can activate. As such, the receptor has been labelled a 'molecular coincidence detector' (Bliss and Collingridge, 1993), and is believed to be critically important for a number of neurological processes, including synaptic plasticity and long-term potentiation (Collingridge, 1987).

Although glycine was originally considered to be the principal co-agonist at the glycine binding site, D-serine has more recently been shown also to be a functional ligand at the site (e.g. Mothet *et al*., 2000), and its regional expression resembles that of NMDA receptors in the rat brain (Schell *et al*., 1997). As such, D-serine is now widely speculated to be the major endogenous agonist at the glycine site of the NMDA receptor (see

Wolosker, 2006; Oliet and Mothet, 2009). However, D-alanine, another substrate of DAO, is also capable of binding the glycine site (Sakata *et al*., 1999) and is receiving considerable interest as another functional D-amino acid in the mammalian brain. Both D-serine and D-alanine are metabolised by DAO, and as such, DAO levels and D-amino acid availability at the synapse may be important factors in regulating glutamate signalling at the NMDA receptor.

1.4 DAO: distribution

1.4.1 Regional

DAO is conventionally described as a hindbrain enzyme confined to the lower brain stem and cerebellum (Horiike *et al*., 1994). However, expression of both DAO mRNA and protein has more recently been observed in forebrain and midbrain regions (Kapoor *et al*., 2006; Verrall *et al*., 2007), although interestingly with much lower activity than in the cerebellum (Neims *et al*., 1966; Madeira *et al*., 2008). As expected, however, this activity pattern is opposite to the relative levels of D-serine in these areas. Very low levels are found in the cerebellum compared with cortical regions (Table 3).

	D-serine (nmol/g tissue)	DAO activity (nmol/g tissue)
Cerebral cortex	128	8.1
Cerebellum	ND	307

Table 3. D-serine concentrations and DAO activity in the adult human brain. *D-serine values are taken as averages of the summarised data in table 2 of Verrall et al., 2010. Units are nmol/g tissue, converted where necessary from alternative units used in the original publication. DAO activity levels taken from Table 2 of Madeira et al., 2008, and measured using D-serine oxidation. ND=not detectable or trace levels.*

DAO has also been shown to be expressed in dopaminergic midbrain nuclei (Moreno *et al*., 1999; Verrall *et al*., 2007), but its activity therein has yet to be unequivocally demonstrated. However, Fernandez-Espejo and colleagues infused the rat ventral tegmental area (VTA) with either a DAO inhibitor or D-serine, and showed an augmentation of behavioural sensitisation to cocaine as a result (Fernandez-Espejo *et al*., 2008). This process critically involves DA transmission from the VTA (Kalivas and Stewart, 1991), and is accompanied by enhanced glutamate release in the VTA (Kalivas and Duffy, 1998) and increased NMDA (Karler *et al.*, 1989) and AMPA receptor (Li *et al.*, 1997) stimulation. As such, the importance of D-serine activity at the NMDA receptor glycine site in the VTA is strongly implicated by this study, as is the presence and physiological relevance of DAO in the midbrain.

1.4.2 Cellular and subcellular

Traditionally, D-serine is considered to be synthesised (via serine racemase, SRR; Wolosker *et al*., 1999b) and released from glia (Wolosker *et al*., 1999a) at tripartite synapses to potentiate the action of glutamate at the NMDA receptor, after which it is taken back up into glia and metabolised by peroxisomal DAO (Figure 2a). However, more recent evidence suggests that DAO may not be exclusively glial, with immunoreactivity being demonstrated in Golgi and Purkinje cells in the rat cerebellum (Moreno *et al*., 1999), and studies in other brain areas also suggesting a neuronal localisation for DAO (Moreno *et al*., 1999; Verrall *et al*., 2007) – and indeed for SRR (Kartvelishvily *et al*., 2006; Verrall *et al*., 2007). A much more complex system seems possible, and indeed likely, with DAO and SRR present in both neurons and glia, working in conjunction with several other influencing factors.

At the subcellular level, DAO has been shown to be a peroxisomal enzyme, specifically being located to microperoxisomes in Bergmann glial cells and astrocytes of the cerebellum (Horiike *et al*., 1987), and possessing a C-terminal peroxisomal targeting sequence (Gould *et al*., 1988; Fukui *et al*., 1987; Caldinelli *et al*., 2004). However, a detailed colocalisation study using immunofluorescence has revealed that a significant amount of DAO is also present outside peroxisomes in cultured human astrocytes (Sacchi *et al*., 2008), suggesting that DAO is not exclusively expressed therein. Indeed, the C-terminal region of the enzyme is prone to proteolysis (Pollegioni *et al*., 1995), resulting in an absence of the peroxisomal-targeting sequence, which could account for cystosolic DAO.

Figure 2b summarises the potential complexity of D-serine regulation at the synapse, and outlines possible pathways involved (adapted from Verrall *et al*., 2010, upon which I am co-author).

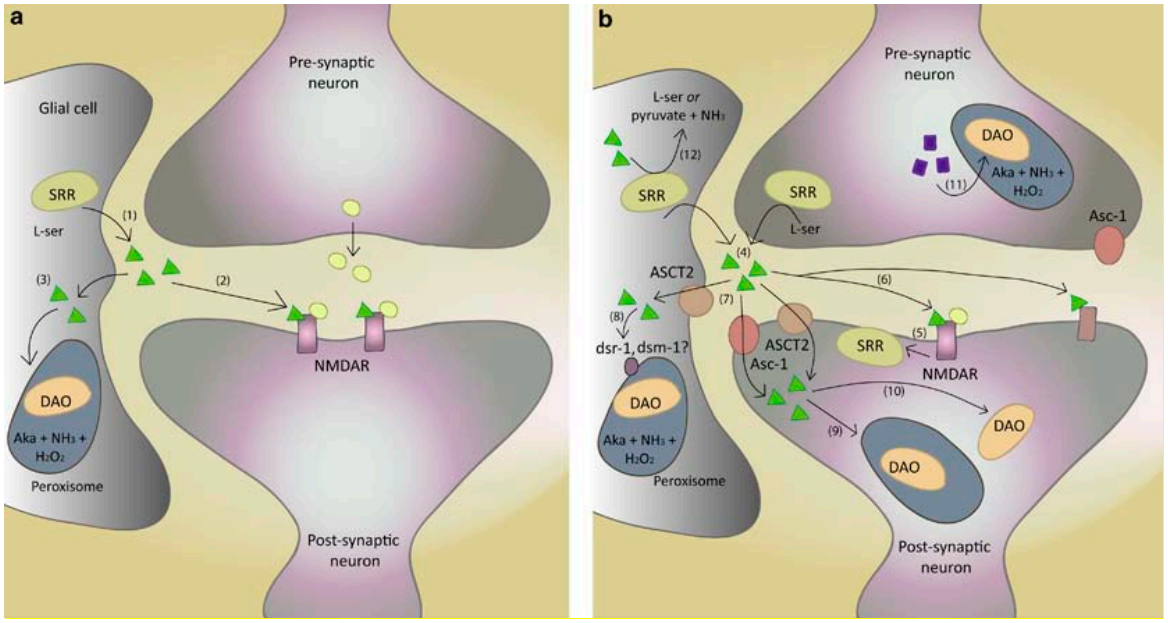


Figure 2. Synaptic regulation and DAO catabolism of D-serine. (a) *Schematic showing a simplified, traditional view of synaptic D-serine and its breakdown by DAO. (1) Glial serine racemase (SRR) synthesises D-serine (green triangles) from L-serine. (2) D-serine is released at tripartite synapses to facilitate the action of synaptic glutamate (yellow circles) at NMDA receptors. (3) Synaptic D-serine is then taken up into glia and broken down by peroxisomal DAO.* ***(b)*** *Schematic incorporating additional players and pathways that may be involved in DAO function and D-serine regulation. (4) SRR may also be localised in neurons, allowing neuronal formation and release of D-serine (5) SRR translocates to the plasma membrane (of neurons or glial cells) after NMDA receptor activation, regulating D-serine synthesis. (6) Synaptic D-serine can have actions additional to potentiating NMDA receptors, including antagonism at NR1/NR3 and AMPA receptors and binding to the GluRd2 receptor. (7) D-serine is removed from the synapse into the glia by ASCT2 (and potentially other transporters), and into neurons predominantly through Asc-1 and also through ASCT2. (8) Intracellular D-serine may enter peroxisomes through a transporter, for which dsr-1 and dsm-1 are potential candidates. (9) D-serine taken up into neurons may be broken down by neuronal DAO. (10) DAO may exist, and be functional, outside peroxisomes. (11) In addition to D-serine, DAO also breaks down certain other D-amino acids, including D-alanine (dark blue squares). (12) D-serine may be catabolised through DAO-independent mechanisms, including SRR-mediated eliminase or reverse racemase functions. It is unclear how many of steps (4)–(12) exist in vivo, and their relative importance in different cellular and synaptic populations. However, the schematic emphasises the likely complexities involved in understanding the neurobiology of DAO and its relationships with other components of D-serine regulation.*

Interestingly, a recent set of immunoprecipitation experiments revealed 24 putative DAO-interacting proteins, including Bassoon, a protein enriched in the presynaptic active zone (Popiolek *et al*., 2011). The authors also found both DAO and Bassoon in a pure synaptic membrane protein fraction without catalase, suggesting that DAO may localise at the synaptic junction, where it may interact with presynaptically located Bassoon (Popiolek *et al*., 2011). Furthermore, Bassoon was shown to inhibit DAO activity (Popiolek *et al*., 2011), suggesting a potentially important role for the Bassoon-DAO interaction in modulating synaptic D-serine and D-alanine levels.

The cellular and regional distribution of DAO is complex, and the original view that it is a peroxisomal, glial, hindbrain enzyme is too simplistic. The presence of DAO appears not to necessarily mean it is functional, at least in the breakdown of D-serine, adding further complexities to the neurobiology of the enzyme. As part of this thesis further investigations will be carried out into the distribution of DAO at both the regional and cellular level, with a view to probing some important questions to which the answers currently remain unknown or equivocal.

1.5 Schizophrenia

1.5.1 Overview of schizophrenia

Schizophrenia is a major psychiatric disorder that affects around 1% of the population worldwide and represents a significant economic burden (Wu *et al*., 2005). It is a debilitating psychiatric syndrome that typically manifests in adolescence or early adulthood and usually has a chronic course. The symptoms of schizophrenia are grouped into two categories: positive and negative. Positive symptoms include those that represent an excess or distortion of normal functions, and include delusions, hallucinations (including auditory), thought disorder and paranoia. Conversely, those symptoms termed

'negative' reflect a reduction of normal function, and include social withdrawal, emotional flattening, anhedonia and alogia. Cognitive symptoms may also be present, and include memory loss and attentional difficulties, making it difficult for patients to function in everyday life, even when their other symptoms are under control. Mood symptoms are also present in many cases, and can make accurate diagnosis of schizophrenia difficult.

Schizophrenia is diagnosed based on standardised criteria outlined in either the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR, published by the American Psychiatric Association) or in the International Statistical Classification of Diseases and Related Health Problems (ICD-10, published by the World Health Organization). European countries usually diagnose according to ICD-10, while the DSM-IV criteria are used in the USA and the rest of the world. Briefly, according to DSM-IV-TR, diagnosis requires the presence of two or more characteristic symptoms, each for at least 1 month, coupled with social or occupational dysfunction and evidence of overall disturbance for at least 6 months. The schizophrenia can then be further classified into one of paranoid type, disorganised type, catatonic type, undifferentiated type or residual type. A diagnosis of schizophrenia cannot be made if the symptoms are likely to be a result of another medical condition or are linked to medication or substance abuse (DSM-IV-TR, 2000).

The exact neuropathological basis of schizophrenia remains unclear, but several different neurotransmitter systems have been implicated in the pathogenesis of the disorder. Classically, the DA system has been most extensively studied, largely based on the finding that the vast majority of antipsychotic medications act on DA receptors. The DA hypothesis of schizophrenia suggests that the positive symptoms are associated with a hyperfunctional mesolimbic DA pathway, and the cognitive and negative symptoms are associated with a hypofunctional mesocortical DA pathway (Davis *et al.*, 1991). However, the glutamate system is now also under considerable investigation, and is believed to play a role through hypoactivity. This is largely based on the finding that administration of

glumatergic NMDA receptor antagonists, such as phencyclidine and MK-801, induce psychosis in healthy individuals (see Javitt and Zukin, 1991), suggesting a diminished activation of NMDA receptors in schizophrenia.

Current treatments for schizophrenia include a combination of antipsychotic medications and psychological treatments. First generation antipsychotics include chlorpromazine and haloperidol, which act mainly at dopaminergic D2 receptors (Chlorpromazine hydrochloride SPC, Rosemont Pharmaceuticals Ltd., 2012; Dolpin SPC, Pinewood Healthcare, 2011). Many second generation antipsychotics, however, also appear to have significant actions at other receptors, including the serotonergic 5-HT_2 receptors, and have fewer extrapyramidal side effects (Risperdal SPC, Janssen-Cilag, 2012; Olanzapine SPC, Accord Healthcare Ltd., 2012). These include risperidone and olanzapine. These medications are usually coupled with psychological treatments, which can include family intervention, cognitive behavioural therapy and art therapy.

1.5.2 Schizophrenia genetics

Schizophrenia is a highly heritable disorder, as demonstrated by repeated family, twin and adoption studies. Estimates of the heritability of risk lie at around 80% (Cardno and Gottesman, 2000; Sullivan *et al*., 2003), with twin studies suggesting concordance rates of around 50% for monozygotic twins and around 20% for dizygotic twins (Cardno and Gottesman, 2000). As such, the genetic basis of schizophrenia has been the focus of considerable research effort.

Candidate genes for schizophrenia have been identified using a variety of methods, which have evolved significantly in recent years. Originally, many putative risk genes for schizophrenia were identified in hypothesis-driven candidate gene association studies (see e.g. Harrison and Weinberger, 2005), in which specific genes were selected based on their predicted potential involvement in the pathogenesis of schizophrenia, and then

compared for variation between patients with schizophrenia and controls. Genome-wide linkage studies were also used to try to identify genomic regions whose inheritance was correlated with developing schizophrenia. Together, these studies identified many potential candidate genes for schizophrenia.

However, more recently, the emergence of genome-wide association studies (GWAS; studies scanning the entire genome for disease association) has led to considerable questioning of the validity of these original identifications. Those genes historically identified as candidate genes for schizophrenia using hypothesis-driven approaches (e.g. COMT, DRD2, NRG1, BDNF) have been analysed relative to GWAS results, and almost no overlap has been found (Collins *et al*., 2012). GWAS results have, however, allowed for the identification of new candidate schizophrenia genes, including ZNF804A (O'Donovan *et al*., 2008). Interestingly, ZNF804A has recently been shown to regulate transcription levels of four putative schizophrenia-associated genes (PRSS16, COMT, PDE4B and DRD2; Girgenti *et al*., 2012), suggesting that it may modulate a network of genes associated with schizophrenia. GWAS analyses of the International Schizophrenia Consortium have supported a polygenic model for schizophrenia, in which susceptibility potentially involves hundreds of common alleles across many domains of brain development and function (Purcell *et al*., 2009). Each individual variant may have little effect, and confer little predictive value, but when combined together, they collectively make considerable contribution to disorder risk.

However, GWAS studies also have limitations. Firstly, there is the need to correct for large numbers of statistical comparisons, and so thresholds of significance must be very conservative (Gejman *et al*., 2011). There are also considerable difficulties in determining potentially significant environmental influences within the study cohort (Gejman *et al*., 2011). Furthermore, rare single nucleotide polymorphisms (SNPs) often remain undetected in GWAS arrays, and many SNPs identified as a result of GWAS do not

appear to be located in coding sequences of the genome – rather, they are intronic, or of unclear function (see Gejman *et al*., 2011).

Recent interest has also focused on copy number variants (CNVs), in which sections of the genome (ranging from 1kb to several Mb) are deleted or duplicated. There has emerged supporting evidence for the association of specific CNVs with schizophrenia; particularly noteworthy is an unusually large deletion at 22q11.21, which has long been known to cause velocardiofacial syndrome. 30% of carriers of this deletion develop psychosis, 80% of which presents as schizophrenia (Murphy *et al*., 1999). This confers the largest known individual risk factor for schizophrenia (next to having an identical twin with the disorder). Other identified CNVs that are associated with schizophrenia include deletions in 2p16.*3 (NRXN1)*, 1q21.1 and 15q13.3, and duplications in 16p11.2 (Levinson *et al*., 2011). CNVs are rare but strong (genotypic relative risks of 7–20; Kim *et al*., 2011), and confer increased risk for multiple psychiatric and general medical disorders (Sebat *et al*., 2009).

Together, studies into the genetic basis of schizophrenia have provided considerable evidence that genetic predisposition contributes significantly to the risk of developing schizophrenia. Although some studies have generated conflicting results, and each of the different study designs has its own limitations, together they point to a complex genetic architecture. It seems likely that the overall genetic risk for schizophrenia is dependent on the presence of both common SNPs of little effect, and on the presence of much rarer CNVs, which are stronger predictors of the disorder. However, the relative contribution of each remains unclear. Furthermore, there is the question of the so-called 'missing heritability'. Clearly, genetics alone does not fully account for predisposition to schizophrenia. In addition to the widely cited gene-environment interactions in schizophrenia, there are likely to be additional gene-gene interactions. Whereas each of the risk variant SNPs are likely to be relatively common in the general population, each

with little individual effect (Picchioni and Murray, 2007), epistatic interactions between such genes, and the interplay between their products, are likely to contribute to the pathogenesis of the disorder. It is also possible that buffering mechanisms that arise in response to potentially detrimental SNPs may themselves play a role (Gejman *et al*., 2011). As such, the genetic basis of schizophrenia is highly complex, and it appears to be a relatively highly polygenic disease (Kim *et al*., 2011).

1.5.3 Schizophrenia and DAO

DAO itself was originally identified as a putative susceptibility gene for schizophrenia in 2002 via genetic and physiological interaction with G72, its putative binding partner (Chumakov *et al*., 2002). Further association studies of DAO with schizophrenia have yielded both positive (e.g. Liu *et al*., 2004; Schumacher *et al*., 2004; Wood *et al*., 2007; Corvin *et al*., 2007; Kim *et al*., 2010) and negative (e.g. Yamada *et al*., 2005; Liu *et al*., 2006; Vilella *et al*., 2008; Shinkai *et al*., 2007; Bass *et al*., 2009) results. However, DAO has not been identified as a candidate gene for schizophrenia in any GWAS studies. A meta-analysis of published association studies for DAO and schizophrenia is available in the SZGene Database (http://www.szgene.org; Allen *et al*., 2008), and when accessed in Q1 2012, revealed only one DAO SNP that is significantly associated with schizophrenia (Table 4; SZGene Database accessed 29 February 2012). Interestingly, this SNP appears to be protective against schizophrenia. No identified CNVs that have been associated with schizophrenia to date appear to span the DAO locus. Overall, DAO is currently the number 40 top schizophrenia gene in the SZGene Database, as ranked by the statistical significance of its significant SNP (http://www.szgene.org/TopResults.asp; updated 23 December 2011, accessed 29 February 2012). The positions of the SNPs analysed in the SZGene Database in the DAO gene are shown in Figure 3.

SNP	Odds Ratio	95% Confidence Interval
rs2070586	1.00	[0.79, 1.26]
rs2070587	0.97	[0.84, 1.11]
rs2111902	1.03	[0.90, 1.18]
rs3741775	0.93	[0.82, 1.07]
rs3825251	1.03	[0.94, 1.12]
rs3918346	1.01	[0.86, 1.18]
rs3918347	1.17	[0.98, 1.41]
rs4623951	0.88	[0.79, 0.98]

Table 4. Association of single nucleotide polymorphisms in the DAO gene with schizophrenia.

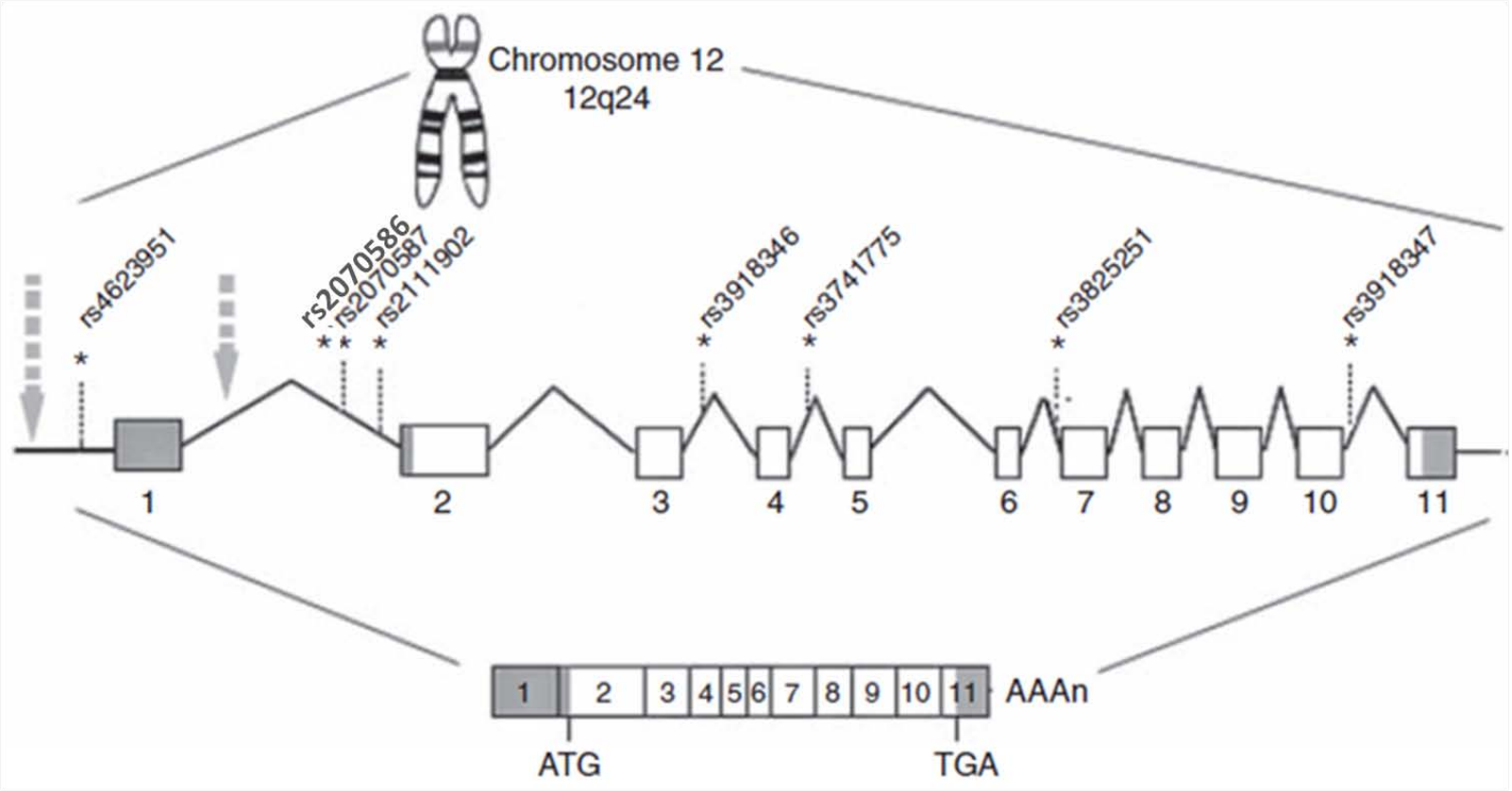


Figure 3. The DAO gene and transcript. *The gene comprises 11 known exons. Exon 1 plus 9 base pairs of exon 2 encode the 5'-untranslated region (shaded grey) of the DAO transcript, exons 2–11 encode the open-reading frame, and exon 11 encodes the 3'-untranslated region (shaded grey). The transcriptional start site is in exon 1. The positions of the DAO SNPs analysed in the SZGene Database are shown. Figure adapted from Verrall et al., 2010.*

There remains scepticism as to the reliability of the identification of DAO as a susceptibility gene for schizophrenia. However, at the time of conception of this thesis, neither the positive nor negative results were overwhelming, and as such, the positive results (Chumakov *et al*., 2002; Corvin *et al*., 2007; Schumacher *et al*., 2004; Wood *et al*., 2007; Liu *et al*., 2004) were still considered sufficient evidence with which to at least consider DAO as a potential gene. Furthermore, a meta-analysis was published by Shi and colleagues in 2008, just prior to the commencement of the current study, in which case-control and family-based studies were combined and a DAO SNP was found to be significantly associated with schizophrenia (Shi *et al*., 2008). This provided further evidence for DAO as a schizophrenia candidate gene.

The mechanism by which DAO may be associated with schizophrenia remains unclear, however, since studies have failed to directly associate DAO single nucleotide polymorphisms (SNPs) with any effect on DAO expression and activity (Burnet *et al*., 2008) or on D-serine levels (Yamada *et al*., 2005). Furthermore, questions have arisen as to the significance of the proposed G72-DAO interaction, which was originally used to identify DAO as a candidate gene (Chumakov *et al*., 2002). The most significant of these, perhaps, comes from a report failing to identify any significant G72 mRNA or protein expression in the human brain (Benzel *et al*., 2008), suggesting not only that any interaction between DAO and G72 does not occur *in vivo*, but potentially that G72 itself is not robustly expressed as a functional molecule. As such, the DAO-G72 interaction is questionable, and whether or not SNPs in the DAO gene lead to schizophrenia remains unclear.

Despite the genetic complexities described above, evidence still points to a role of DAO in the pathophysiology of schizophrenia. DAO shows increased expression and activity in the disorder (Kapoor *et al*., 2006; Verrall *et al*., 2007; Burnet *et al*., 2008; Madeira *et al*., 2008). Although evidence across all regions is inconclusive, increased DAO mRNA and

activity in the cerebellum has been confirmed in several studies (Kapoor *et al*., 2006; Verrall *et al*., 2007; Burnet *et al*., 2008). Subject to the caveats of post-mortem studies, these data suggest that DAO may play a contributing role in the pathogenesis of schizophrenia; in the simplest model, it may be hypothesised that an increase in the expression and activity of DAO may reduce the availability of D-serine in the synapse, contributing to the hypofunction of the glutamatergic NMDA receptor, which itself has long been implicated in the pathogenesis of schizophrenia (Kim *et al*., 1980; Javitt and Zukin, 1991; Moghaddam, 2003; Coyle *et al*., 2003; Marek *et al*., 2010). Consistent with this, decreased D-serine levels have been observed in the serum and cerebrospinal fluid of patients with schizophrenia (Hashimoto *et al*., 2003; Hashimoto *et al*., 2005b), although interestingly not in brain tissue (Bendikov *et al*., 2007; Kumashiro *et al*., 1995). Regionally, it may be considered surprising that the changes in DAO expression and activity are only convincingly observed in the cerebellum, as this brain area is not traditionally associated with schizophrenia. Nevertheless, evidence is growing to suggest that the cerebellum has an important role in cognition, via connections with the cerebral cortex (see (Andreasen and Pierson, 2008), and cerebellar malfunction could help to explain the diverse symptomatology of schizophrenia (Andreasen and Pierson, 2008).

1.6 The DA system

1.6.1 DA synthesis and metabolism

The neurotransmitter dopamine (DA) is a member of the catecholamine family, which is synthesised via the hydroxylation of L-tyrosine to L-DOPA by tyrosine hydroxylase, followed by decarboxylation of L-DOPA by DOPA decarboxylase. DA is released from neurons via vesicular exocytosis. DA can then be metabolised in one of two ways: first, via degradation to dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase (MAO) and aldehyde dehydrogenase, followed by further metabolism to homovanillic acid (HVA)

by catechol-O-methyltransferase (COMT); or second, by inital degradation by COMT to 3-methoxytyramine (3-MT), then further metabolism by MAO and aldehyde dehydrogenase to HVA (Figure 4). Both pathways converge on HVA.

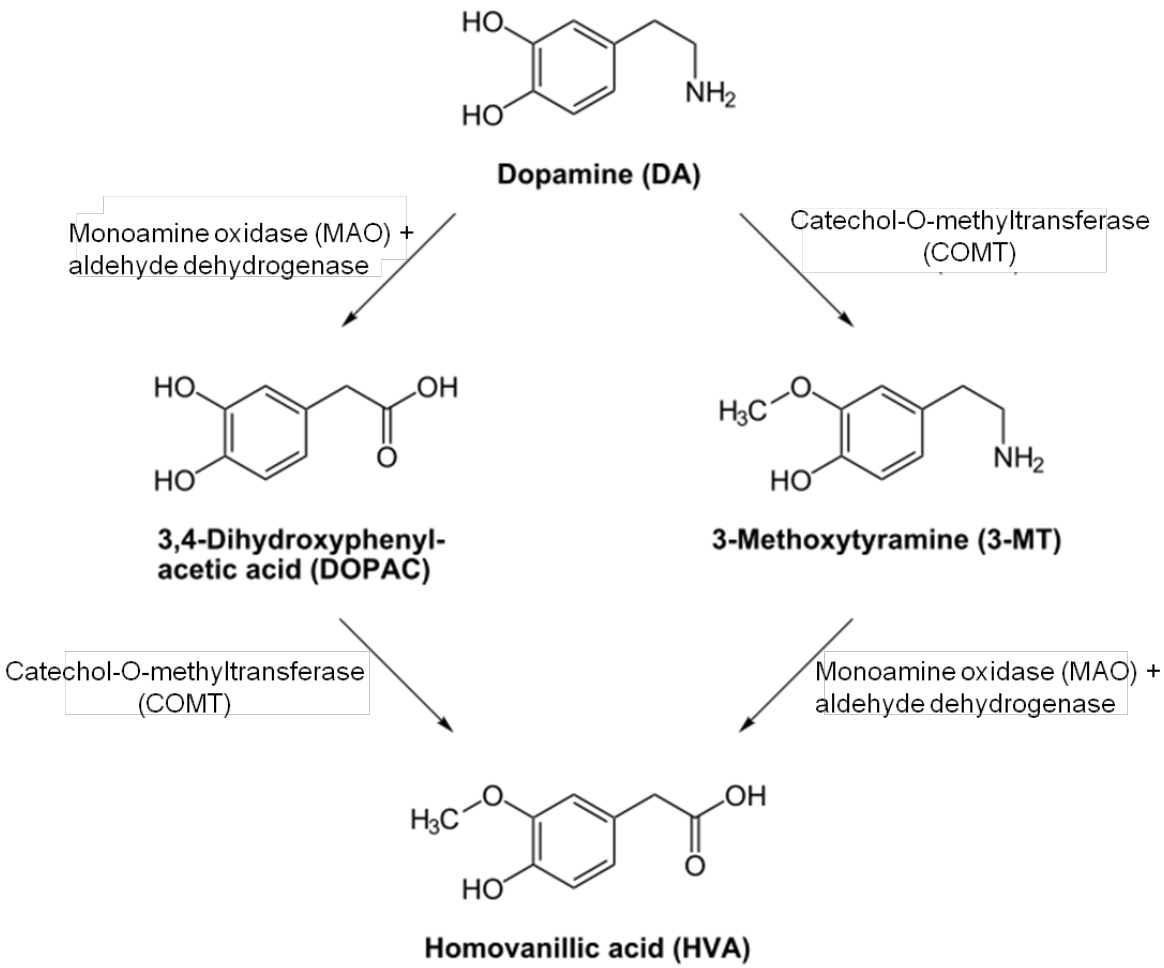


Figure 4. The DA metabolic pathway.

1.6.2 DA pathways and receptors

There are four major DA pathways in the brain: the mesolimbic pathway, which projects from the VTA to the nucleus accumbens; the mesocortical pathway, which projects from the VTA to the prefrontal cortex (PFC); the nigrostriatal pathway, which projects from the substantia nigra to the striatum; and the tuberoinfundibular pathway, which projects from the hypothalamus to the pituitary gland. DA has several important functions in the brain, including the regulation of cognition, voluntary movement and reward. DA receptors are G-protein coupled receptors, of which there are at least five subtypes (D_1–D_5) and which are differentially expressed throughout the brain.

1.6.3 Regulation of the DA system in the VTA

The VTA is the source of the mesocortical and mesolimbic DA systems in the human brain. The VTA receives innervation from the cerebellum (Snider *et al.*, 1976) and the PFC, which provides one of the principal glutamate inputs to the VTA (Sesack and Pickel, 1992). DA neurons in the VTA express glutamatergic NMDA receptors (Seutin *et al.*, 1990), and considerable evidence suggests that dopaminergic output from the VTA is regulated by these receptors (e.g. Bennett and Gronier, 2007). Burst firing of midbrain DA neurons has been shown to result from the tonic activation of NMDA receptors *in vivo* (Chergui *et al*., 1993). However, noncompetitive NMDA receptor antagonists, such as PCP and MK-801, have also been shown to produce dose-dependent increases in the firing rate of ventral tegmental DA neurons in the rat, which are accompanied by increases in burst activity (French *et al.*, 1993). Modulators of the NMDA receptor glycine site, such as kynurenic acid, can also tonically modulate the activity of these neurons, with some studies suggesting that antagonists of the site produce increases in firing rate and burst firing activity (Schwieler et al., 2006), and others suggesting that antagonists produce inhibition of burst firing activity (Charlety *et al.*, 1991). As such, NMDA receptors

appear to be important regulators of DA output from the VTA, although the mechanism may be complex.

1.7 DAO and schizophrenia: link with the DA system

The DA system has long been implicated in schizophrenia; hyperfunction of the mesolimbic DA pathway is believed to be responsible for the positive symptoms of the disorder, and conversely, hypofunction of the mesocortical pathway is thought to contribute to the negative and cognitive symptoms (Davis *et al*., 1991; Howes and Kapur, 2009; Weinberger *et al*., 2001). The vast majority of classical antipsychotic drugs are DA receptor antagonists, and evidence suggests that their neuroleptic effects are achieved by reducing DA activity in the mesolimbic system (see Davis *et al*., 1991). However, the battery of negative and cognitive symptoms associated with hypofunction of the mesocortical pathway currently remains largely untreated.

Disruption of both the mesolimbic and mesocortical systems in schizophrenia suggests that aberrant regulation of a common feature of their pathways may be involved in the pathogenesis. Both of these systems originate in the VTA, and as discussed above, the DA output from the VTA is mediated by NMDA receptor activation. It is therefore possible that disrupted NMDA receptor mediation may play a role in schizophrenia. If DAO is present and active in the VTA, as suggested above (Fernandez-Espejo *et al*., 2008), aberrant DAO activity, altered D-serine levels and consequent effects on NMDA receptor function in the VTA could contribute to DA system dysfunction in schizophrenia. Manipulation of DAO and D-serine levels in the midbrain could thus alter DA levels in projection areas, including in the cortex.

Interestingly, evidence also suggests that the cerebellum can modulate dopaminergic function in the PFC (Mittleman *et al*., 2008), and that disrupted Purkinje cell output from

the cerebellum may result in abnormal DA transmission in the PFC (Mittleman *et al*., 2008). If, indeed, the cerebellum is at least partially responsible for the regulation of cortical DA function, cerebellar malfunction, again as a result of aberrant DAO and D-serine levels, may also be a contributing factor to the deficits in cortical DA signalling observed in schizophrenia. Furthermore, a more recent study also showed that the cerebellar-PFC connection is also partially reliant on NMDA receptor function in the VTA, with intra-VTA infusion of the NMDA receptor antagonist kynurenate reducing cerebellar stimulation-invoked cortical DA release by around 50% (Rogers *et al*., 2011). Clearly, both the cerebellum and VTA have a potential role in the modulation of cortical DA function, and as such, levels of D-serine and DAO, and NMDA receptor function, in these areas may play an important part in this modulation.

1.8 DAO and schizophrenia: therapeutic potential

The reduced D-serine levels found in patients with schizophrenia (Hashimoto *et al*., 2003; Hashimoto *et al*., 2005b) may be therapeutically relevant. Increasing D-serine levels in these patients and augmenting NMDA receptor function could potentially be of benefit. Indeed, some clinical trials with D-serine as an add-on treatment have shown a favourable effect, including on negative and cognitive symptoms (Coyle *et al*., 2002; Javitt, 2006; Kantrowitz *et al*., 2010; Heresco-Levy *et al*., 2005). Given that these symptoms currently remain largely untreated by existing drugs, this makes D-serine therapy very attractive. However, large doses of D-serine are required, which may cause nephrotoxicity (Ganote *et al*., 1974; Krug *et al*., 2007). Other methods of potentiating NMDA receptor glycine site function have also been investigated, including the use of other glycine site agonists D-cycloserine (a partial agonist; Heresco-Levy and Javitt, 2004), glycine (Heresco-Levy and Javitt, 2004; Heresco-Levy *et al*., 1999; Javitt *et al*., 1994) and D-alanine (Tsai *et al*., 2006), with full agonists at the glycine site being more

effective in producing clinical improvement than partial agonists (Heresco-Levy and Javitt, 2004). Glycine transporter inhibitors are under investigation, which increase synaptic levels of glycine through prevention of its reuptake from the synaptic cleft. One such inhibitor, sarcosine (N-methyl glycine), has been shown to induce significant reductions in negative and cognitive symptoms of schizophrenia, in addition to positive symptoms, when taken in combination with standard antipsychotic regimens (Tsai *et al*., 2004). DAO inhibitors have also been identified as a potential alternative method of augmenting glycine site function, via the elevation of levels of D-amino acids as a result of inhibition of their metabolism. No clinical trials of DAO inhibitors in schizophrenia have been published to date, although some pre-clinical studies have investigated their potential antipsychotic and cognitive-enhancing effects *in vivo* (Adage *et al*., 2008; Smith *et al*., 2009), with one showing promising results (Adage *et al*., 2008). However, another study suggested that DAO inhibition alone may not be sufficient to produce similar effects to those seen with D-serine (Smith *et al*., 2009). It is attractive to speculate, however, that administration of the two substances in conjunction may allow for synergistic therapeutic effects while not needing to increase the D-serine dose further.

Studies of DAO mutant mice also support the hypothesis that DAO inhibition may be useful in treating schizophrenia. For example, ddY/DAO- mice have been shown to have a reduction in stereotypy and ataxia induced by the NMDA receptor antagonist MK-801 (Hashimoto *et al*., 2005a), which is widely used as a model of schizophrenia (see Moghaddam and Jackson, 2003). Furthermore, the mice have been shown to have improved spatial learning in the Morris water maze and increased hippocampal long-term potentiation (Maekawa *et al*., 2005), suggesting a pro-cognitive effect of the knockout. Together, these data suggest that a reduction in DAO activity may have beneficial effects in schizophrenia, including on the cognitive symptoms.

However, despite the evidence presented above suggesting that D-amino acid elevation or DAO inhibition may be useful in the treatment of schizophrenia, not all studies have reported positive findings. Some studies in DAO mutant mice have failed to show differences between wild-types and knockouts in some behavioural tests (e.g. Almond *et al*., 2006), and furthermore, some clinical studies with glycine site agonists have failed to show beneficial effects (Goff *et al*., 2005; Tuominen *et al*., 2005). There also remain further caveats; not least the fact that whereas D-serine levels are reduced in the serum and cerebrospinal fluid of patients with schizophrenia, as noted above, levels of D-serine in the brain itself do not seem to be decreased (Bendikov *et al*., 2007; Kumashiro *et al*., 1995). Nevertheless, the body of evidence suggesting that manipulation of DAO and/or D-amino acid levels may have a beneficial effect in some cases may be considered sufficient to support further research into this therapeutic approach.

1.9 Summary and thesis aims

In summary, the expression pattern and physiological role of DAO in the brain remains unclear. Whereas DAO was traditionally considered to be an enzyme expressed in peroxisomes of the glia in the hindbrain, it now seems that it may also be expressed outside peroxisomes, it may be expressed in neurons as well as glia, and it may be present in other brain regions in addition to the hindbrain. Furthermore, given that DAO activity, and its effects on levels of D-amino acids, can potentially affect the function of NMDA receptors, its regional expression and activity may be of great functional importance. The VTA is the source of the mesocortical and mesolimbic DA systems in the mammalian brain, and dopaminergic output along these systems is regulated by NMDA receptors. As such, if DAO is expressed and active in the VTA, its regulation of D-amino acid availability at the glutamatergic synapse may have a functional impact on NMDA receptor glycine site occupancy, and hence activation of the receptor and stimulation of the DA system. DAO may, therefore, be an indirect modulator of DA function in the brain, and may have a role in those psychiatric disorders involving the DA system, including schizophrenia.

In light of these considerations, this thesis will have three main focal points:

1. *Investigations into the regional and cellular expression of DAO in the rat brain.* The main focus will be the question of whether or not DAO is present in the VTA, and if so, in which type of cell? DAO expression will also be investigated in the cerebellum, to assess the cell type in which it is expressed therein. Both *in situ* hybridisation and immunofluorescence techniques will be used to investigate mRNA and protein expression, respectively.

2. *Investigations into the potential links between DAO activity, D-amino acid levels, NMDA receptor activation and mesocortical DA system function in the rat brain.* How the manipulation of DAO activity and/or D-serine levels in the rat brain affects levels of DA in the PFC will be studied. *In vivo* microdialysis will be used to measure levels of DA in the PFC, while systemic or intra-VTA injections of D-serine and/or DAO inhibitors are administered.

3. *Investigations into the effects of genetically abolishing DAO activity on markers of DA system function.* The expression of DA receptors and metabolising enzymes will be investigated in the PFC of DAO knockout mice. RT-PCR will be used to quantify the expression of the dopaminergic Drd1a and Drd2 receptors, in addition to catechol-O-methyl transferase (COMT; a metabolising enzyme of DA), in tissue taken from the PFC of DAO knockout, heterozygote, and wild-type mice. The three groups will then be compared, and differences in the DA markers investigated as possible evidence for altered mesocortical DA function in the knockout mice.

Chapter 2:

The regional and cellular expression of DAO in the developing and adult rat brain

2.1 Background

Although DAO is classically considered to be a glial hindbrain enzyme, it may have important functions in other brain regions. For example, DAO has been shown to be expressed in dopaminergic midbrain nuclei (Moreno *et al*., 1999; Verrall *et al*., 2007). Verrall and colleagues reported strong DAO immunoreactivity in large neurons in the substantia nigra pars compacta of the human brain, which were believed to be putative dopaminergic neurons (Verrall *et al*., 2007). Similarly, Moreno and colleagues reported dense staining in the pars compacta and pars lateralis regions of the substantia nigra of the rat brain, with even denser staining in the pars reticula (Moreno *et al*., 1999). These results all suggest the presence of DAO in dopaminergic midbrain nuclei. Furthermore, Moreno reported some DAO immunoreactivity in the VTA of the rat brain, which is very interesting in the context of the VTA as the source of the mesocortical and mesolimbic dopaminergic pathways in the brain (Moreno *et al*., 1999).

Despite these expression studies, the activity of DAO in the midbrain has yet to be unequivocally demonstrated. However, Fernandez-Espejo and colleagues infused the rat VTA with either sodium benzoate (a DAO inhibitor) or D-serine, and showed an augmentation of behavioural sensitisation to cocaine (Fernandez-Espejo *et al*., 2008), a process known to involve VTA NMDA receptor activation. As such, the importance of D-serine activity at the glycine site of NMDA receptors in the VTA is strongly implicated, as is its regulation by DAO. It seems possible, therefore, that DAO has an important role in the midbrain.

Traditionally, D-serine is considered to be synthesised and released from glia at tripartite synapses to potentiate the action of glutamate at the NMDA receptor, after which it is taken back up into glia and metabolised by peroxisomal DAO. However, more recent evidence suggests that DAO may not be exclusively glial, with two studies suggesting a

neuronal localisation for DAO (Moreno *et al*., 1999; Verrall *et al*., 2007). DAO may also not be exclusively peroxisomal (Popiolek *et al*., 2011). A much more complex system seems possible, and indeed likely, with DAO present in both neurons and glia, working in conjunction with several other influencing factors. In the cerebellum, for example, DAO has been detected numerous times exclusively in glia, across many decades of research (Weimar and Neims, 1977b; Horiike *et al*., 1987; Gaunt and de Duve, 1976; Moreno *et al*., 1999; Ono *et al*., 2009). However, in 1999 Moreno and colleagues reported it to be present in Purkinje cells, raising the question as to whether it was, indeed, exclusively glial in this brain region. This possibility may be of particular interest in the cerebellum, given the high DAO activity levels reported there in the adult (Neims *et al*., 1966).

Interestingly, D-serine levels in the cerebellum are known to change throughout development (Schell *et al*., 1997), suggesting that DAO levels may also change with time in order to regulate the D-serine. For example, D-serine levels in the rat cerebellum showed a decrease over time from P12 onwards, reaching undetectable levels by P26 (Schell *et al*., 1997). Similarly in the mouse cerebellum, Wang and Zhu (2003) reported high levels of D-serine at P7, but a dramatic decline after P12, reaching trace levels by P17 (Wang and Zhu, 2003). No DAO activity was detected in the developing mouse cerebellum until P12, after which it increased dramatically, reaching adult levels at P49 (Wang and Zhu, 2003). Together, these studies suggest that D-serine and DAO are expressed in a reciprocal fashion throughout development, and that DAO may work to control levels of D-serine in the developing brain. It is interesting to note that D-serine is detectable in the developing cerebellum, but not in the adult cerebellum (Schell *et al*., 1997). As such, D-serine may serve an important function in normal cerebellar development, and there may be interplay between NMDA receptors, D-serine and DAO in the cerebellum before the adult brain is fully formed. Aberrant levels of D-serine, and perhaps DAO, during development may therefore affect cerebellar formation.

2.2 Summary and aims

The regional and cellular distribution of DAO is complex, and the original view that it is a glial hindbrain enzyme is too simplistic. The aim of this chapter was to carry out investigations into the distribution of DAO at both the regional and cellular level, with a view to probing some important questions to which the answers currently remain unclear. Specifically, is DAO expressed in the midbrain, and if so, in what type of cell? Is DAO present in neurons in the cerebellum, or is it confined to glia, as traditionally thought? Do the levels of DAO change in the rat cerebellum during development, in order to control levels of D-serine?

First, an *in situ* hybridisation histochemistry (ISHH) riboprobe was created to investigate the expression of DAO mRNA in coronal sections taken from the rat VTA. The riboprobe was tested using northern blotting, in order to verify the size of the transcript target and confirm the specificity of the probe binding. Immunofluorescent histochemistry was then employed to investigate the corresponding protein expression. Sections were probed with a DAO antibody in combination with either a tyrosine hydroxylase antibody (TH; a marker of dopaminergic neurons) or a glial fibrillary acidic protein antibody (GFAP; an established marker of glia) in order to probe the potential expression of DAO in dopaminergic neurons and/or glia in the VTA.

The expression pattern of DAO mRNA and protein was also investigated in coronal and sagittal sections taken from the rat cerebellum, again using ISHH and immunofluorescent histochemistry. Sections were first labelled with both GFAP and calbindin, a marker of Purkinje cells, in order to define and understand the Purkinje cell/glial architecture in the region. Further sections were then incubated with DAO and GFAP antibodies, and inspected for potential colocalisation.

The focus then moved to the expression of DAO mRNA throughout development. ISHH using the probe created above was used to investigate the expression of DAO mRNA in coronal cerebellar rat brain sections from ages postnatal day (P) 7 (n=4), P21 (n=4) and P56 (n=4). The sections were opposed to photographic film before being inspected using a lightbox and camera, and the density of the mRNA signal in the Purkinje cell layer at different ages quantified and compared.

2.3 Methods

2.3.1 In situ hybridisation

DAO cDNA was amplified from rat cerebellar cDNA using forward and reverse primers (forward sequence: GTGATGCGCGTGGCCGTGAT; reverse sequence: GGAATACACCTCCGAGTGTA), purified and ligated into pGEM-T Easy Vector. Plasmids were transformed into *E. Coli*, grown up overnight and positive colonies selected using blue-white reagent. Plasmids were extracted and purified using ChargeSwitch®-Pro Plasmid Miniprep Kit and eluted with 50µl elution buffer. 10µg plasmid was then digested with either SacII or PstI to linearise the construct.

To create the riboprobe, approximately 1µg of linearised plasmid was dried down with [^{35}S]UTP and then incubated with NTPs, RNAsin, reaction buffer, dithiothreitol (DTT) and either SP6 or T7 RNA polymerase to transcribe the SacII and PstI linearised constructs, producing sense or antisense sequences, respectively. The plasmid DNA template was then removed using DNase. The probe was hydrolysed by adding hydrolysis buffer and tRNA and incubating at 60C for 34 minutes. The hydrolysed probe was purified using NICK columns (GE Healthcare) and activity measured using a scintillation counter.

Sections (14 microns) were taken from the adult male rat VTA and cerebellum, and from the male rat cerebellum at ages P7, P21 and P56 (n=4 for each). Sections were taken across the whole structure in each case and were stored at -80°C. Six sections were selected from each structure in each animal, covering both dorsal and ventral regions. These sections were allowed to thaw for 15 minutes and then fixed in 4% formaldehyde (in diethylpyrocarbonate [DEPC]-treated PBS) before being treated with DEPC-treated triethanolamine containing 0.25% acetic anhydride. They were then dehydrated in graded ethanols before soaking in chloroform to remove lipids, then rehydrated to 95% ethanol and air-dried.

The probe was diluted to 1.2 x 10^4cpm/µl in hybridisation buffer containing DTT and 200µl added to each section. Sections were coverslipped and incubated overnight at 45˚C in trays prepared with filter paper soaked in 4x saline-sodium citrate buffer (SSC) containing 50% formamide. Sections were rinsed twice in 2x SSC at room temperature before sequential stringency washes were carried out to remove adventitiously bound probe: RNase A buffer, 30 minutes at room temperature; 2x SSC, 10 minutes at 55˚C; 0.5x SSC, 10 minutes at 55˚C; 0.1x SSC, 3 x 20 minutes at 55˚C; 0.1x SSC, 45 minutes at room temperature. Sections were rinsed in dH_20 and opposed to photographic film (Kodak) at room temperature for 24–72 hours.

2.3.1.1 Emulsion dipping

In order to assess the cellular localisation of the riboprobe binding, sections taken from the adult cerebellum and VTA were then dipped in autoradiography emulsion (Amersham, UK) and left to incubate for 2 months at 4˚C. This was carried out by Mary Walker. Sections were then developed and counterstained using cresyl violet.

2.3.1.2 Quantification of mRNA expression

Films were magnified and inspected using a lightbox and camera. MCID Elite software was used to measure the density of the DAO mRNA signal in cerebellar sections at ages P7, P21 and P56 by drawing multiple small boxes over the Purkinje cell layer and measuring the average signal density therein. Grey density values were converted to nCi/mg tissue using commercial ^{14}C microscales. Statistical analysis was carried out with SPSS, using a univariate ANOVA followed by Bonferroni post-hoc testing, to compare signal density at different ages.

2.3.2 Northern blotting

Total RNA was isolated from adult rat cerebellum (n=2) using Tri®-Reagent (Sigma T9424), suspended in 50µl nuclease-free water and quantified using a Nanodrop

spectrophotometer. From this, poly(A)-rich mRNA was extracted using GenElute mRNA Miniprep kit (MRN10-1KT) and eluted in 100µl elution buffer before again being quantified using spectrophotometry.

A 1.5% agarose northern gel was prepared in 1x MESA (MOPS-EDTA-sodium acetate buffer, Sigma M-5755) containing formaldehyde, and was placed in a gel tank treated with RNaseZap (Sigma R-2020), containing 1x MESA. 20-30µg total RNA or 2-3µg mRNA was mixed with a denaturing solution (containing glycerol loading buffer, formaldehyde and formamide in 1x MESA), heated to 65˚C for 5 minutes and quenched on ice. Samples were loaded onto the gel in duplicate and ethidium bromide added to one of each. The gel was run at 120V for 2 hours before being visualised under UV light. Overnight capillary transfer was then carried out onto Hybond™-N+ nylon transfer membrane (Amersham Pharmacia Biotech, RPN119B) using 40mM NaOH.

The DAO cDNA sequence was amplified via PCR of plasmids containing a DAO insert (see previous ISHH protocol) using forward and reverse primers (forward sequence: GTGATGCGCGTGGCCGTGAT; reverse sequence: GGAATACACCTCCGAGTGTA) and purified using a QIAquick PCR Purification Kit. The DAO cDNA was incubated for 2 hours at 37˚C in the presence of Klenow DNA polymerase, [^{32}P]dATP, dNTPs, random hexamers and polymerase buffer in order to produce a ^{32}P-labelled DAO cDNA northern probe. The probe was purified using NICK columns (GE Healthcare).

A hybridisation buffer was prepared containing formamide, 20x SSC, Denhardt's solution, ssDNA (10mg/ml) and 10% SDS, and incubated with the membrane at 42˚C for 1.5–2 hours (with rotation). The purified probe was heated to 90˚C for 5 minutes, quenched on ice and mixed with further hybridisation buffer, before being incubated overnight (approx. 18 hours) with the membrane at 42˚C.

The membrane was rinsed twice in 2x SSC + 0.1% SDS before sequential stringency washes were carried out to remove adventitiously bound probe (2x SSC + 0.1% SDS for 15 minutes at 60˚C, followed by 1x SSC + 0.1% SDS for 30 minutes at 60˚C, and finally 0.2% SSC + 0.1% SDS for 1 hour at 60˚C). The membrane was placed against MS film (Kodak) and exposed overnight at -70˚C.

2.3.3 Immunofluorescence – VTA colocalisation

A rabbit polyclonal antibody against a C-terminal human DAO sequence (peptide H-CGRILEEKKLSRMPPSHL-OH; N-terminal Cys added for coupling, GlaxoSmithKline) was used to carry out immunofluorescence studies. This antibody had been used previously by Verrall and colleagues (2007) and has been verified to detect DAO, including in rat cerebellar tissue, using Western blotting (Verrall *et al*., 2007).

VTA sections were taken from adult rat brain (perfused with paraformaldehyde and cryoprotected in sucrose solution) in the coronal plane using a cryostat and left free-floating. Sections were washed in PBS, then incubated in 50mM ammonium chloride for 10 minutes. Further washing was carried out once in PBS, and twice in PBS containing Triton X-100 at 0.2% (PBSX). Following blocking of non-specific sites for 1 hour in 6% normal donkey serum in PBSX, the sections were incubated overnight at 4˚C in rabbit primary antibody to DAO at 1:500 in 2% normal donkey serum in PBSX, and either mouse primary anti-TH antibody (Sigma T-2928) at 1:1000 or goat primary anti-GFAP antibody (Abcam ab53554) at 1:2000.

Following washes in PBS, sections were soaked for 1 hour in secondary donkey anti-rabbit IgG at 1:1000 (Alexa Fluor® 488, A-21206, Invitrogen), and either goat anti-mouse IgG at 1:1000 (Alexa Fluor® 594, A-11005, Invitrogen) or donkey anti-goat IgG at 1:1000 (Cy3, 705-166-147, Jackson Immunoresearch). Sections were then washed further, once in PBSX, once in PBS and once in PB (saline), and mounted onto slides using coverslips

and Vectashield mounting fluid. Sections were viewed and photographed under a microscope (Axio Imager, Zeiss, with Axioscope software).

Control experiments were carried out concurrently following the above method, but without the addition of the primary antibody.

2.3.4 Immunofluorescence - cerebellum

Cerebellar sections were taken from adult rat brain (perfused with paraformaldehyde and cryoprotected in sucrose solution) using a sledge microtome and left free-floating. Sections were washed in ice-cold PBS, followed by 50mM ammonium chloride for 10 minutes and further washing in PBS. Cells were permeabilised by soaking for 1 hour in 0.5% Triton X-100 in PBS at 4˚C and further washes in PBS carried out. Following blocking of non-specific sites for 1 hour in 5% normal goat serum and 0.5% Triton X-100 in PBS at 4˚C, the sections were incubated overnight at 4˚C in primary DAO antibody at 1:500. Following washes in PBS, sections were soaked for 1 hour in secondary goat anti-rabbit IgG at 1:100 (Alexa Fluor 568, Invitrogen), washed further and mounted onto slides using coverslips and Vectashield mounting fluid. Control experiments were carried out concurrently without the addition of the primary antibody.

2.3.5 Immunofluorescence – cerebellar colocalisation

This work was carried out some 18 months later than the original immunofluorescence studies in the cerebellum, and some modifications were made to the protocol after re-optimisation.

Cerebellar sections were taken from adult rat brain (perfused with paraformaldehyde and cryoprotected in sucrose solution) in the sagittal plane using a cryostat and left free-floating. Sections were washed in PBS, then incubated in 50mM ammonium chloride for 10 minutes. Further washing was carried out once in PBS, and twice in PBS containing Triton X-100 at 0.2% (PBSX). Following blocking of non-specific sites for 1 hour in 6%

normal donkey serum in PBSX, the sections were incubated overnight at 4˚C in goat primary antibody to GFAP at 1:2000 in 2% normal donkey serum in PBSX, and either rabbit primary anti-DAO antibody at 1:500 (as described previously) or rabbit primary anti-calbindin antibody (AB1778, Chemicon) at 1:500.

Following washes in PBS, sections were soaked for 1 hour in secondary donkey anti-goat IgG at 1:1000 (Cy3, 705-166-147, Jackson Immunoresearch) and donkey anti-rabbit IgG at 1:1000 (Alexa Fluor® 488, A-21206, Invitrogen). Sections were then washed further, once in PBSX, once in PBS and once in PB (saline), and mounted onto slides using coverslips and Vectashield mounting fluid. Sections were viewed and photographed under a microscope (Axio Imager, Zeiss, with Axioscope software). Control experiments were carried out concurrently without the addition of the primary antibody.

2.4 Results

2.4.1 ISHH probe verification – northern blotting

A single band was detected at approximately 2kb following northern blot analysis of poly(A)-rich mRNA incubated with the ISHH riboprobe (Figure 5). The DAO transcript is approximately 2kb (Momoi *et al*., 1988); as such, the transcript target was of the expected size, and the probe considered to be verified.

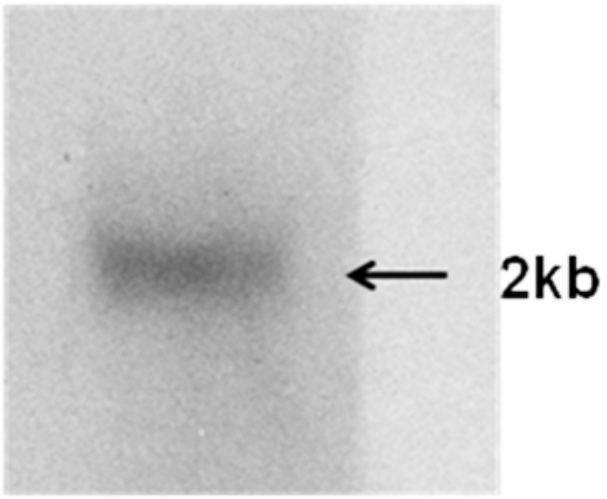


Figure 5. Northern blot analysis of poly(A)-rich mRNA extracted from rat cerebellum. *A single band was detected at around 2kb (arrow).*

2.4.2 Expression of DAO in the adult VTA

Incubation of sections from the adult rat VTA with a DAO T7 antisense riboprobe and subsequent exposure to photographic emulsion revealed a pattern of clustered granules over putative neurons throughout the VTA (Figure 6). This suggested that DAO mRNA was present in the adult rat VTA, including in putative neurons. No specific region of the VTA showed more expression of the mRNA than others. Incubation with a DAO SP6 sense probe resulted in the presence of very few granules, demonstrating minimal non-specific binding (Figure 6, inset).

When corresponding DAO protein expression was investigated, immunoreactivity could clearly be seen in putative neurons across the VTA (Figure 7a, Figure 8a), although the cell nuclei appeared not to be stained. Some of these neurons were also TH immunopositive (Figure 7b, Figure 8b), suggesting that they were dopaminergic. However, not all DAO-positive neurons were TH-positive, and similarly, not all TH-positive neurons were DAO-positive (Figure 7c, Figure 8c). Taken together, these findings suggested that DAO was expressed in, but not confined to, TH-positive neurons in the adult rat VTA.

When VTA sections were incubated with both a DAO antibody and a GFAP antibody, the two signals were shown to colocalise (Figure 9, Figure 10). This suggested that DAO was also expressed in astrocytes in the adult rat VTA, and was not confined solely to neurons.

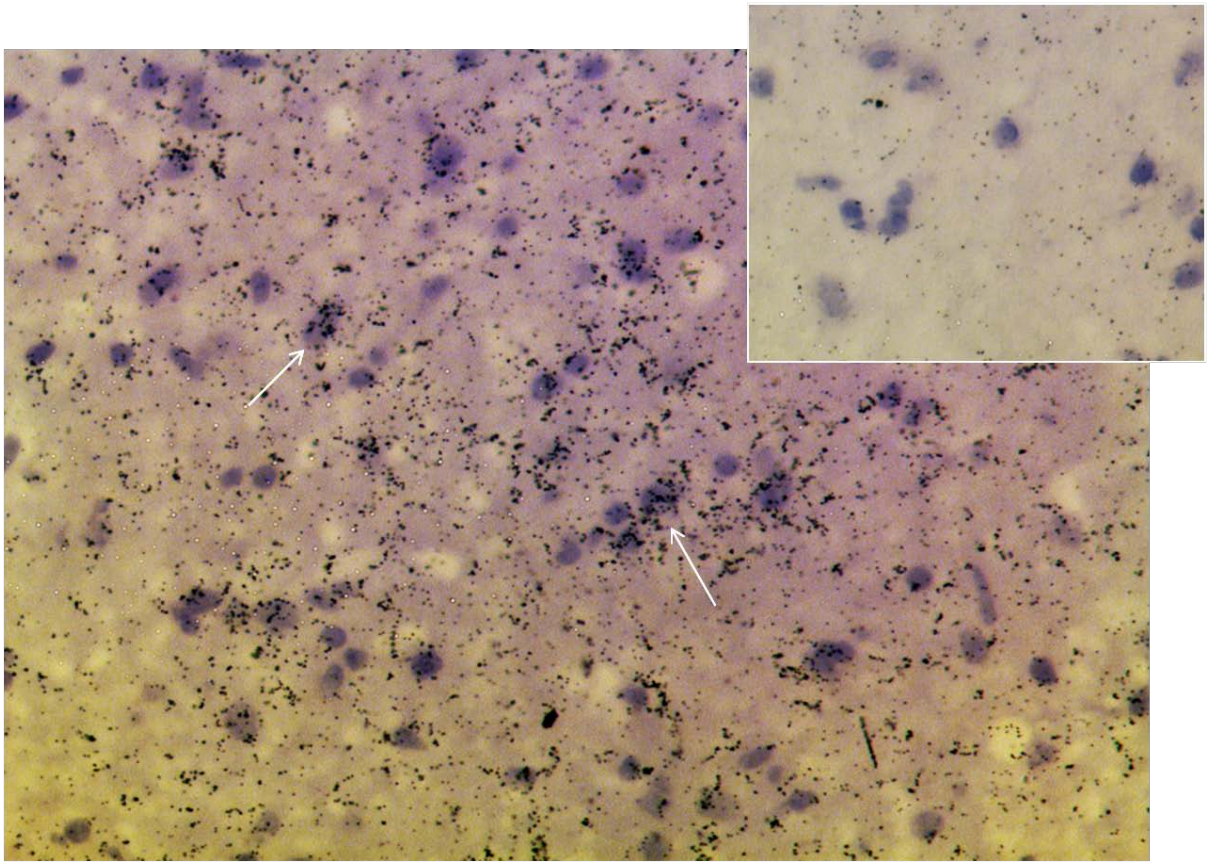


Figure 6. DAO mRNA expression in the adult rat VTA, detected using ISHH with a T7 antisense riboprobe, and a corresponding SP6 sense riboprobe (inset). *Magnification 20x.Granule clusters can be seen over putative neurons with the T7 probe. Arrows indicate examples of clustering. Despite some background signal, there is no clear clustering with the SP6 sense probe, demonstrating little non-specific binding of the riboprobes.*

.

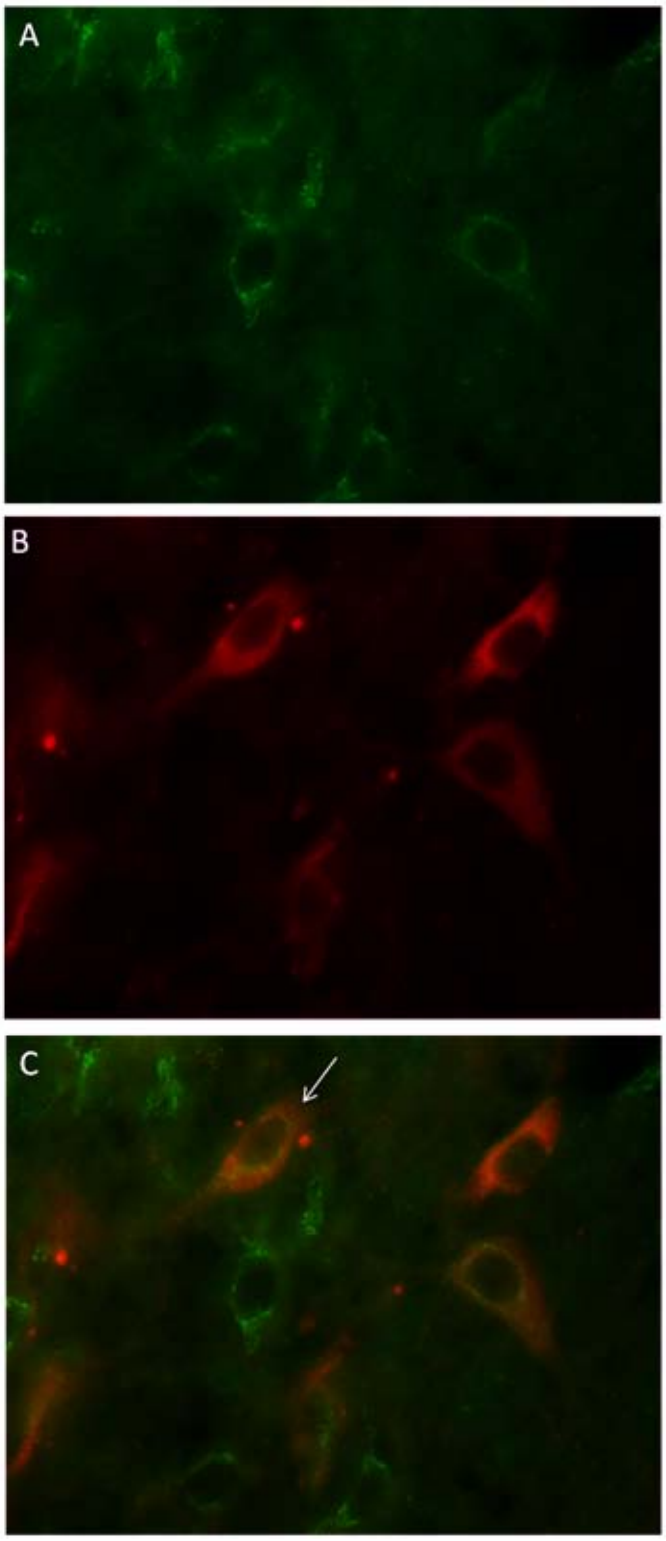


Figure 7. Expression of DAO (green, panel A), TH (red, panel B) and the two combined (panel C) in a coronal section taken from the adult rat VTA. *Magnification 40x. Arrow indicates an example of a putative neuron clearly expressing both DAO and TH.*

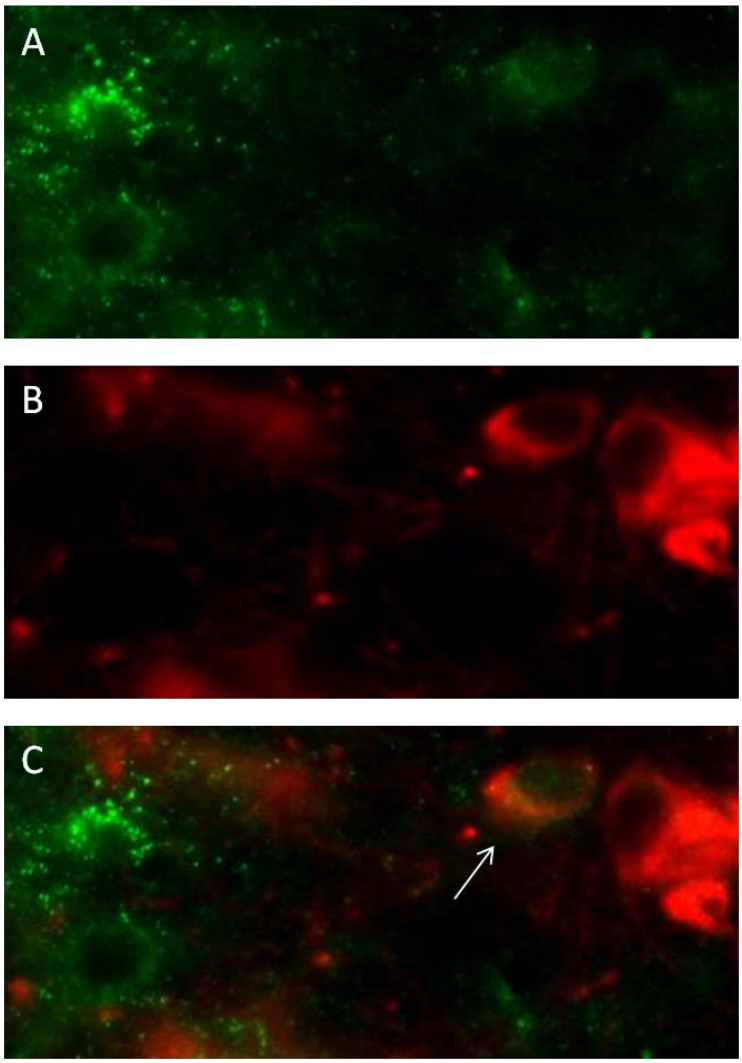


Figure 8. Expression of DAO (green, panel A), TH (red, panel B) and the two combined (panel C) in a coronal section taken from the adult rat VTA. *Magnification 40x. Arrow indicates an example of a putative neuron clearly expressing both DAO and TH. Examples of DAO-positive neurons not expressing TH (left of picture), and TH-positive neurons not expressing DAO (right of picture), are also clearly visible.*

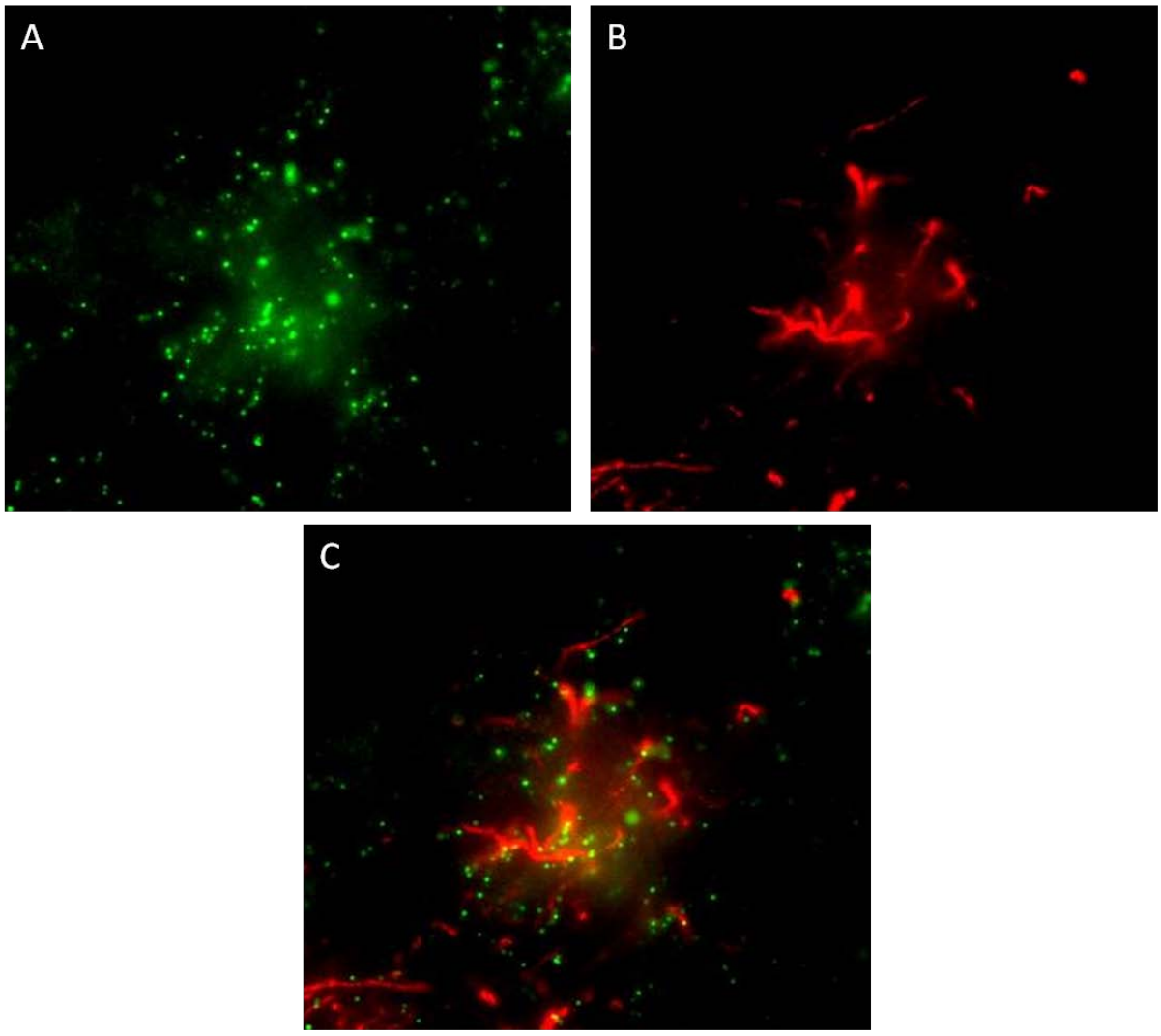


Figure 9. Expression of DAO (green, panel A), GFAP (red, panel B) and the two combined (panel C) in a putative astrocyte in a coronal section taken from the adult rat VTA. *Magnification 63x. Clear colocalisation is visible.*

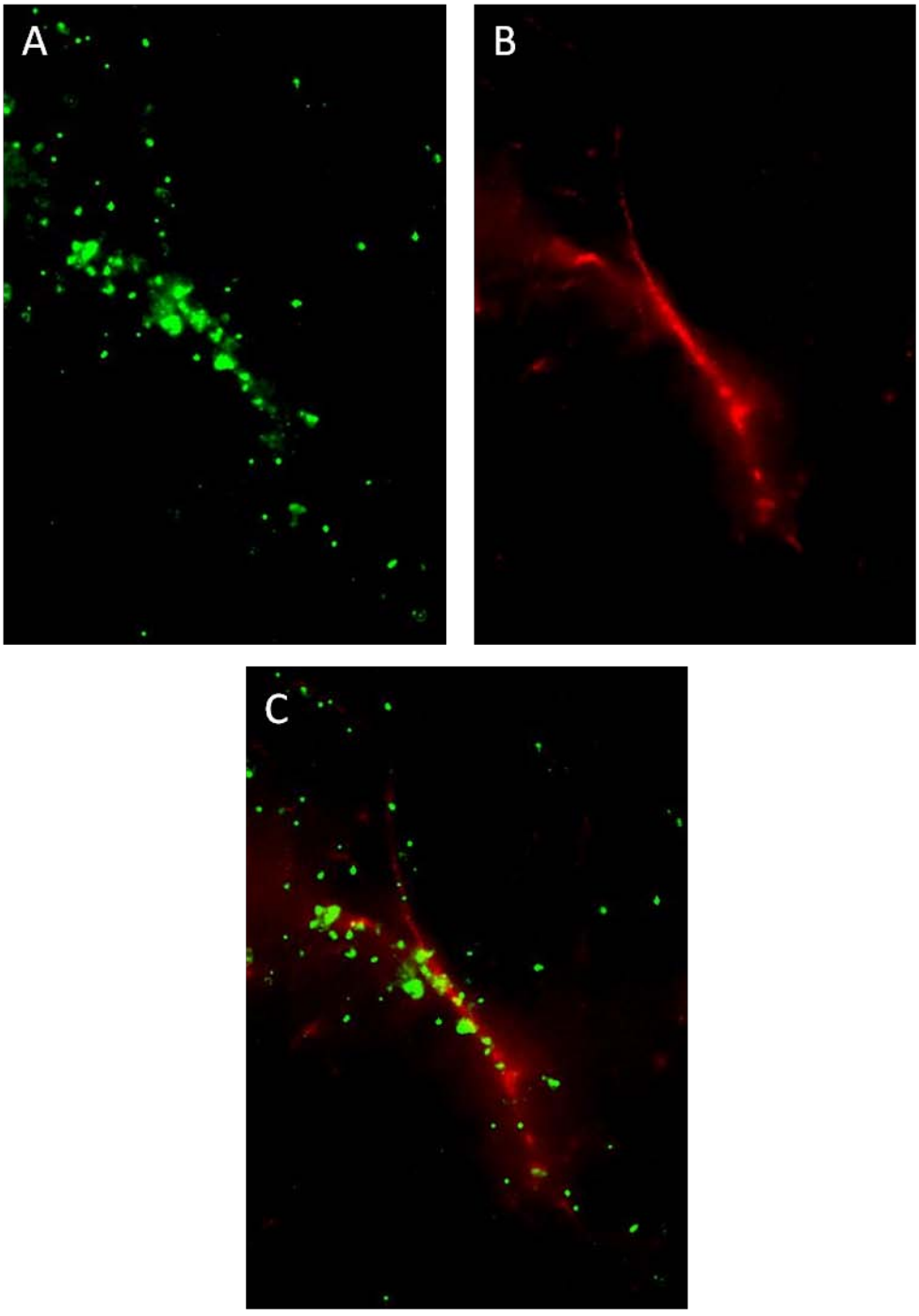


Figure 10. Expression of DAO (green, panel A), GFAP (red, panel B) and the two combined (panel C) in a putative astrocyte in a coronal section taken from the adult rat VTA. *Magnification 63x. Clear colocalisation is visible.*

2.4.3 Expression of DAO in the adult cerebellum

Upon inspection of coronal cerebellar sections incubated with an ISHH T7 antisense riboprobe for DAO and dipped in photographic emulsion, clusters of granules could be seen in the Purkinje cell layer (Figure 11). This suggested that DAO mRNA expression was concentrated in the Purkinje cell layer of the cerebellum. Incubation of the sections with an SP6 sense riboprobe revealed minimal non-specific binding (Figure 12).

When corresponding DAO protein expression was investigated using a primary C-terminal DAO antibody and secondary fluorescence, punctate staining was seen around the periphery of putative Purkinje cells in the coronal plane, but again not over the cell bodies (Figure 13). No punctate staining was seen when sections were incubated with secondary fluorescent antibody, in the absence of the DAO antibody (Figure 14).

Staining of cerebellar sections in the sagittal plane with calbindin and GFAP antibodies (markers of Purkinje cells and glia, respectively) revealed clear enveloping of Purkinje cells with glial processes (Figure 15, inset), which then extended in a linear and parallel fashion into the molecular layer. When the DAO expression was investigated in sections taken in the sagittal plane, punctate staining was again visible around the periphery of the Purkinje cells (but not over the cell bodies), and also in linear processes projecting into the molecular layer (Figure 16). Staining with GFAP and DAO antibodies then showed colocalisation of DAO and GFAP expression round the periphery of Purkinje cells in the same location as the glial ‘envelopes’ [Figure 17 (inset), Figure 18], and also along apparent linear glial processes in the molecular layer (Figure 17).

Together, these results suggest that DAO is not expressed in the cell bodies of Purkinje cells; rather, that expression occurs in glia surrounding the Purkinje cells, and extends into glial processes in the molecular layer.

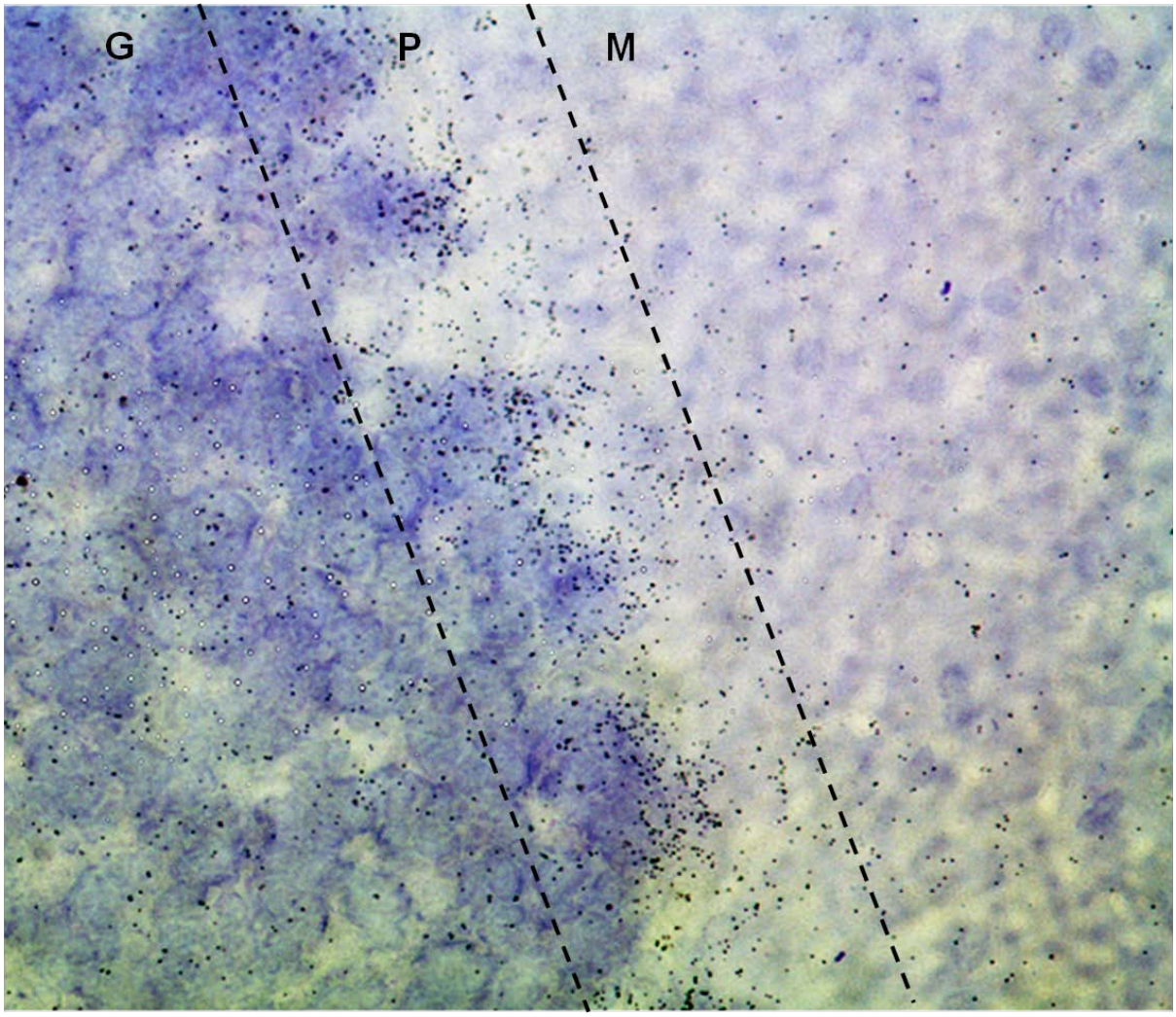


Figure 11. DAO mRNA expression in the adult rat cerebellum, detected using ISHH. *A T7 antisense riboprobe was used to probe the section. Magnification 20x. Granule clusters are concentrated in the Purkinje cell layer (P), with little clustering in the molecular (M) and granule cell (G) layers.*

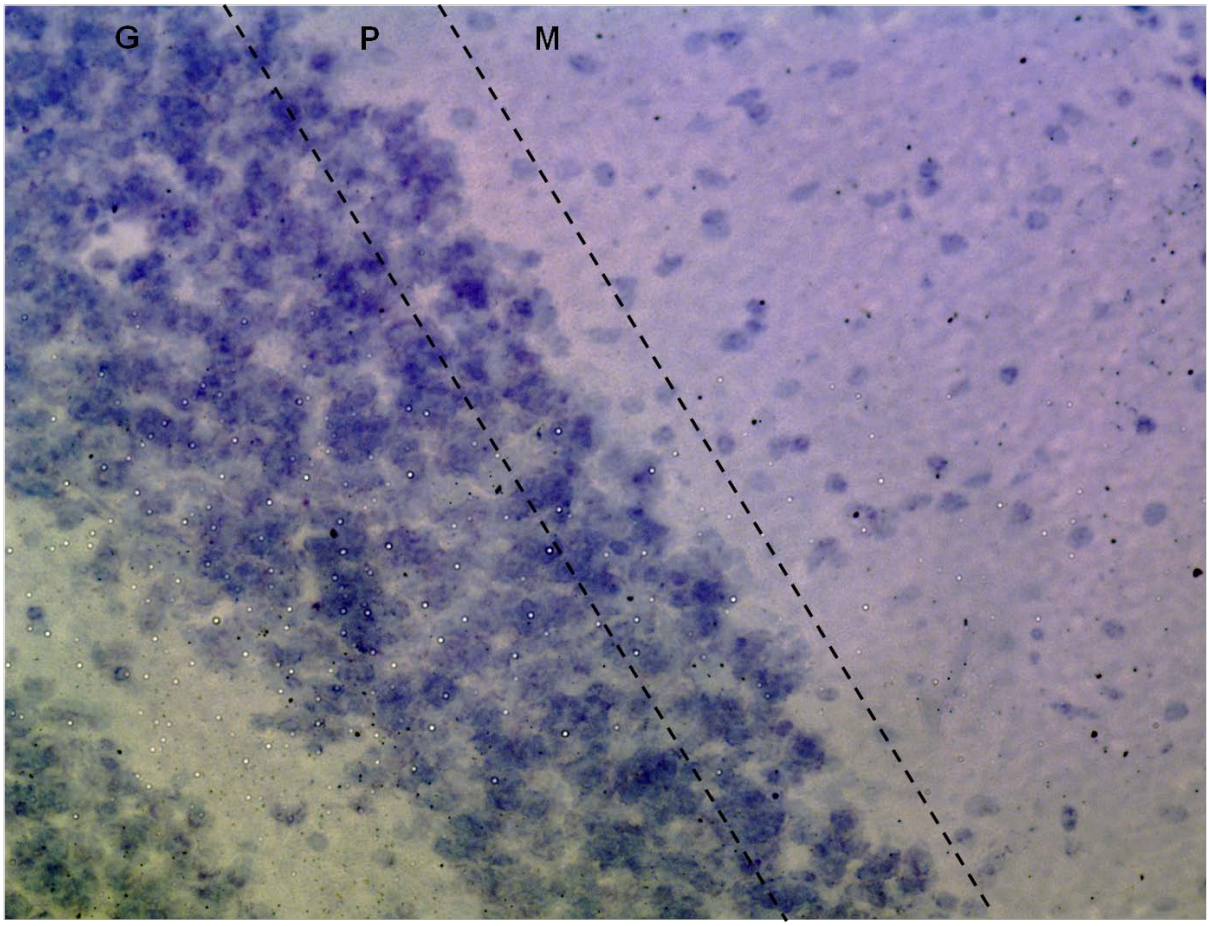


Figure 12. A section taken from the adult rat cerebellum, incubated with an SP6 sense ISHH riboprobe. *Magnification 20x. No clear granule clusters are visible, demonstrating very little non-specific binding of the riboprobes. G=granule cell layer, M=molecular layer, P=Purkinje cell layer.*

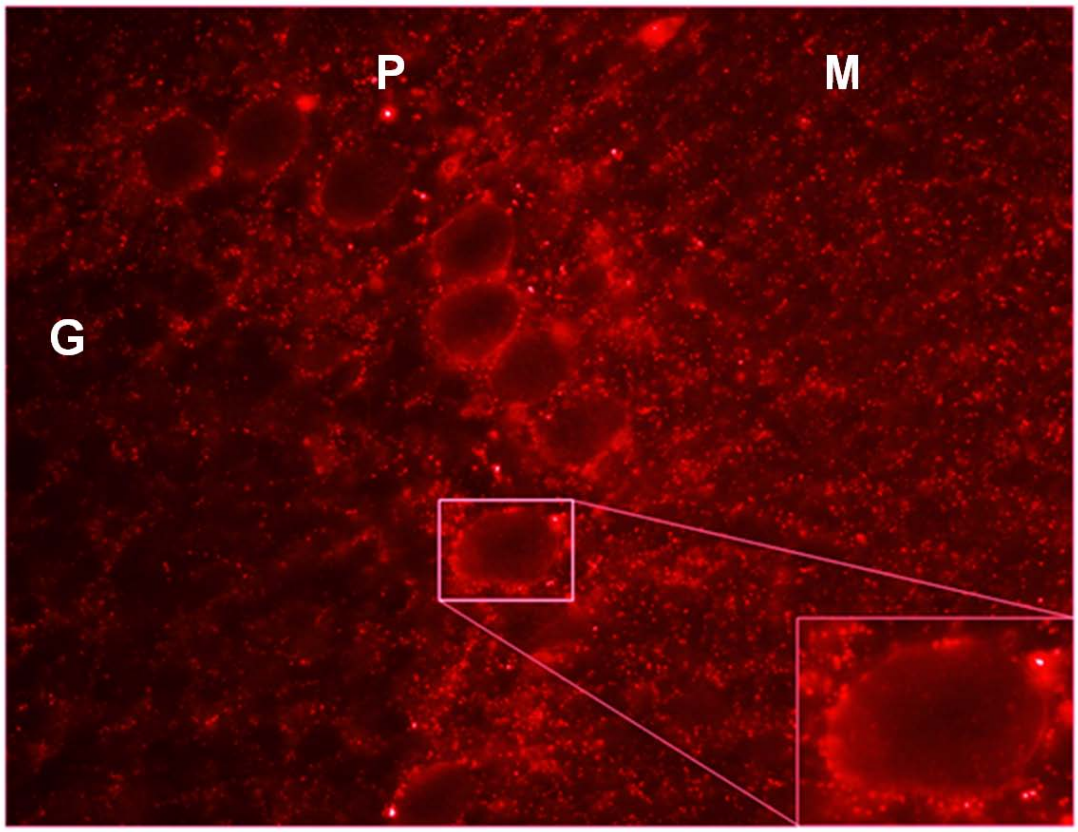


Figure 13. DAO expression in a coronal section from the adult rat cerebellum, detected using a primary C-terminal DAO antibody and secondary fluorescence. *Magnification 40x. Punctate staining is seen around the periphery of Purkinje cells (see inset), but not over Purkinje cells themselves. G=granule cell layer, M=molecular layer, P=Purkinje cell layer.*

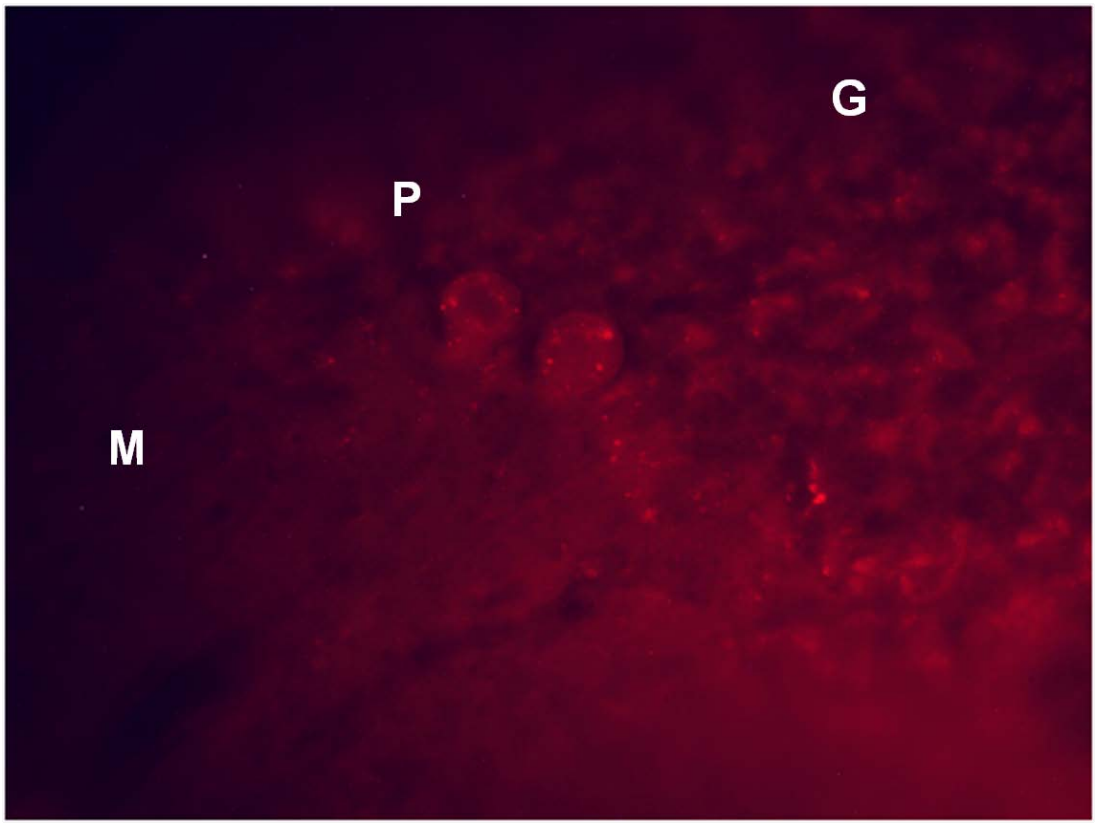


Figure 14. A coronal section from the adult rat cerebellum, incubated with a secondary fluorescent antibody, but in the absence of the primary DAO C-terminal antibody. *Magnification 40x. No punctate staining is seen. G=granule cell layer, M=molecular layer, P=Purkinje cell layer.*

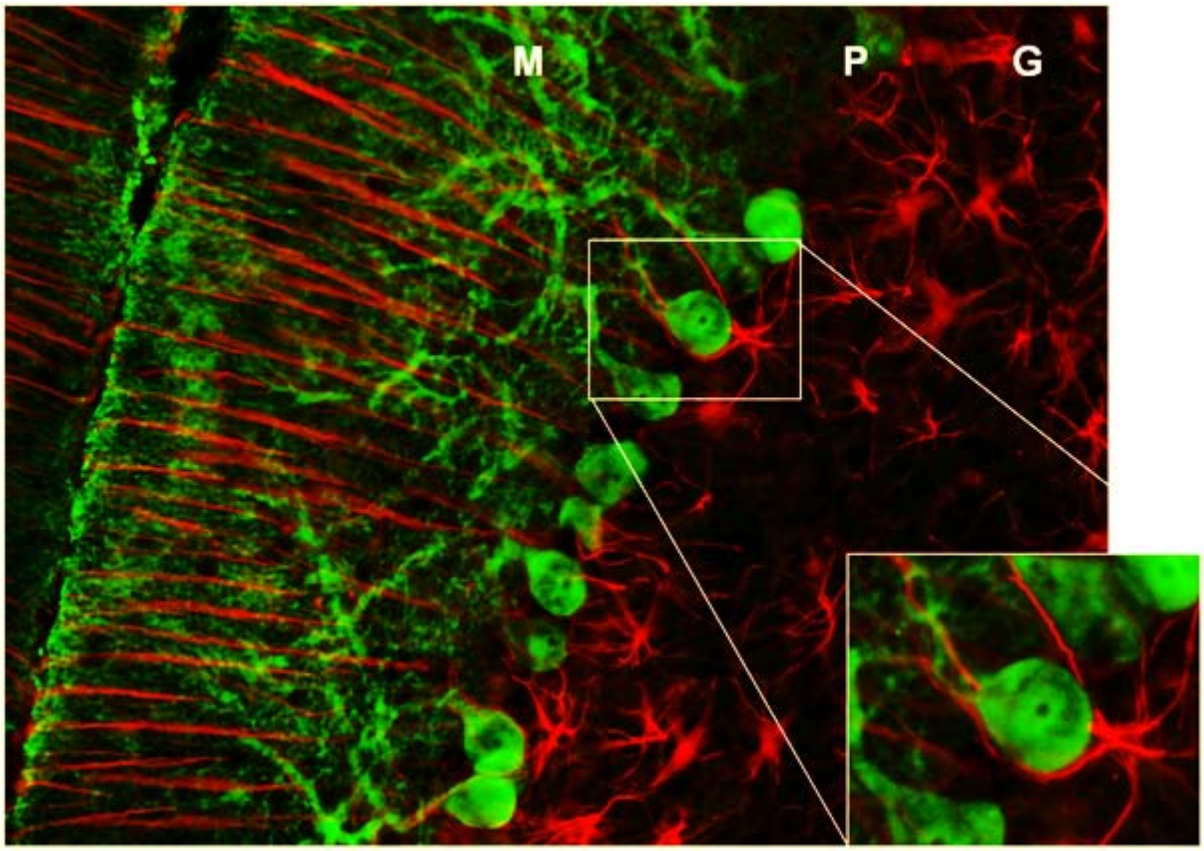


Figure 15. A sagittal section of adult rat cerebellum showing expression of calbindin (green) and GFAP (red), for reference. *Magnification 40x. Purkinje cells can be clearly seen (green), along with their extensive arborisations in the molecular layer (M). GFAP labelling shows some clear enveloping of Purkinje cells by glial processes (inset). These processes are then seen to continue in a linear and parallel fashion through the molecular layer. G=granule cell layer, M=molecular layer, P=Purkinje cell layer.*

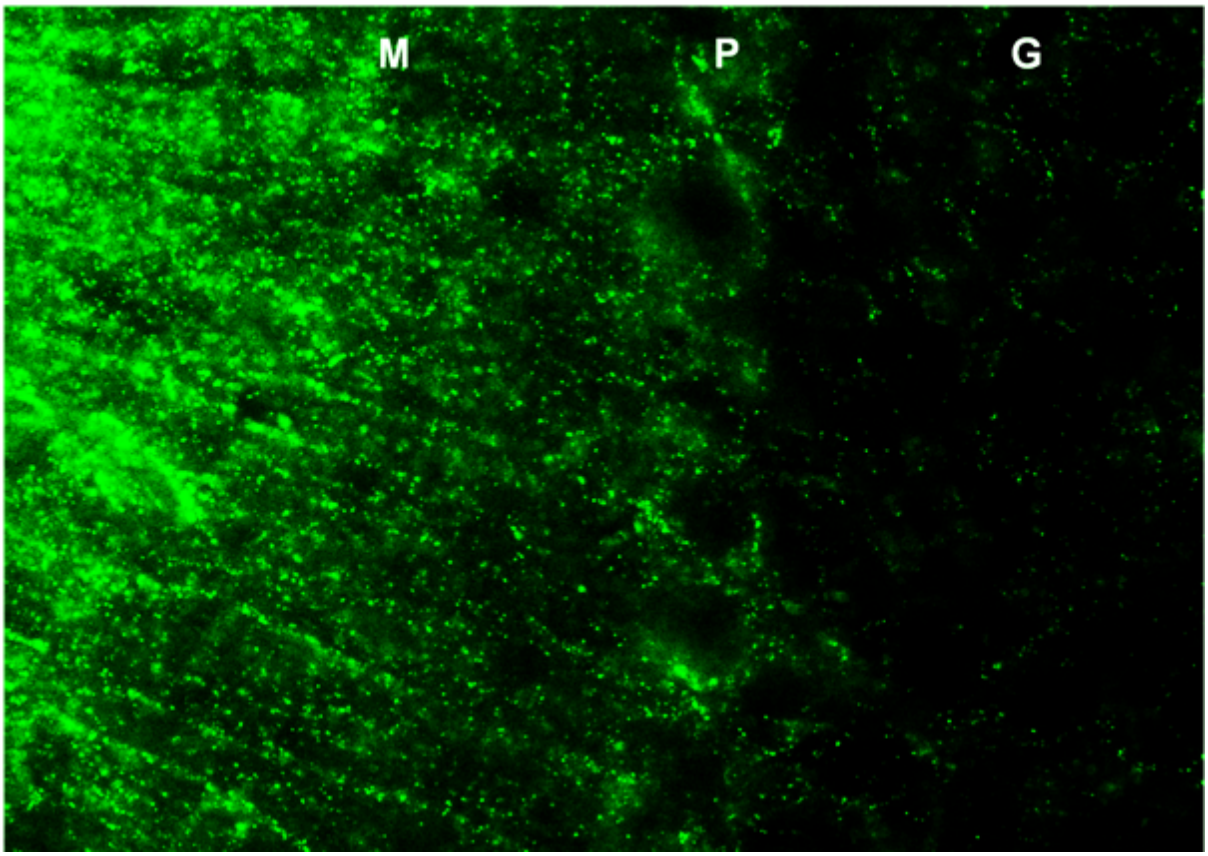


Figure 16. DAO expression in a sagittal section taken from the adult rat cerebellum. *Magnification 40x. Punctate staining is seen around the periphery of Purkinje cells, and along linear processes in the molecular layer, but not over Purkinje cells themselves. G=granule cell layer, M=molecular layer, P=Purkinje cell layer.*

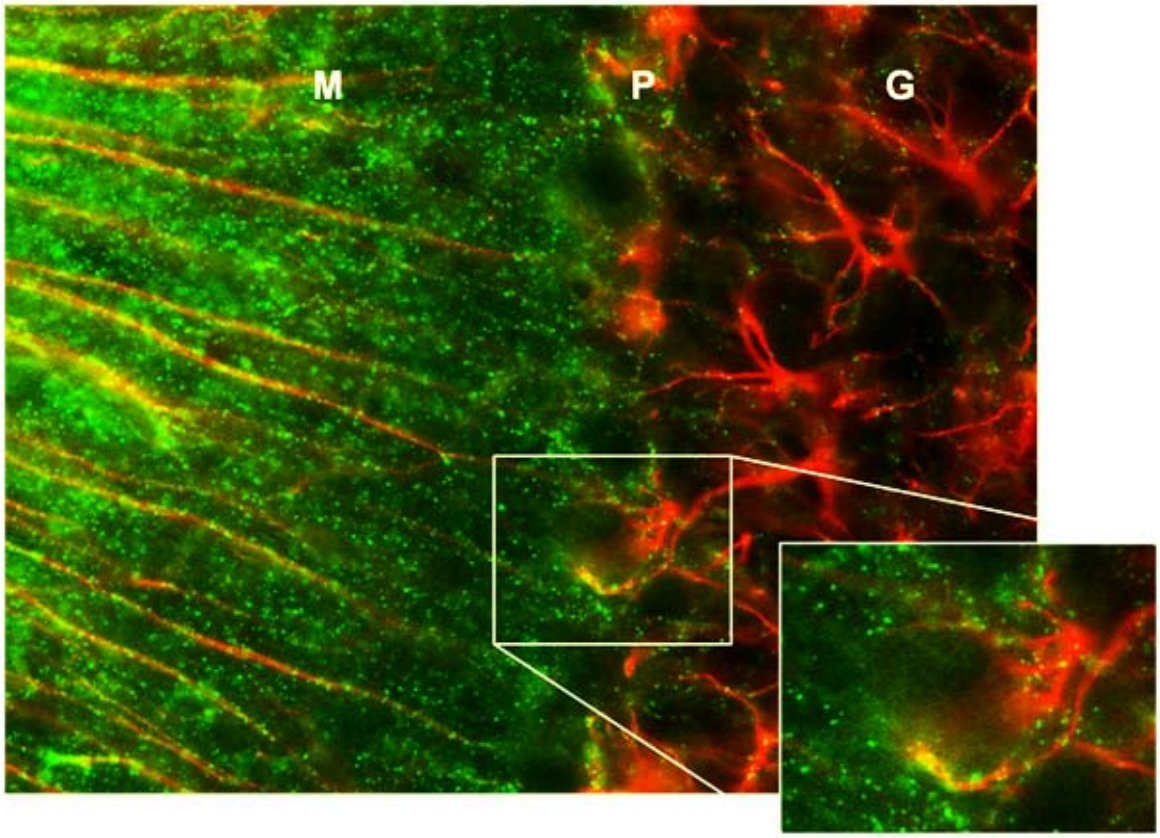


Figure 17. DAO (green) and GFAP (red) expression in a sagittal section of the adult rat cerebellum. *Magnification 40x. Punctate DAO staining is again seen around the periphery of putative Purkinje cells (which are unmarked), seemingly colocalising with GFAP expression (inset). DAO expression also appears visible along linear glial processes in the molecular layer (M). G=granule cell layer, M=molecular layer, P=Purkinje cell layer.*

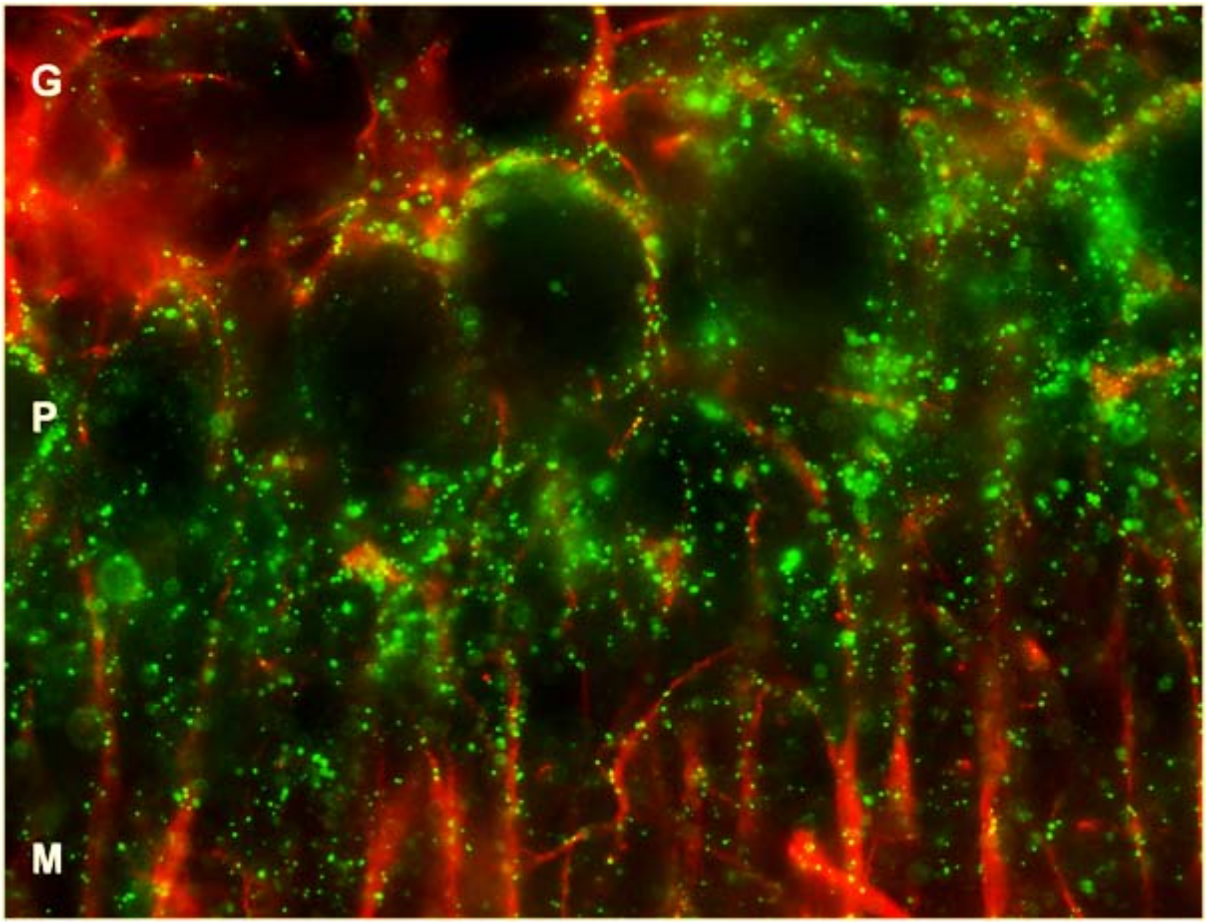


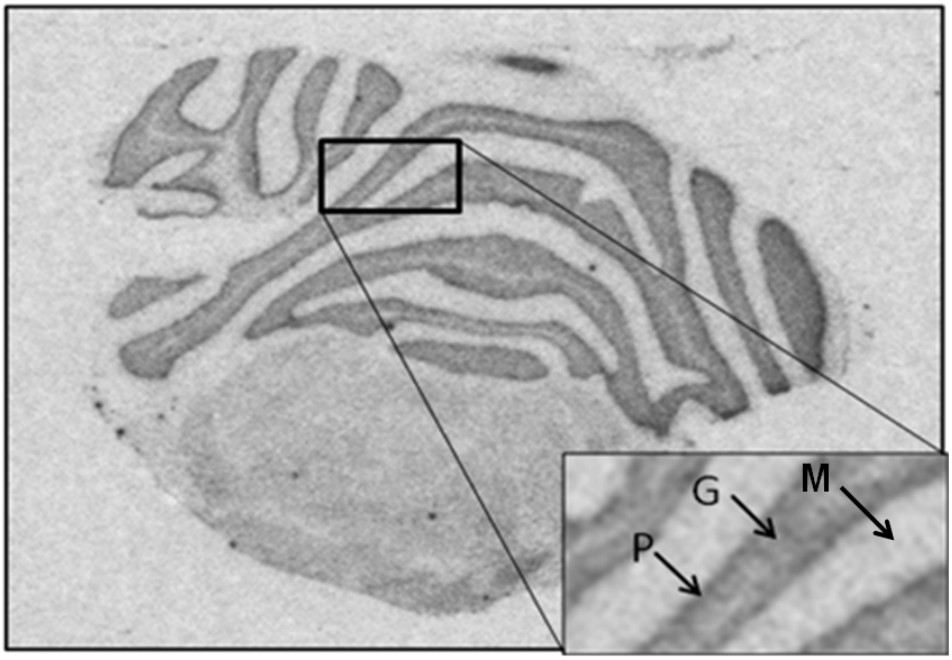
Figure 18. DAO (green) and GFAP (red) expression in a sagittal section taken from the adult rat cerebellum. *Magnification 63x. Punctate DAO staining is again seen around the periphery of Purkinje cells, seemingly colocalising with GFAP expression. G=granule cell layer, M=molecular layer, P=Purkinje cell layer.*

2.4.4 Expression of DAO in the developing cerebellum

Inspection of films incubated with ISHH-probed sections from the rat cerebellum at P56 revealed DAO mRNA expression in the granule cell layer, with particularly dense staining in the Purkinje cell layer, when the section was probed with an antisense T7 riboprobe (Figure 19a). No specific marking of DAO mRNA was visible in sections incubated with a sense SP6 riboprobe (Figure 19b).

Quantification of the ISHH signal at ages P7, P21 and P56 revealed a significant increase in DAO mRNA expression over time (Figure 20). There was a main effect of age on DAO mRNA signal in the cerebellum [$F(2,9)=7.457$, $p=0.012$]. Bonferroni post-hoc testing revealed a significant difference between DAO expression at P7 and P21 ($p=0.018$), and P7 and P56 ($p=0.042$), but not between P21 and P56 ($p=1.000$).

A)



B)

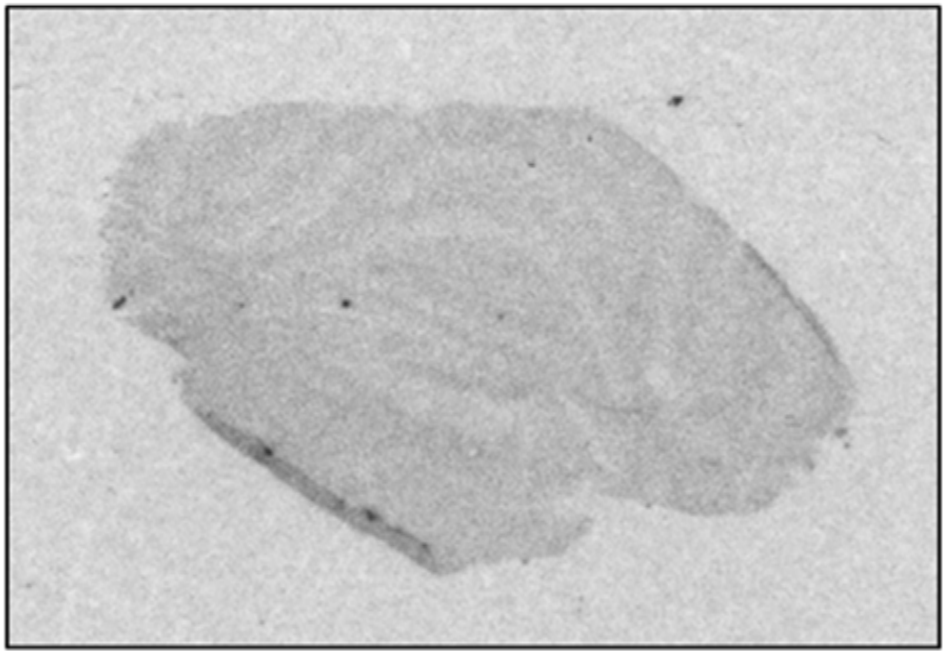


Figure 19. Example sections from the rat cerebellum at P56 probed with a T7 antisense ISHH probe (A) and an SP6 sense probe (B). *(A) DAO mRNA expression is seen in the granule cell layer, with particularly dense staining in the Purkinje cell layer. (B) No specific marking of DAO mRNA is visible. G=granule cell layer, M=molecular layer, P=Purkinje cell layer.*

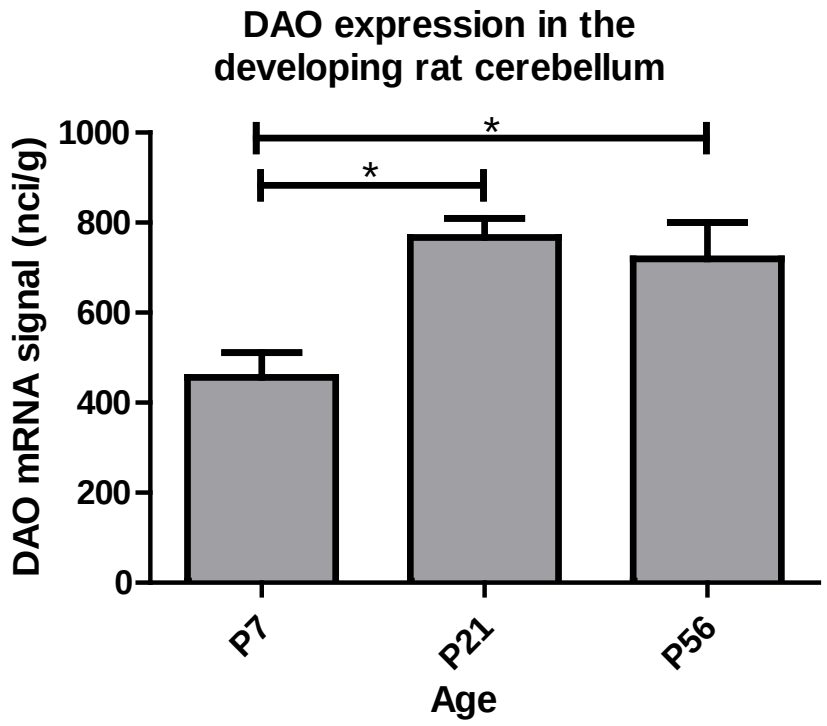


Figure 20. DAO expression in the Purkinje cell layer of the developing rat cerebellum, as measured by ISHH. *Plot shows mean with S.E.M., n=4 in each group. *p<0.05.*

2.5 Discussion

In this chapter, several key findings were demonstrated with regard to the expression of DAO in the rat brain at both the regional and cellular level. Firstly, DAO was expressed in the adult rat VTA, in both TH-positive and TH-negative neurons, as well as in astrocytes. Secondly, DAO was expressed predominantly in glia in the adult rat cerebellum, and appeared not to be expressed in Purkinje cells, but rather in the glial processes enveloping the Purkinje cell bodies and extending into the molecular layer. Finally, the expression of DAO mRNA changed during development of the rat cerebellum, increasing with time and inversely correlating with the levels of D-serine previously recorded (Schell *et al*., 1997).

In the first study of this chapter, the presence of both DAO mRNA and protein were demonstrated in the adult rat VTA, using ISHH and immunofluorescence, respectively. DAO protein was shown in three cell types in the VTA; TH-positive neurons, TH-negative neurons, and astrocytes. This is the first known study to date to confirm the expression of DAO in the rat VTA, and to explore its cellular localisation therein. These results are physiologically interesting in the context of the VTA as the source of the mesocortical and mesolimbic DA pathways in the brain, and the potential association with schizophrenia. Dopaminergic output from the VTA is regulated by NMDA receptors (Bennett and Gronier, 2007), and as such, it is conceivable that D-serine, and its regulation by DAO, plays a pivotal role in the modulation of these VTA receptors, culminating in the modulation of mesocortical and mesolimbic DA function. DAO expression and activity has been reported to be increased in some (though not all) brain regions in schizophrenia (Kapoor *et al*., 2006; Verrall *et al*., 2007; Burnet *et al*., 2008; Madeira *et al*., 2008), and D-serine levels are reduced in serum and cerebrospinal fluid (Hashimoto *et al*., 2003; Hashimoto *et al*., 2005b). Although DAO activity in the VTA has yet to be directly demonstrated, and any activity changes in schizophrenia in the region not yet investigated, it is conceivable that

insufficient activation of VTA NMDA receptors as a result of increase DAO expression and reduced D-serine levels may contribute to the mesocortical and mesolimbic DA dysfunctions seen in the disorder. As such, the expression of DAO in the VTA may provide a potential link between DAO activity, NMDA receptor hypofunction and DA system dysregulation in schizophrenia.

DAO immunoreactivity was demonstrated in both neurons and glia in the VTA in this chapter. This is one of few studies to report the expression of DAO in neurons as well as glia, and provides support for the notion that DAO is not an exclusively glial enzyme. However, it may not necessarily be considered surprising that DAO is also expressed in neurons, given that Asc-1, the major D-serine transporter, is expressed predominantly in neurons (Helboe *et al*., 2003), whereas Asc-2, another D-serine transporter, has been reported to be expressed in both neurons and glia (Dolinska *et al*., 2004; Gliddon *et al*., 2009). It is conceivable that both transporters are active in the uptake of D-serine in the VTA, and that DAO is present and active in both neurons and glia, serving to metabolise D-serine in either compartment.

However, the current studies of DAO expression in the adult rat cerebellum, both in terms of mRNA and protein, consistently revealed DAO signal in glia, but not in neurons. This is in accordance with numerous other studies, and was first reported 35 years ago (Gaunt and de Duve, 1976). DAO positivity has since been repeatedly reported in glia in the cerebellum, which have largely been assigned to be putative Bergmann glia (Weimar and Neims, 1977b; Weimar and Neims, 1977a; Horiike *et al*., 1987; Verrall *et al*., 2007; Ono *et al*., 2009; Moreno *et al*., 1999). This has been based on their location surrounding Purkinje cells, since Bergmann glia have their cell bodies in the Purkinje cell layer. They also feature radial processes which extend into the molecular layer, and indeed, DAO signal has been repeatedly reported in ascending parallel processes of putative Bergmann glia in the molecular layer (Weimar and Neims, 1977b; Horiike *et al*., 1987;

Verrall *et al*., 2007; Moreno *et al*., 1999). These studies have used a range of techniques, including ISHH, immunohistochemistry and coupled peroxidation, and all seem to point to the same conclusion; that DAO is glial in the cerebellum, which the current findings support. The only known study with which these results do conflict is by Moreno and colleagues (1999), who reported immunoreactivity in Golgi and Purkinje cells of the rat cerebellum (Moreno *et al*., 1999). They did, however, also describe 'intense positivity' in Bergmann glia, and positively stained Bergmann glial processes in the molecular layer. The antibody used in this study has since come into question, as it has subsequently been suggested to cross-react with D-aspartate oxidase (Shleper *et al*., 2005), which is expressed in Purkinje cells (Zaar *et al*., 2002). Given the striking number of other studies using different techniques all showing expression in Bergmann glia but not Purkinje cells, this seems a viable explanation for the contrasting results reported in the Moreno paper. Laying this study aside, therefore, the data collected in this chapter support previous findings that DAO is expressed in glia in the cerebellum, and not in Purkinje cells. However, it must be noted that none of the imaging performed in this chapter used a confocal microscope, and so whereas signals of immunoreactivity may appear to colocalise, this has not been unequivocally proven. Furthermore, although one can hypothesise that the expression is in Bergmann glia, based on the location of the signal, this again has not been definitively shown.

In the final study of this chapter, levels of DAO mRNA expression in the cerebellum increased from P7–P56, with adult levels being reached by P21 (Figure 20). This increase is consistent with existing data on the concentrations of D-serine present in the rat cerebellum; there is a decrease in D-serine over time from P12 onwards, reaching undetectable levels by P26 (Schell *et al*., 1997). Similarly in the mouse cerebellum, no DAO activity was detected until P12, after which it increased dramatically, reaching adult levels at P49 (Wang and Zhu, 2003). Conversely, high levels of D-serine were observed

at P7, but there was a dramatic decline after P12, reaching trace levels by P17 (Wang and Zhu, 2003). As such, the two appear to vary reciprocally, suggesting that increasing DAO expression through development may serve to limit D-serine levels. However, several factors may potentially confound the developmental mRNA expression data collected in this chapter. Firstly, no normalisation factor has been applied to the measurements. It must be taken into consideration that no other cerebellar mRNAs have been measured concurrently, and thus one cannot rule out the possibility that all cerebellar mRNAs increase during development, and it is not specific to DAO. Furthermore, no consideration has been made of the relative cell densities in the cerebellar sections at different ages. It is possible that the reported increase in mRNA expression merely reflects an increase in cell density with age, and not a change in expression of mRNA itself.

It is interesting to consider, however, the potential importance of changing DAO expression in cerebellar glia, especially putative Bergmann glia, and the role it may have in NMDA receptor modulation and development. NMDA receptors are essential for healthy synapse formation in the cerebellum, as demonstrated by connectivity studies using NMDA receptor blockers (Rabacchi *et al*., 1992), but they are absent from Purkinje cells in the adult cerebellum (Garthwaite *et al*., 1987), and D-serine is undetectable (Schell *et al*., 1997). Given the close proximity of the Bergmann glia and Purkinje cell body, it is attractive to assume that the expression of DAO in the Bergmann glia functions to control the levels of D-serine at the Purkinje cell synapse, and hence to modulate NMDA receptor function during synaptogenesis. Once development is complete, DAO levels are high, keeping D-serine levels low, and NMDA receptors become obsolete.

It is also interesting to consider the relevance of DAO reactivity being observed in glial processes in the molecular layer in the cerebellum, both in the current study and in several others, including during development (Weimar and Neims, 1977b; Horiike *et al*.,

1987; Verrall *et al*., 2007; Moreno *et al*., 1999). Schell and colleagues (1997) reported the presence of D-serine-producing radial processes of Bergmann glia in the molecular layer from around P7 (Schell *et al*., 1997). This coincides with the migration of granule cells to the internal granule cell layer along processes of the Bergmann glia, a process which is known to be dependent on NMDA receptors (Komuro and Rakic, 1993). Schell and colleagues thus proposed D-serine as a strong candidate for the regulation of NMDA receptor glycine sites involved in the migration of granule cells. It is therefore possible that DAO expression in Bergmann glial processes may serve to limit the D-serine activity here once the migration process is complete.

It is also possible that the changing levels of DAO and D-serine during development serve to modulate other receptors, as well as NMDA receptors. Recently, a study was published by Kakegawa and colleagues which considered the role of D-serine in binding to the δ2-type glutamate receptor (GluD2, encoded by Grid2), a member of the ionotropic glutamate receptor (iGluR) family, rather than to the NMDA receptor (Kakegawa *et al*., 2011). This receptor is selectively expressed in Purkinje cells of the cerebellum, and crystallographic studies have shown the ability of D-serine to induce closure of the ligand-binding domain of GluD2 (Naur *et al*., 2007), suggesting a role for D-serine in the modulation or activation of the receptor. This is interesting when considering that the receptor appears to have crucial roles in cerebellar functioning; indeed Grid2-null mice display severe impairments in motor coordination and impaired long term depression (LTD) at synapses between Purkinje cells and parallel fibres (Kashiwabuchi *et al*., 1995). It is therefore conceivable that the high levels of D-serine seen in the immature rat cerebellum have a pivotal role in signalling through GluD2 to regulate, for example, the induction of LTD. Indeed, the authors used a transgenic rescue approach to investigate the importance of D-serine binding to the receptor, and found that in mice with disrupted binding, motor dyscoordination and impaired LTD were present during development (Kakegawa *et al*., 2011). As such, it seems that D-serine, and its regulation by DAO, may

not only be important for NMDA receptor-related developmental processes, but also those of other receptor types.

In summary, this chapter has generated some interesting findings with regard to the regional and cellular expression of DAO in the rat brain. Perhaps most notably, both DAO mRNA and protein were expressed in the adult VTA, in both neurons and glia. This finding may provide a potential physiological link between high DAO activity and dysfunctional mesocortical and mesolimbic DA signalling seen in schizophrenia. The cellular expression of DAO appears to be region-specific, with the enzyme appearing to be present in both neurons and glia in the VTA, but only glia in the cerebellum. Finally, changing levels of DAO in the cerebellum during development may serve to temporally regulate D-serine levels, controlling important developmental processes such as granule cell migration and synaptogenesis.

Chapter 3:

The effect of systemic pharmacological manipulation of D-serine on extracellular DA and its metabolites in the PFC

3.1 Background

In schizophrenia, there is evidence that DAO expression and activity in the brain are increased (Kapoor *et al*., 2006; Verrall *et al*., 2007; Burnet *et al*., 2008; Madeira *et al*., 2008), and D-serine levels in the serum and cerebrospinal fluid are decreased (Hashimoto *et al*., 2003; Hashimoto *et al*., 2005b). Therapeutically this is very interesting, as increasing D-serine levels and augmenting NMDA receptor function could potentially be of benefit. Indeed, some clinical trials with D-serine as an add-on treatment have shown a favourable effect, including relief of negative and cognitive symptoms (Coyle *et al*., 2002; Javitt, 2006; Kantrowitz *et al*., 2010; Heresco-Levy *et al*., 2005). Given that these symptoms currently remain poorly treated, D-serine therapy may therefore be very relevant. However, large doses of D-serine are required to induce therapeutic effects, which may cause nephrotoxicity (Krug *et al*., 2007). Other pharmacological strategies aimed to increase levels of synaptic D-serine have been the focus of recent studies. As such, DAO inhibitors are becoming attractive candidates for increasing D-serine levels in the treatment of schizophrenia.

Some pre-clinical studies have investigated DAO inhibitors for their potential effects. Adage and coworkers (2008) investigated the potential antipsychotic effects of the selective DAO inhibitor AS057278 in preclinical models (Adage *et al*., 2008). The inhibitor normalised PCP-induced prepulse inhibition, both after acute and chronic dosing, and also attenuated PCP-induced hyperlocomotion after chronic administration. These results provided support for the antipsychotic potential of DAO inhibitors. However, these findings contrasted with those of another study which focused on the effects of the DAO inhibitor 4*H*-Thieno [3,2-*b*]pyrrole-5-carboxylic Acid (Compound 8) in comparison with D-serine (Smith *et al*., 2009). Acute administration of Compound 8 had no effect on either amphetamine-induced locomotor activity or MK-801 impaired novel object recognition in rats, although D-serine significantly reduced the effects of both amphetamine and

MK-801. The authors concluded that increased D-serine in the brain *in vivo* produces antipsychotic effects and enhances cognition, presumably via augmentation of NMDA receptor function. However, they speculated that the elevation in D-serine achieved by acute DAO inhibition alone would be insufficient, and propose chronic DAO inhibition as a way to more robustly increase D-serine levels.

Together, these two studies provide complementary evidence that increasing D-serine *in vivo* may have both antipsychotic and cognitive-enhancing effects. However, whether or not the elevation of brain D-serine achieved by acute DAO inhibition is sufficient remains unclear.

The negative and cognitive symptoms of schizophrenia are thought to originate from the PFC, with hypofunction of the mesocortical dopaminergic pathway being a major contributor (Davis *et al*., 1991). The improvements in these symptoms seen with D-serine therapy may therefore be mediated via enhanced dopaminergic transmission in the mesocortical pathway. However, at the time of conception of this thesis, the effect of pharmacological D-serine elevation on cortical DA had not been investigated. Having said that, one preclinical study assessed the effects of DAO inhibition or D-serine administration on the mesolimbic DA pathway, and measured extracellular DA levels in the nucleus accumbens shell using *in vivo* microdialysis. Administration of D-serine significantly attenuated amphetamine-induced DA efflux, whereas administration of a DAO inhibitor had no effect, despite increasing brain D-serine levels (Smith *et al*., 2009). These data supported the notion that modulation of NMDA receptors can affect the function of the mesolimbic DA pathway. However, it appears that acute systemic administration of a DAO inhibitor alone may be insufficient to affect DA.

Overall, the data suggest that D-serine administration, and perhaps DAO inhibition, has the potential to modulate DA pathways and thereby have a beneficial effect on cognitive and negative symptoms in patients with schizophrenia. However, the effect of D-serine on the mesocortical DA pathway is unknown. This chapter set out to investigate the effects of systemic administration of D-serine and a DAO inhibitor on extracellular DA and its metabolites in the medial PFC (mPFC), as measured using *in vivo* microdialysis.

3.2 Summary and aims

The aim of this study was to investigate the effect of systemic administration of D-serine and a DAO inhibitor on extracellular levels of DA and its metabolites in the mPFC *in vivo. In vivo* microdialysis coupled with high-performance liquid chromatography (HPLC) was used to measure levels of DA and its metabolites in the mPFC of anaesthetised rats. First, pilot studies using local infusions of high potassium and systemic injection of amphetamine were carried out in order to validate the experimental method. Then, the effects of systemically administered D-serine and sodium benzoate (a DAO inhibitor) were investigated. Levels of the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) and the noradrenaline metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) were also measured to assess the potential effects of the D-serine treatment on the other monoamine systems.

3.3 Methods

3.3.1 *In vivo* microdialysis

Adult male Sprague-Dawley rats (Harlan, UK) were anaesthetised with chloral hydrate (500mg/kg i.p.) and mounted in a stereotaxic frame in the flat skull position. Anaesthesia was maintained with 20–40mg i.p. doses of chloral hydrate, and body temperature monitored and maintained at 36–37°C. Hydration was maintained using 0.5ml s.c. injections of 4% glucose in 0.18% saline as required. A hole was made in the skull using a dental drill (Foredom®, Bethel, USA) and an intracerebral guide cannula (CMA/11, CMA/Microdialysis AB, Stockholm, Sweden) was implanted into the PFC at the following coordinates relative to bregma: ML +0.6mm; AP +3.2mm; DV -2.0mm (from dura), according to the rat brain atlas of Paxinos and Watson (1998). The guide cannula was secured using a cranial screw (2mm, Royem Scientific, Luton, UK) and dental cement (Simplex Rapid™, Kemdent®, Swindon, UK). A commercial microdialysis probe (CMA/11, 2mm membrane, CMA/Microdialysis AB, Stockholm, Sweden) was then introduced into the guide cannula and perfused with artificial CSF (containing 140.0mM NaCl, 3.0mM KCl, 1.2mM $Na_2HPO_4.2H_2O$, 0.27mM $NaH_2PO_4.H_2O$, 1.0mM $MgCl_2.6H_2O$, 2.4mM $CaCl_2$ and 7.2mM glucose) at a flow rate of 1µl/min. Samples were collected every 20 minutes in small tubes containing 5µl of 0.1M perchloric acid, analysed immediately using HPLC. Samples were collected until a stable baseline was achieved (typically 2–3 hours), before any drugs were administered.

First, several pilot studies were carried out. The following substances were administered:

- 100mM potassium (K^+; local, administered in the aCSF via the probe) followed by amphetamine (1.5mg/kg s.c.; n=1)
- 100mM K^+, followed by local calcium (Ca^{2+}) depletion (achieved via perfusing aCSF containing no $CaCl_2$), and then further K^+ (100mM; n=1)

Subsequently, the main experiments were performed:

- D-serine (1280mg/kg s.c.) plus sodium benzoate (500mg/kg i.p.) dissolved in 0.9% NaCl (n=5)
- 0.9% NaCl (5ml/kg s.c. plus 2ml/kg i.p.; n=5).

Following drug administration, samples were collected every 20 minutes for a further 4 hours in each case (in the main study; shorter time durations for pilot studies), before the animals were euthanased and the brains harvested. Probe placements were checked histologically, and data from any placed incorrectly excluded from the analysis.

3.3.2 Localisation of cannula and probe placement

Figure 21 shows a schematic representation of expected probe placements. Each individual brain was histologically assessed for placement accuracy, and the data from those not matching this schematic removed from the analysis.

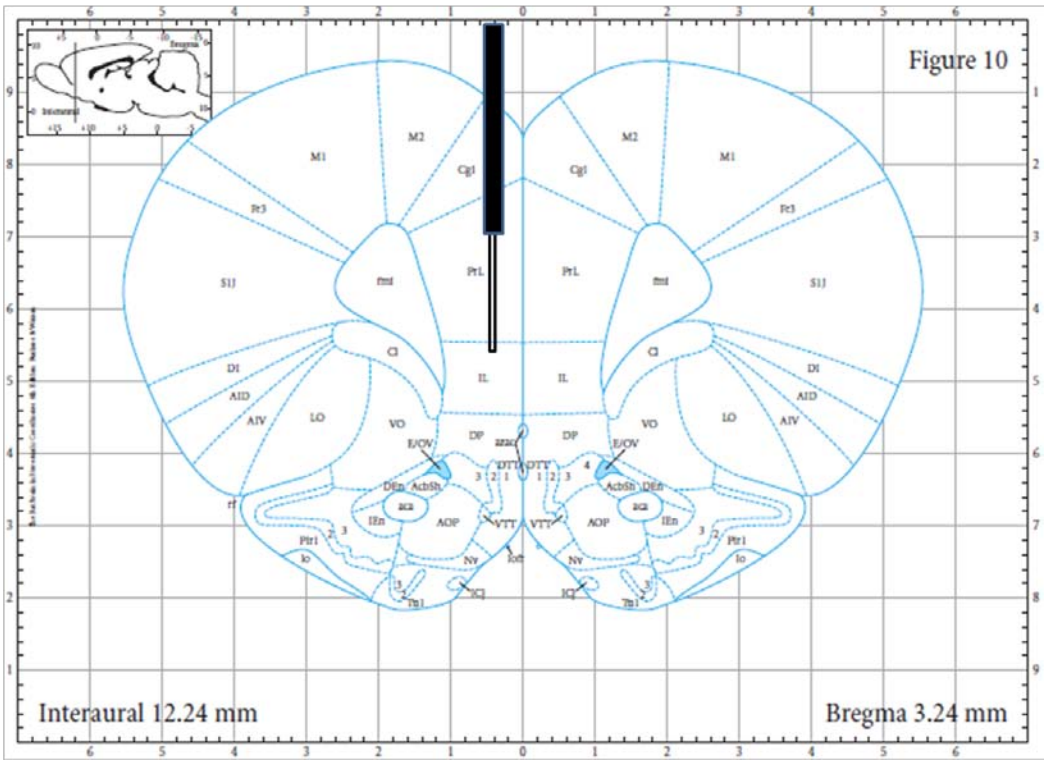


Figure 21. Schematic representation of observed mPFC probe placement in sections of brain taken from animals used for microdialysis. *Brain atlas images taken from Paxinos and Watson, 2007.*

3.3.3 Drug preparation and dosing

Given the possibility that acute administration of a DAO inhibitor alone is unable to produce physiologically relevant increases in D-serine levels (Smith *et al*., 2009), both a DAO inhibitor and exogenous D-serine were administered together in order to produce the largest increase in central D-serine possible. Sodium benzoate was selected as the DAO inhibitor, since it has been described as a 'prototypical competitive DAO inhibitor' (Vanoni *et al*., 1997; Gong *et al*., 2011). Furthermore, it is both soluble and commercially available.

3.3.3.1 D-serine

Smith and colleagues (2009) used several subcutaneous doses of D-serine in their studies – 320, 640 and 1280mg/kg. The dose of 1280mg/kg dose was selected for the current study as it was shown by Smith to be able to both attenuate amphetamine-induced psychomotor activity and reverse MK-801 impaired novel object recognition. Moreover, this dose was shown to attenuate amphetamine-induced DA efflux in the nucleus accumbens shell (Smith *et al*., 2009). D-serine was dissolved in 0.9% NaCl and administered subcutaneously at 5ml/kg.

3.3.3.2 Sodium benzoate

In a detailed study of the effects of sodium benzoate on D-serine-induced renal toxicity in the rat, Williams and colleagues (2005) noted that sodium benzoate administered intraperitoneally at 500mg/kg produced the greatest mean inhibition of peripheral DAO activity, while producing only mild perturbations in diuresis and levels of urea (which were prominent in higher doses; Williams and Lock, 2005). Furthermore, Moses and co-workers (1996) investigated the effects of systemic sodium benzoate on circling behaviour induced by L- and D-DOPA in the hemi-parkinsonian rat (Moses *et al*., 1996). Circling in this model is believed to be a result of L-DOPA conversion to DA (although D-DOPA can also produce circling). DAO is required for the conversion of D-DOPA to L-DOPA, and so the authors hypothesised that circling induced by D-DOPA could be prevented with inhibition of DAO. Indeed, a dose of 400mg/kg sodium benzoate completely blocked rotation induced by D-DOPA. This suggests that sodium benzoate is able to inhibit DAO-mediated processes in the brain *in vivo*, and that these effects can be seen when a systemic dose of 400mg/kg is given. Taking these two results together, 500mg/kg was selected as the sodium benzoate dose for the current study, in order to be confident that the dose would have a central effect, while trying to maximise the inhibition of DAO and

minimise renal problems. The sodium benzoate was dissolved in 0.9% NaCl and administered at 2ml/kg.

3.3.4 Analysis of DA and monoamine metabolites using HPLC

High performance liquid chromatography coupled with electrochemical detection was employed to analyse the samples. Samples (25µl) were injected onto a Dynamax microsorb column (100 x 4.6mm, 100-3 C18, Varian Inc, Middelburg, The Netherlands) and mobile phase containing 15% methanol, 0.12M $NaH_2PO_4.H_2O$, 0.002M NaCl, 0.1mM ethylenediaminetetraacetic acid (EDTA) and 0.5mM 1-octanesulphonic acid sodium salt (OSA; overall pH4.17) pumped through the column at a rate of 1ml/min (JASCO PU-1585 Intelligent HPLC Pump, JASCO, Essex, UK). A VT-03 electrochemical flow cell with a 2mm glassy carbon (GC) working electrode and *in situ* Ag/AgCl (ISAAC) reference electrode (Antec Leyden, ARC Sciences, Hampshire, UK) was used to analyse the column eluate. Electrode settings were as follows: voltage=+0.6V, range=5nA, filter= 0.5s. JASCO ChromPass Chromatography Data System software was used to plot the chromatograms and analyse the data; Figure 22 shows an example chromatogram of a 25µl sample taken from the mPFC of an anaesthetised rat.

Drug effects were measured on prefrontal levels of DA, and the DA metabolites DOPAC and HVA were also monitored for assessment of DA metabolism. The 5-HT metabolite 5-HIAA and the noradrenaline metabolite MHPG were also monitored for comparison. Analyte peaks were measured and quantified when the signal to noise ratio exceeded a value of 2.

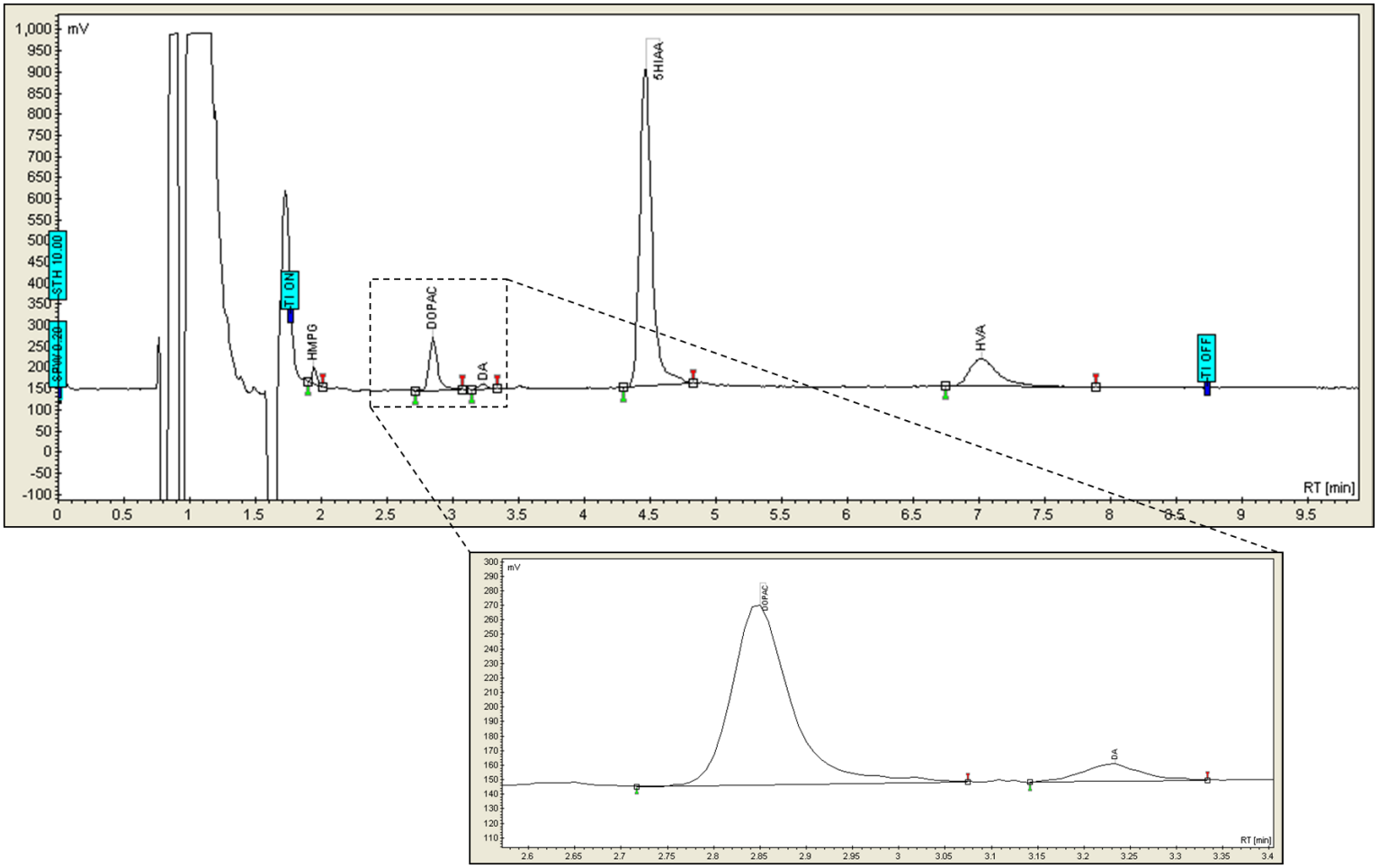


Figure 22. Example chromatogram of a 25µl sample taken from the mPFC of an anaesthetised rat. *Peak heights are compared with those of the reference standards, and quantities of each molecule calculated. Magnified box demonstrates relative sizes of DOPAC and DA peaks.*

3.3.5 Measurement of brain D-serine

The brains from the animals used for microdialysis were harvested at the end of each experiment, and the tissue D-serine levels were measured. Measurements were performed in the cerebellum, which is rich in DAO, and also in the PFC, where DAO levels are much lower.

Half the cerebellum (cut in the coronal plane) and the left PFC (contralateral to probe placement) were weighed and homogenised in 500µl iced water, and the supernatant was extracted following centrifugation for 1 minute. Protein concentration was then measured using a spectrophotometer (NanoDrop® ND-1000, LabTech International). The supernatant from the cerebellum was diluted to 5mg/ml in 100µl. All PFC supernatant was run at its original concentration without further dilution. D-serine standards were made up by serial dilution from 2–0.016nmol/ml in PBS containing 0.2mg/ml BSA.

25µl glutaraldehyde was added to 100µl of each supernatant, or D-serine standards. The solutions were mixed thoroughly and 50µl duplicates added to an ELISA plate. After a 2-hour incubation at room temperature, wells were washed five times with PBS and air dried. Non-specific binding sites were blocked by incubation of the wells with blocking buffer (2% non-fat milk in PBS, 50µl) for 30 minutes at room temperature, followed by three washes with PBST (PBS + 0.1% Tween20). Rabbit polyclonal primary antibody to D-serine (Abcam ab6472-50) was diluted 1:500 in PBS containing 1% BSA and 50µl added to each well for a 1 hour incubation. Following three washes with PBST, HRP-linked goat anti-rabbit IgG (BioRad 172-1019) at 1:2000 was added for 25 minutes. The wells were washed a further three times in PBST. A colorimetric reaction was initiated by the addition of 50µl ABTS to each well and was stopped after 8 minutes by the addition of 50µl 4% oxalic acid. The plate was measured using a spectrophotometer (SpectraMax 190, Molecular Devices Ltd) at 415nm.

Pilot experiments were carried out to determine optimal reagent concentrations.

3.3.6 Data analysis

Statistical analyses of microdialysis were carried out using SPSS software (version 17.0). A repeated measures ANOVA was performed to assess the effects of time and drug on DA, DOPAC, HVA, 5-HIAA and MHPG dialysate levels (relative to baseline). Simple main effects tests were then carried out if the ANOVA was significant. Time points were included from time point 20 onwards.

Analysis of brain D-serine levels in animals was also carried out using SPSS software (version 17.0), using a univariate ANOVA to compare levels between treatment groups.

3.4 Results: Part 1

3.4.1 Pilot study: Effect of local K+ application and amphetamine injection on extracellular levels of DA and its metabolites in the mPFC

Upon infusion of potassium (100mM) into the mPFC via the probe perfusate, there was a large increase in extracellular levels of DA in the mPFC, occurring within 20 minutes of the infusion (Figure 23). This was accompanied by a decrease in levels of both DOPAC and HVA, again within 20 minutes. After the potassium infusion was removed, DA levels returned to baseline after a further 40 minutes. Levels of DOPAC and HVA, however, remained reduced, and did not return to baseline.

Subsequent administration of amphetamine (1.5mg/kg s.c.) resulted in another increase in levels of extracellular DA in the mPFC, peaking 40 minutes after administration (Figure 23). This was accompanied by a small and progressive decrease in DOPAC and a small decrease in HVA after the first hour.

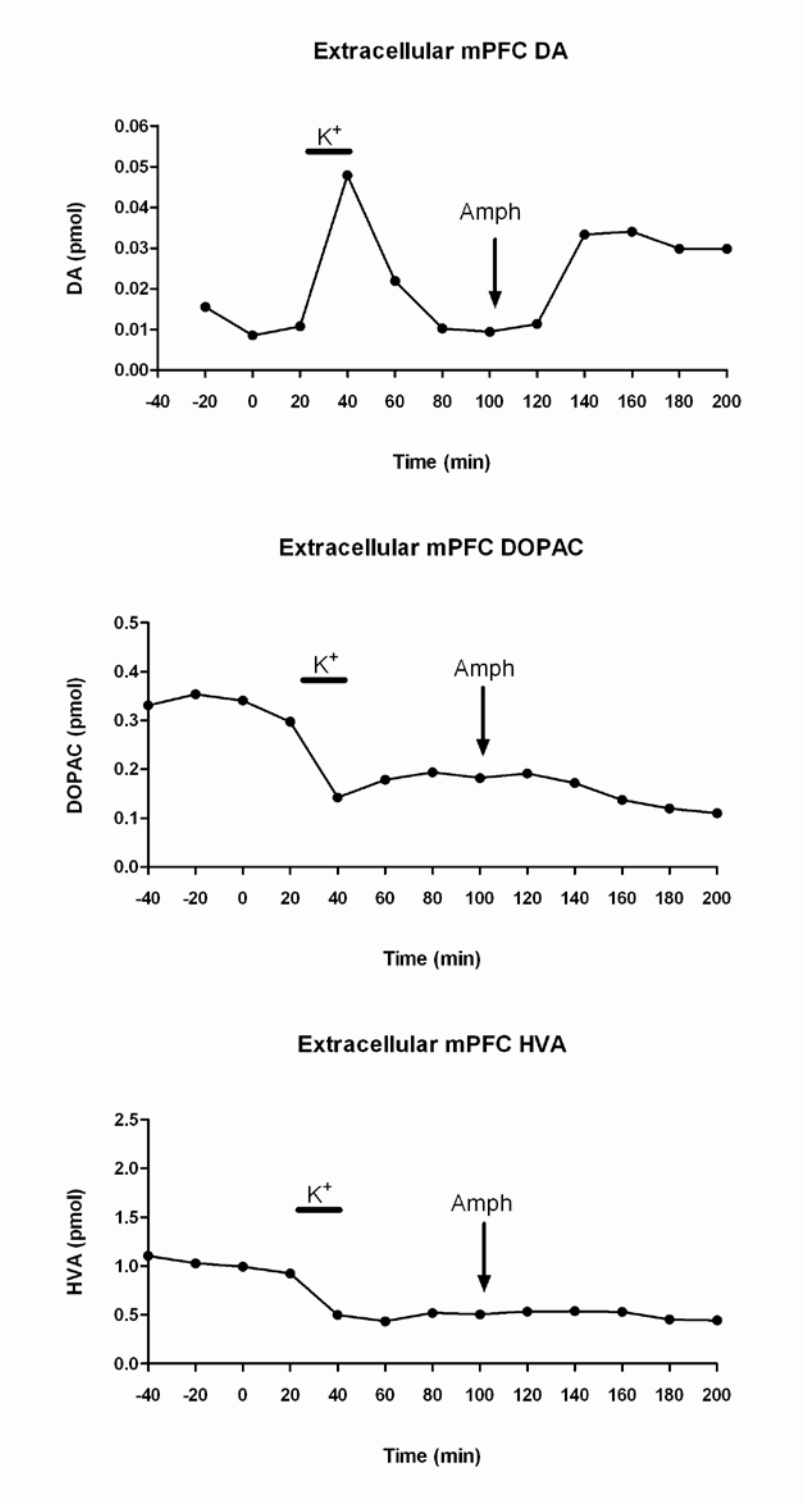


Figure 23. Effect of local K^+ application and amphetamine injection on extracellular levels of DA and its metabolites in the mPFC. *Time 0 represents the time point at which the baseline was stable. Bar represents K^+ administration via probe perfusate, and arrow represents the time of amphetamine injection (1.5mg/kg s.c.).*

3.4.2 Pilot study: Effect of local K^+ application and Ca^{2+} depletion on extracellular levels of DA and its metabolites in the mPFC

Local application of K^+ (100mM, via the probe perfusate) resulted in an increase in levels of extracellular DA in the mPFC, detectable within the first 20 minutes after the onset of infusion (Figure 24). This was accompanied by decreases in levels of both DOPAC and HVA, which were also visible within the first 20 minutes.

Once the K^+ was removed, DA levels decreased, returning to baseline within 40 minutes. Levels of DOPAC and HVA also began to recover, although did not return to baseline levels, and remained reduced by around 50%. Omission of Ca^{2+} from the aCSF resulted in a further decrease in extracellular levels of DA, but had no apparent effect on levels of DOPAC or HVA. Further application of K^+, while maintaining a depletion of Ca^{2+}, resulted in an increase in levels of DA, although to a lesser extent than when calcium levels were normal. This was once again accompanied by a decrease in levels of DOPAC and HVA. All changes were visible within 20 minutes of the application of K^+ (Figure 24).

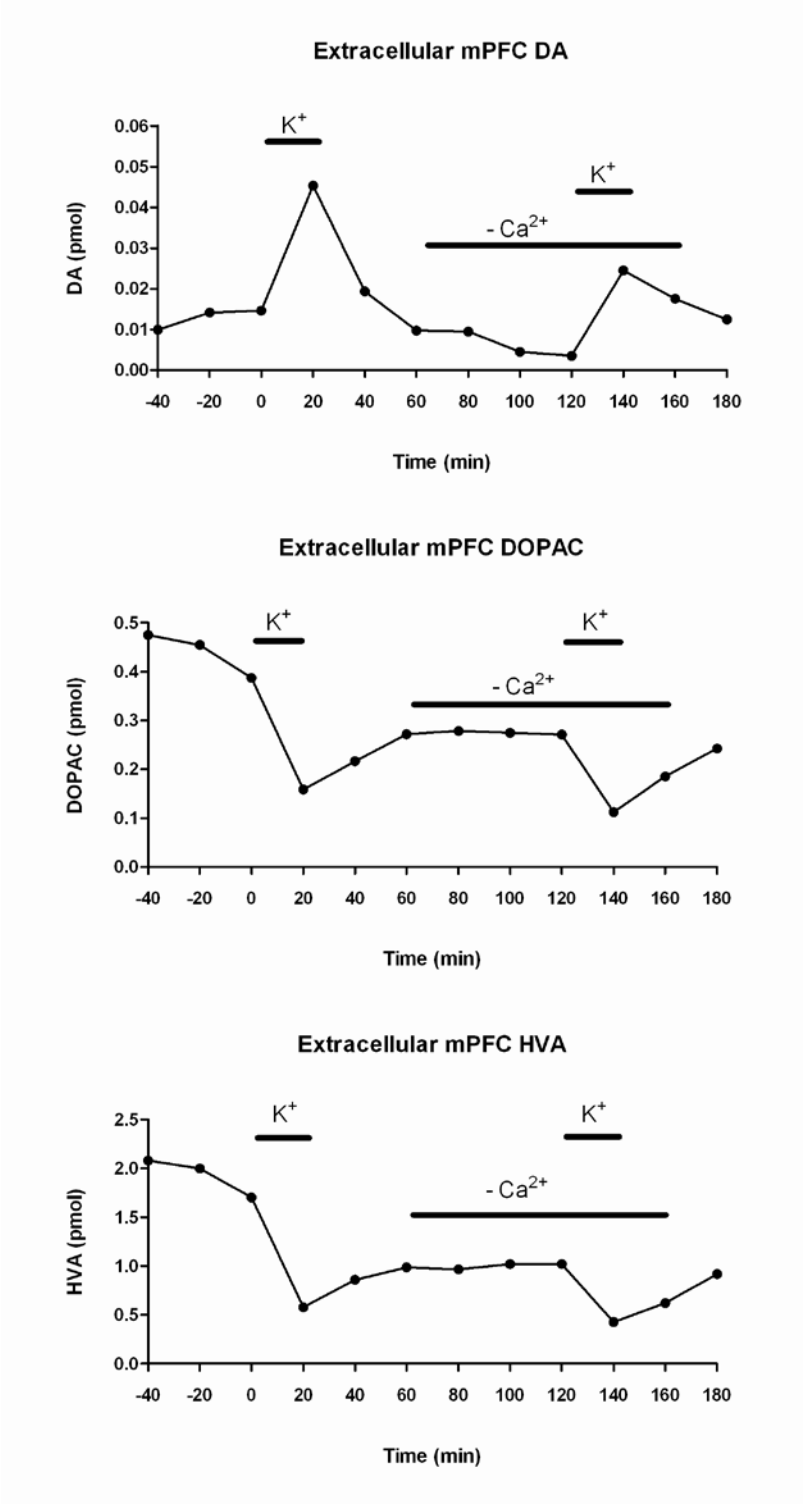


Figure 24. Effect of local K^+ application and Ca^{2+} depletion on extracellular levels of DA and its metabolites in the mPFC. *Time 0 represents the time point at which the baseline was stable. Bars represent length of time over which substances were administered or depleted. All substances were administered locally via the probe perfusate.*

3.4.3 Effect of systemic administration of D-serine and sodium benzoate on levels of D-serine in the cerebellum

Systemic administration of D-serine and sodium benzoate resulted in a significant increase in D-serine levels in the cerebellum when compared with vehicle controls [F(1,4)=11.893, p=0.026; Figure 25].

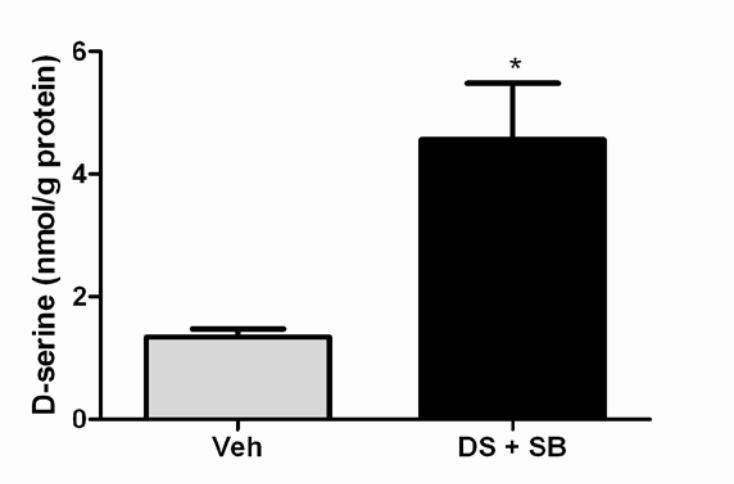


Figure 25. Effect of systemic administration of D-serine and sodium benzoate on cerebellar levels of D-serine. *Plot shows mean ± S.E.M., n=3 in each group. *p<0.05.*

3.4.4 Effect of systemic administration of D-serine and sodium benzoate on extracellular levels of DA and its metabolites in the mPFC

Table 5 shows the average raw baseline levels of DA, DOPAC, HVA, 5-HIAA and MHPG found in the final three baseline dialysis samples collected in each group before administration of either D-serine and sodium benzoate or vehicle (0.9% NaCl). Statistical analysis revealed no difference between baseline levels of DA, HVA, 5-HIAA or MHPG between the two treatment groups, but a statistically significant difference between levels of DOPAC in the two groups ($p = 0.044$).

Treatment Group	DA	DOPAC	HVA	5-HIAA	MHPG
D-serine + sodium benzoate	0.010 ± 0.001 (n=5)	0.180 ± 0.027 (n=5)	1.182 ± 0.376 (n=5)	2.232 ± 0.347 (n=5)	0.213 ± 0.103 (n=5)
Vehicle (0.9% NaCl)	0.016 ± 0.003 (n=5)	0.776 ± 0.207 (n=5)	1.928 ± 0.391 (n=5)	4.775 ± 1.665 (n=5)	0.500 ± 0.162 (n=5)

Table 5. Raw baseline levels of DA, DOPAC, HVA, 5-HIAA and MHPG in dialysates from the mPFC. *Values given are mean ± S.E.M. (pmol) in each treatment group, and are calculated from the final three baseline samples collected from each animal before any drugs were administered.*

Systemic administration of D-serine and sodium benzoate had no effect on extracellular levels of DA in the mPFC [effect of drug $F(1,8)=2.824$, $p=0.131$; effect of time $F(11,88)=0.669$, $p=0.764$; drug x time interaction $F(11,88)=0.630$, $p=0.7995$; Figure 26]. There was also no significant main effect of drug on levels of DOPAC [$F(1,8)=3.841$, $p=0.086$]. However, there was a significant effect of time on DOPAC levels [$F(11,88)=1.998$, $p=0.038$] and a significant drug x time interaction [$F(11,88)=2.888$, $p=0.003$]. Individual analysis of the two groups for the main effects of time revealed a significant effect in the D-serine and sodium benzoate treatment group

[F(11,44)=2.486, p=0.016] that was not seen in the vehicle group [F(11,44)=1.854, p=0.073]. This demonstrates that the significant drug x time interaction is driven by a clear increase in DOPAC levels over time seen in the treatment group that is not seen in the vehicle control group (Figure 26).

There was no effect of treatment on extracellular levels of HVA in the mPFC [effect of drug F(1,8)=0.077, p=0.789]. However, there was a significant effect of time [F(11,88)=2.015, p=0.036] and an interaction between time and drug [F(11,88)=2.009, p=0.037], although simple main effects revealed no significant time points (Figure 26).

Systemic administration of D-serine and sodium benzoate had no effect on extracellular levels of 5-HIAA in the mPFC [effect of drug F(1,8)=0.155, p=0.704; effect of time F(11,88)=0.656, p=0.776; drug x time interaction F(11,88)=0.780, p=0.659; Figure 27]. However, the treatment resulted in a clear significant increase in levels of extracellular MHPG in the mPFC that was not seen in the vehicle control group [effect of drug F(1,8)=7.301, p=0.027; effect of time F(11,88)=2.143, p=0.025; drug x time interaction F(11,88)=2.273, p=0.017; Figure 27].

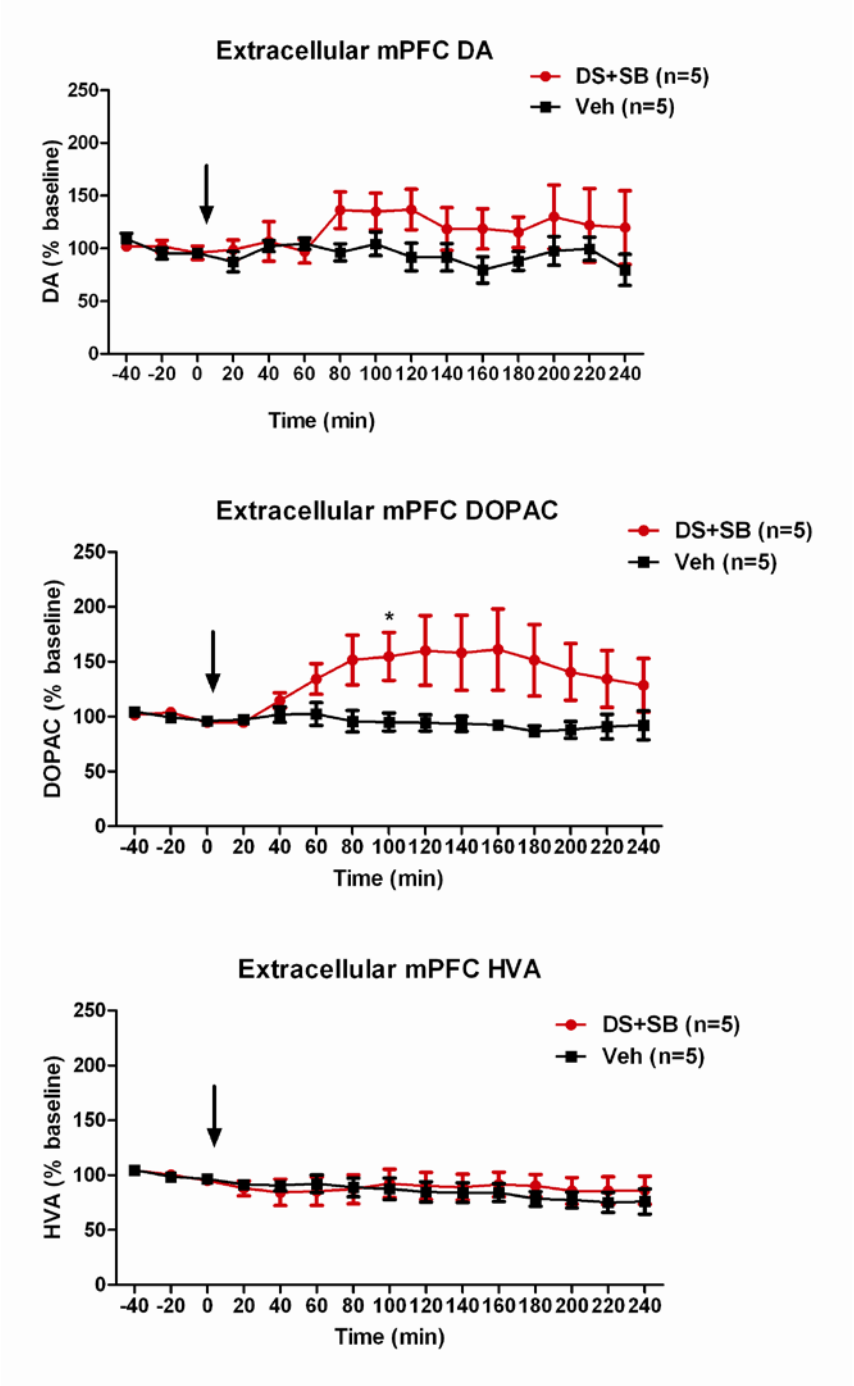


Figure 26. Effect of systemic administration of D-serine and sodium benzoate on extracellular DA and its metabolites in the mPFC. *Time point 0 indicates the time at which the baseline was stable and systemic injection of D-serine and sodium benzoate was administered. Points represent mean ± SEM, and are expressed as a percentage of the final three baseline samples. Statistical analysis carried out with repeated measures ANOVA. *p<0.05.*

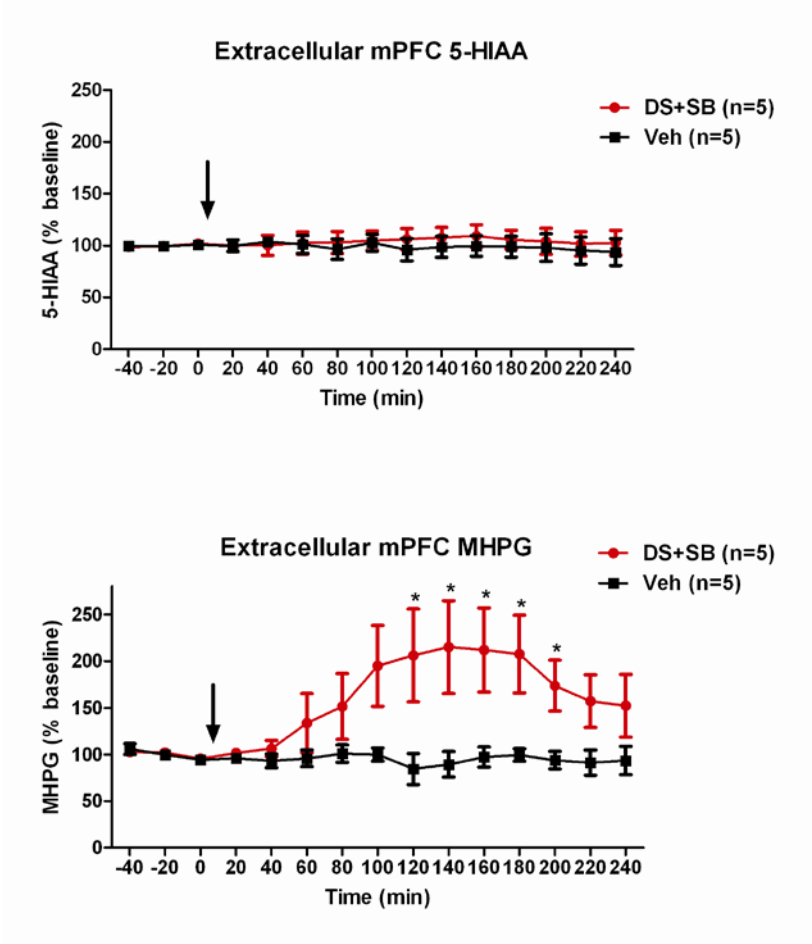


Figure 27. Effect of systemic administration of D-serine and sodium benzoate on extracellular 5-HIAA and MHPG in the mPFC. *Time point 0 indicates the time at which the baseline was stable and systemic injection of D-serine and sodium benzoate was administered. Points represent mean ± SEM, and are expressed as a percentage of the final three baseline samples. Statistical analysis carried out with repeated measures ANOVA. *p<0.05.*

3.5 Protocol refinements

Detected DA levels in the current study were low (~0.01pmol/sample), and baseline levels of DOPAC were significantly different in the two treatment groups. This suggested that the measurement technique used was not sufficiently accurate, and that interpretations of the data collected may be invalid. As such, two protocol refinements were made in order to ensure maximal sensitivity of the measurements. Then, the experiments were repeated. First, probes were assembled in-house, and the membrane length increased to 3mm in order to improve recovery. Second, nomifensine, an inhibitor of the DA transporter (DAT1) and the noradrenaline transporter (NET), was added to the perfusing aCSF.

3.5.1 Addition of nomifensine

It is common practice in microdialysis studies for a reuptake blocker to be included in the perfusing aCSF (see e.g. Fuchs and Hauber, 2004). This is in order to ensure that any subtle changes in the neurotransmitter levels are detected before the transporters remove the molecules from the extracellular space. In most brain regions, DA undergoes reuptake via DAT1. However, in the PFC, DAT1 is only expressed in very low levels, and instead DA is reuptaken by NET (Moron *et al*., 2002). Nomifensine was added to the perfusing a CSF at a concentration of 3μM, in order to inhibit both transporters during the experiment, following the protocol of Fuchs and Hauber (2004).

3.5.2 Microdialysis probes

Probes were assembled in-house. Two 40mm lengths of 40μm/140μm fused silica tubing (SGE Analytical, Milton Keynes, UK) were inserted through a 22mm long 23G steel cannula (Coopers Needleworks, Birmingham, UK) and left with approximately 10mm protruding at each end. A 4mm long 25G steel cannula was then threaded onto each of the lengths of the silica tubing at the top end of the long cannula, in order to form a Y-shaped structure. One of the lengths of silica tubing (marked at the top end as the ‘outlet’) was then retracted such that it ended 3mm from the bottom end inside the long

cannula. The three steel cannulae were then glued together using Loctite® acrylic adhesive (RS Components, Corby, UK), and the assembled structure left to dry overnight.

The single length of silica tubing protruding from the lower end of the long steel cannula was trimmed to approximately 2.5mm, and a short length of dialysis membrane (Filtral™ 16, AN69 HF™, Hospal, Cambridgeshire, UK) was threaded over it, and continued such that it went inside the steel cannula as far as it would go. The membrane was then retracted 1mm and a small amount of quick set epoxy adhesive (RS Components, Corby UK) applied at the membrane/cannula interface, before it was pushed back into place. The adhesive was left to dry. The dialysis membrane was trimmed such that it extended 1mm beyond the end of the silica tubing, and a tiny amount of quick set epoxy adhesive was applied to the end in order to seal it, before the whole structure was left to dry.

At the top end of the long cannula, the 'outlet' silica tubing was trimmed such that it just extended beyond the small steel cannula. A 2cm length of 0.38mm/1.09mm Portex polythene tubing (Smiths Medical, Kent, UK) was pushed over the small steel cannula, and marked as the outlet. This was repeated with the other protruding silica tubing and small steel cannula. Finally, the two pieces of polythene tubing were secured in place using Loctite® acrylic adhesive (RS Components, Corby, UK). The finished probe was left to dry overnight (as a minimum) before use.

Figure 28 shows a schematic of the assembled probe.

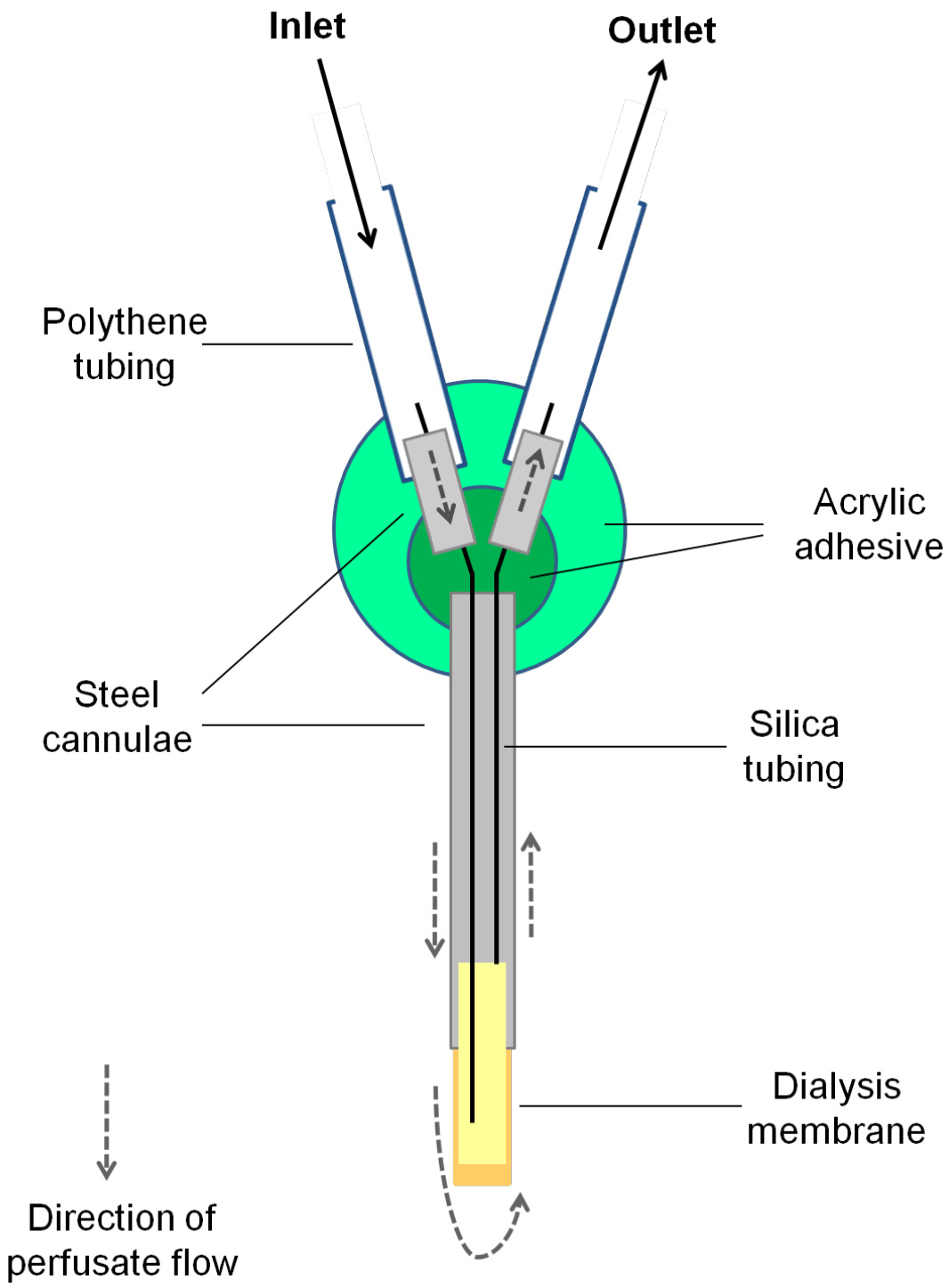


Figure 28. Schematic diagram of an assembled probe made in-house. *N.B. Diagram is a representation and is not to scale.*

3.6 Results: Part 2

3.6.1 Effect of systemic administration of D-serine and sodium benzoate on levels of D-serine in the cerebellum and PFC, in the presence of nomifensine

Systemic administration of D-serine and sodium benzoate resulted in a significant increase in D-serine levels in both the cerebellum [F(1,12)=5.917, p=0.032] and PFC [F(1,12)=18.845, p=0.001] when compared with vehicle controls (Figure 29).

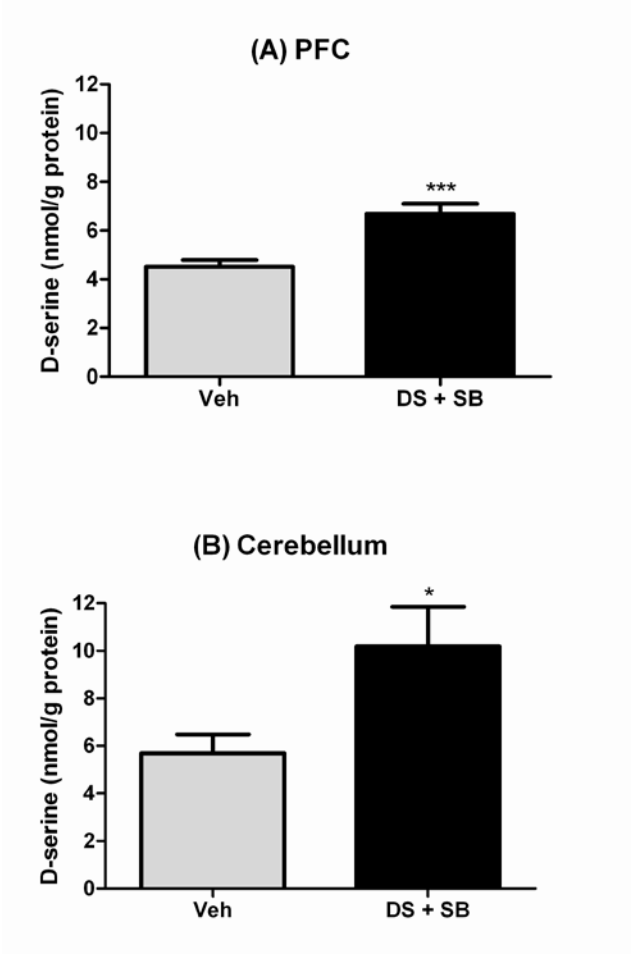


Figure 29. Effect of systemic administration of D-serine and a DAO inhibitor on levels of D-serine in (A) PFC and (B) cerebellum, in the presence of nomifensine. *Plot shows mean ± S.E.M., n=7 in each group. *p<0.05, ***p<0.005.*

3.6.2 Effect of systemic administration of D-serine and sodium benzoate on extracellular levels of DA and its metabolites in the mPFC, in the presence of nomifensine

Table 6 shows the average raw baseline levels of DA, DOPAC, HVA, 5-HIAA and MHPG found in the final three baseline dialysis samples collected in each group before administration of either D-serine and sodium benzoate or 0.9% NaCl. In both treatment groups, 3μM was contained in the perfusing aCSF. Statistical analysis revealed no difference between baseline levels of DA, DOPAC, HVA, 5-HIAA or MHPG between the two treatment groups.

Treatment Group	DA	DOPAC	HVA	5-HIAA	MHPG
D-serine + sodium benzoate	0.060 ± 0.009 (n=5)	0.509 ± 0.193 (n=6)	2.708 ± 0.623 (n=7)	2.029 ± 0.162 (n=6)	0.122 ± 0.013 (n=7)
Vehicle (0.9% NaCl)	0.060 ± 0.006 (n=5)	0.480 ± 0.140 (n=5)	2.464 ± 0.458 (n=5)	2.332 ± 0.243 (n=5)	0.145 ± 0.014 (n=5)

Table 6. Raw baseline levels of DA, DOPAC, HVA, 5-HIAA and MHPG in the dialysates from male Sprague-Dawley rats. *Values given are mean ± S.E.M. (pmol) in each treatment group, and are calculated from the final three baseline samples collected from each animal before any drugs were administered.*

There was no detectable effect of systemic administration of D-serine and sodium benzoate on levels of extraclleular DA in the mPFC (Figure 30), in the presence of nomifensine. There was, however, a significant increase in extracellular levels of DOPAC, HVA, 5-HIAA and MHPG (Figure 30, Figure 31).

Statistical analysis revealed no significant effect of D-serine and sodium benzoate administration on levels of extracellular DA in the mPFC [effect of drug $F(1,8)=0.141$, $p=0.717$; drug x time interaction $F(11,88)=0.329$, $p=0.977$; Figure 30]. There was a significant effect of time on levels of DA [$F(11,88)=11.020$, $p<0.001$] that was clearly

driven by an increase in levels of DA seen over time in both the treatment group and the vehicle control group (Figure 30).

Administration of D-serine and sodium benzoate resulted in a significant increase in levels of DOPAC in the mPFC that became significant after 40 minutes, but was not seen in the vehicle control group [effect of drug $F(1,9)=31.275$, $p<0.001$; effect of time $F(11,99)=22.625$, $p<0.001$; drug x time interaction $F(11,99)=20.270$, $p<0.001$; Figure 30]. There was also a significant increase in mPFC levels of HVA in the treatment group that was not seen in the vehicle control group [effect of drug $F(1,10)=13.193$, $p=0.005$; effect of time $F(11,110)=6.715$, $p<0.001$; drug x time interaction $F(11,110)=13.036$, $p<0.001$; Figure 30], although this increase did not become significant until 100 minutes after injection.

Extracellular levels of 5-HIAA were significantly increased in the treatment group, but not in the vehicle group [effect of drug $F(1,9)=20.672$, $p=0.001$; effect of time $F(11,99)=27.247$, $p<0.001$; drug x time interaction $F(11,99)=14.596$, $p<0.001$; Figure 31]. The effect became significant within 60 minutes of injection. Extracellular levels of MHPG were also significantly increased in the treatment group, but not in the vehicle group [effect of drug $F(1,10)=19.782$, $p=0.001$; effect of time $F(11,110)=27.906$, $p<0.001$; drug x time interaction $F(11,110)=16.212$, $p<0.001$; Figure 31]. The effect became significant within 60 minutes of injection.

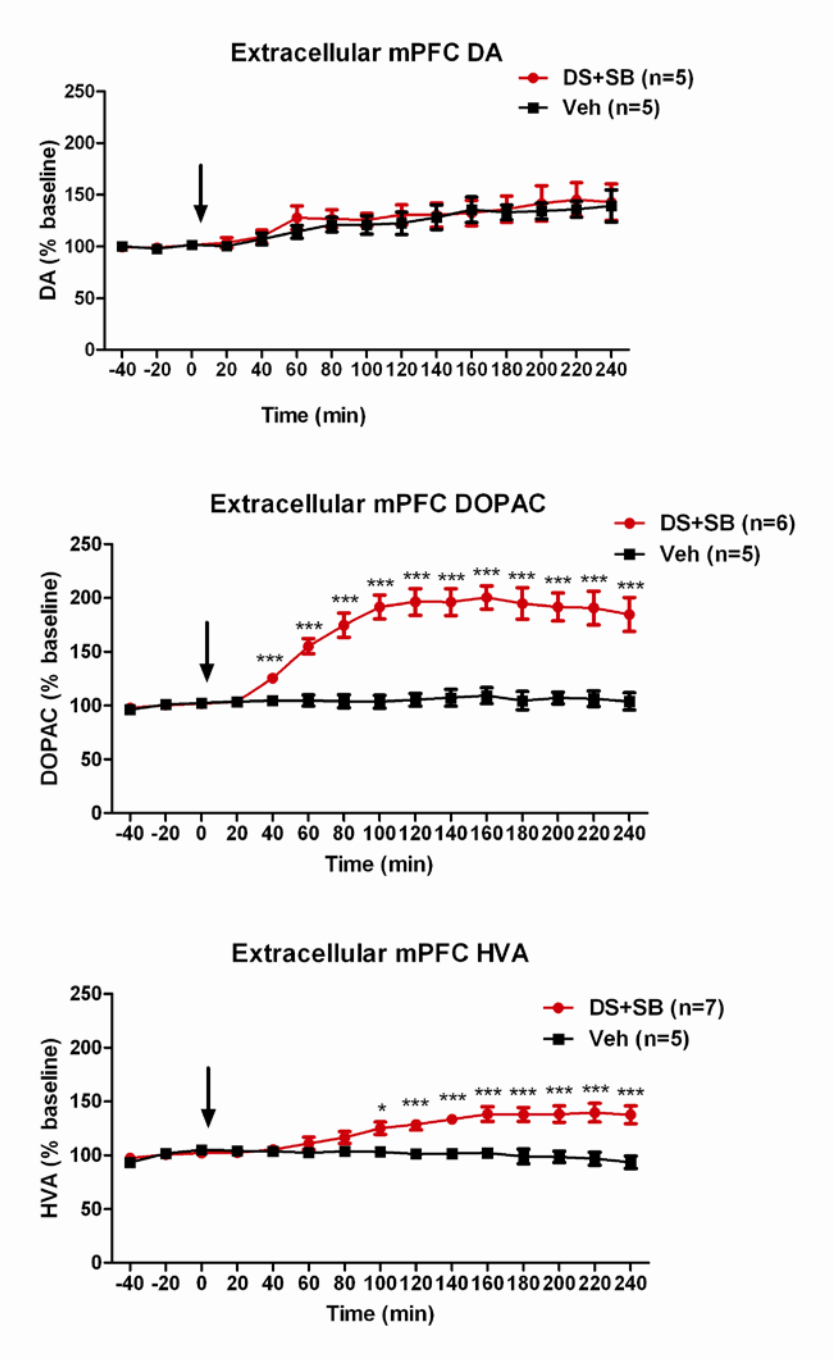


Figure 30. Effect of systemic administration of D-serine and sodium benzoate on extracellular DA and its metabolites in the mPFC, in the presence of 3μM nomifensine. *Time point 0 indicates the time at which the baseline was stable and systemic injection of D-serine and sodium benzoate was administered. Points represent mean ± SEM, and are expressed as a percentage of the final three baseline samples. Statistical analysis carried out with repeated measures ANOVA. *p<0.05, ***p<0.005.*

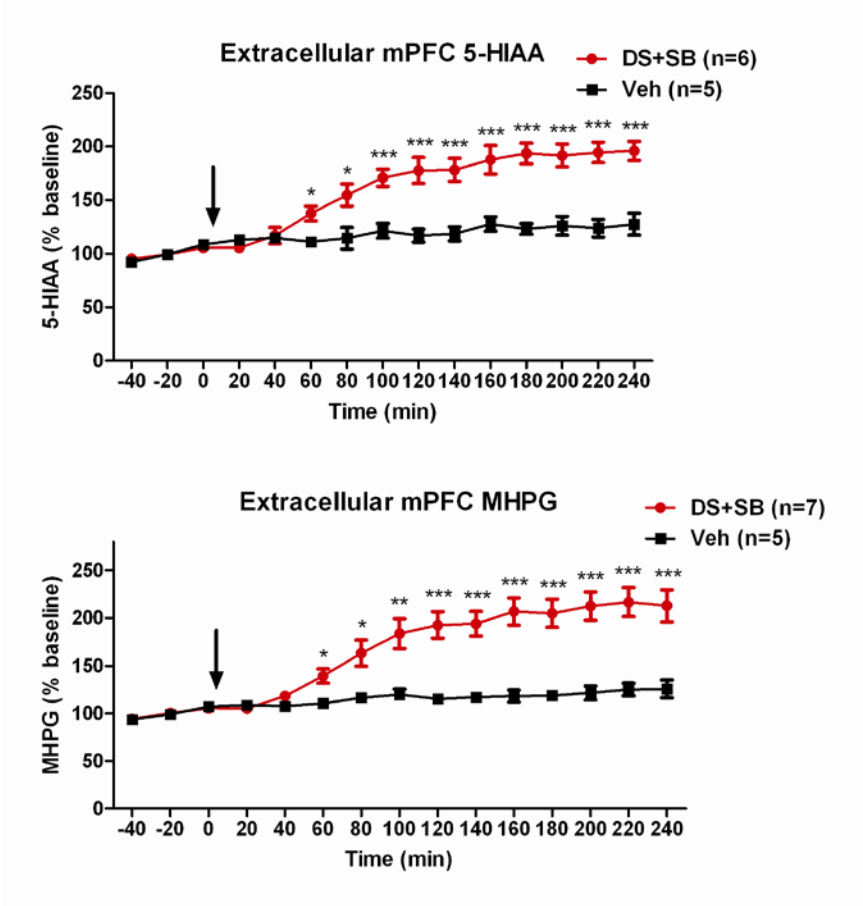


Figure 31. Effect of systemic administration of D-serine and sodium benzoate on extracellular 5-HIAA and MHPG in the mPFC, in the presence of 3μM nomifensine. *Time point 0 indicates the time at which the baseline was stable and systemic injection of D-serine and sodium benzoate was administered. Points represent mean ± SEM, and are expressed as a percentage of the final three baseline samples. Statistical analysis carried out with repeated measures ANOVA. *p<0.05, **p<0.01, ***p<0.005.*

3.7 Discussion

Experiments in this chapter established a protocol in which *in vivo* microdialysis coupled with HPLC was used to measure extracellular levels of DA, DOPAC, HVA, 5-HIAA and MHPG in the mPFC of the anaesthetised rat, while concurrently performing local applications and systemic injections of pharmacological agents.

The results can be summarised as follows:

- In a pilot study, local application of K^+ (100mM, via the probe perfusate) to the mPFC caused an increase in extracellular levels of DA, and a concurrent decrease in levels of DOPAC and HVA

- In a pilot study, systemic administration of amphetamine (1.5mg/kg) caused an increase in extracellular levels of DA in the mPFC, accompanied by small decreases in DOPAC and HVA

- In a pilot study, depletion of Ca^{2+} from the extracellular space (by omitting $CaCl_2$ from the perfusing aCSF) caused a decrease in extracellular levels of DA in the mPFC

- Systemic injection of D-serine (1280mg/kg) and sodium benzoate (500mg/kg) significantly increased levels of D-serine in both the cerebellum and PFC when compared with vehicle

- Systemic injection of D-serine and sodium benzoate had no significant effect at any individual time point on extracellular levels of DA, HVA or 5-HIAA in the mPFC, but resulted in a significant increase in levels of DOPAC and MHPG, when compared with vehicle

- When nomifensine was added locally to the mPFC (via the probe perfusate), and the probe membrane length increased from 2mm to 3mm, significant increases in extracellular levels of DOPAC, HVA, 5-HIAA and MHPG in the mPFC were detected following systemic injection of D-serine and sodium benzoate, but no significant change was detected in levels of DA, when compared with vehicle.

Pilot studies confirmed that the experimental method was suitable for the measurement of extracellular levels of DA and its metabolites in the mPFC of the anaesthetised rat. Local application of high K^+ caused an increase in extracellular DA levels (Figure 23). A high level of K^+ in the synapse causes local depolarisation, resulting in the influx of calcium into the neuron through voltage-gated calcium channels, and consequent exocytosis of intracellular vesicles. As such, extracellular DA levels increased, due to its release from intracellular vesicles into the synaptic cleft. Interestingly, there was a concurrent decrease in levels of DOPAC and HVA. This may be explained, at least in part, by the work of Zetterström and colleagues (1988). These authors proposed that the majority of DOPAC and HVA arise from the breakdown of newly synthesised intracellular pools of DA stores before it is released (Zetterström *et al*., 1988). Therefore, upon elevated DA release, newly synthesised DA stores become depleted, and consequently may lead to a decrease in DOPAC and HVA.

Amphetamine administration also evoked an increase in extracellular DA in the mPFC, accompanied by a small decrease in DOPAC and HVA (Figure 23). Amphetamine enhances synaptic DA via non-exocytic release from neurons. First, it acts to redistribute vesicular DA to the cytosol, increasing free intracellular DA (Sulzer *et al*., 1995). This increase then induces reverse transport of DA via DAT into the synaptic cleft

(Sulzer *et al*., 1995). Consequent depletion of newly synthesised intracellular DA stores again explains the observed decrease in levels of DA metabolites.

Upon omission of $CaCl_2$ from the probe perfusate, there was a decrease in extracellular DA (Figure 24). Depolarisation of the nerve terminal as the result of an action potential induces the opening of voltage-gated calcium channels, which allow an influx of Ca^{2+} into the neuron. This causes the intracellular vesicles to fuse with the plasma membrane, and stored neurotransmitter molecules are released into the synaptic cleft. As such, the presence of Ca^{2+} in the synaptic cleft is essential for normal DA release to occur. A reduction in the availability of Ca^{2+} limits the release of DA into the synaptic cleft. This results in little change in newly synthesised DA stores, and so the metabolite levels are largely unaffected.

Taken together, the series of pilot studies confirmed that the model was displaying the expected characteristics, and that the experimental method was allowing for measurement of DA and its metabolites in the mPFC.

The effect of D-serine in combination with sodium benzoate was tested in two sets of experiments; one in the absence of the DAT and NET inhibitor nomifensine, and the other in its presence. In both sets of experiments, systemic administration of sodium benzoate and D-serine in combination significantly increased the levels of D-serine in the cerebellum, when compared with vehicle (Figure 25, Figure 29). D-serine levels in the PFC were also measured in the second set of experiments, and were shown to be increased following D-serine and sodium benzoate administration when compared with vehicle (Figure 29). These findings verify that systemic administration of the drugs is having an effect on levels of D-serine in the brain.

In the first set of experiments, there was a trend towards an increase in levels of DA in the mPFC following D-serine and sodium benzoate administration, accompanied by a significant increase in levels of DOPAC (Figure 26). There was also an interaction between time and drug in the HVA measurements, but no individual time points showed a significant difference between the vehicle and treatment groups. From these data, it seemed conceivable that the systemic DAO inhibition combined with exogenous D-serine administration was activating the mesocortical DA pathway, but without producing a detectable release of DA.

This procedure was repeated using probes of longer membrane length and with local nomifensine application in the mPFC. DOPAC AND HVA again showed a significant increase following drug treatment when compared with controls, but without a detectable increase in DA (Figure 30). However, DA levels gradually increased in both groups, as demonstrated by the significant effect of time, putatively as a result of the addition of nomifensine and blockade of DAT and NET, preventing DA reuptake. In light of the significant increases observed in extracellular levels of DOPAC and HVA in the mPFC, these results provide evidence of an increase in activation of the mesocortical DA system. However, interpretation of these results must remain constrained by the bounds of reasonable assumption. Although levels of extracellular DOPAC and HVA in the mPFC increased, this is not necessarily evident of an increase in DA release, and one cannot unequivocally conclude that D-serine affects mesocortical DA transmission *per se*. The majority of DOPAC and HVA formed in the brain are as a result of the metabolism of newly synthesised DA (Zetterström *et al*., 1988). It is possible, therefore, that the observed increase in the DA metabolites is representative of an increase in the turnover of DA, and while more DA may be being synthesised, it is not necessarily being released.

Some interesting questions remain as to the origin of the increase in DA metabolites observed in this chapter. D-serine was putatively elevated throughout the brain as a result

of the systemic injections, and so the effects on DA may be caused by several possible processes. Elevated D-serine levels may directly affect DA terminals in the PFC, for example by increasing stimulation of presynaptic NMDA receptors, which may have a knock-on effect on DA metabolism. Another possibility is that the primary effect of the D-serine elevation lies within the cerebellum. Evidence suggests that the cerebellum can modulate dopaminergic function in the PFC, and that disrupted Purkinje cell output from the cerebellum may result in abnormal DA transmission in the PFC (Mittleman *et al*., 2008). In chapter 2 of this thesis, DAO was shown to be present in glia surrounding Purkinje cells, and was suggested to be serving to minimise D-serine levels in the locality. It is therefore possible that elevated D-serine levels in the cerebellum as a result of the systemic injections in this study may result in disrupted Purkinje cell output, and consequently affect levels of DA and its metabolites in the PFC. Furthermore, mesocortical DA neurons have their cell bodies in the VTA, and DA output from the VTA is modulated by NMDA receptors (see e.g. Bennett and Gronier, 2007). It is possible that elevated D-serine levels in the VTA as a result of the systemic injections is resulting in increased NMDA receptor activation on mesocortical DA neurons, culminating in an increase in their activity.

Extracellular 5-HIAA and MHPG levels also significantly increased following injection of D-serine and sodium benzoate in the presence of nomifensine (Figure 31). Given that the injections were given systemically, and levels of D-serine increased in two brain regions investigated, it is likely that the systemic injections resulted in an increase in D-serine levels throughout the brain. NMDA receptors on 5-HT and NA neurons may thus be affected by the increased D-serine levels, likely showing augmented function and consequent increased activation of their host neurons. The PFC receives considerable serotonergic input from the raphe nuclei (e.g. Smiley and Goldman-Rakic, 1996), and noradrenergic innervation from the locus coeruleus (e.g. Radley *et al*., 2008). NMDA

receptors have been shown to regulate the firing of serotonergic neurons in the raphe nuclei (Gartside *et al*, 2007), and noradrenergic neurons in the locus coeruleus (Charléty *et al*, 1993), with application of NMDA to the neurons resulting in increased firing rate in both cases. Increased activation of NMDA receptors on these neurons in the experiments above may therefore lead to increased firing of these neurons and consequent increased levels of 5-HT and noradrenaline in the mPFC. This may explain the increase in the levels of 5-HT and noradrenaline metabolites recorded.

Relatively few studies have considered the effect of systemic application of exogenous D-serine, or systemic DAO inhibition. One study, however, published by Bannai and colleagues (2011; after this study had been completed), investigated how oral administration of D-serine affected DA and 5-HT levels in the PFC of awake Wistar rats. D-serine administration yielded a transient (0-10 mins), yet significant, DA increase, and a similarly transient increase in levels of 5-HT. The authors concluded that both effects were likely as a result of NMDA receptor-modulation of PFC neurons. These results support the theory that enhanced availability of NMDA receptor glycine site agonists in the brain may alter NMDA receptor-mediated modulation of PFC neurons, and consequent extracellular levels of neurotransmitters.

In the current study, both DA metabolites and a 5-HT metabolite were increased following systemic administration of D-serine and a DAO inhibitor. This may be therapeutically relevant, as there is evidence for both 5-HT and DA deficits in the PFC in schizophrenia (Abi-Dargham, 2007; Davis *et al*., 1991), and so the manipulation of both neurotransmitter systems may be therapeutically useful. Increasing NMDA receptor-mediated stimulation of both 5-HT and DA neurons may result in an increased release of both neurotransmitters in the PFC that may synergistically improve the cognitive and negative symptoms of schizophrenia thought to originate therein.

In summary, this chapter has produced some interesting data suggesting that systemic administration of D-serine and a DAO inhibitor has an effect on cortical DA. This may be therapeutically relevant in the context of schizophrenia, in which an enhancement of mesocortical DA function may be beneficial in the treatment of cognitive and negative symptoms.

Chapter 4:

Investigation of the effects of D-serine manipulation in the VTA on extracellular DA in the PFC

4.1 Background

In Chapter 3, the effect of systemic D-serine manipulation on DA and its metabolites in the mPFC of the anaesthetised rat was investigated. The results of these experiments were equivocal; extracellular levels of the DA metabolites DOPAC and HVA were increased after systemic injection of D-serine and a DAO inhibitor, while levels of DA were not significantly changed. Levels of the 5-HT and noradrenaline metabolites 5-HIAA and MHPG (respectively) were also increased, suggesting that the increase in brain levels of D-serine was having an effect on NMDA receptors throughout the brain. This resulted in an increase in levels of multiple neurotransmitters in the mPFC.

Three possible explanations were explored for the effects on DA metabolites observed. First, elevated D-serine levels may directly affect DA terminals in the PFC, for example by increasing stimulation of presynaptic NMDA receptors, which may have a knock-on effect on DA metabolism. Second, the primary effect of the D-serine elevation could lie within the cerebellum, which has a role in the modulation of DA function in the PFC. Disrupted Purkinje cell output from the cerebellum as a result of D-serine elevation may result in a change in DA transmission in the PFC. Finally, mesocortical DA neurons have their cell bodies in the VTA, and DA output from the VTA is modulated by NMDA receptors (see e.g. Bennett and Gronier, 2007). Therefore, elevated D-serine levels in the VTA may increase NMDA receptor activation on mesocortical DA neurons, altering their activity. The third explanation for the observed changes was chosen for further investigation in this chapter.

Although several studies have considered the effect of direct NMDA receptor activation or inhibition on VTA DA neuron firing (e.g. Mathe *et al*., 1999; Svensson *et al*., 1998; Johnson *et al*., 1992; Suaud-Chagny *et al*., 1992; Chergui *et al*., 1993), relatively few have considered a role for the co-agonist glycine site of the NMDA receptor. One study,

however, investigated the effect of elevating levels of kynurenic acid (an endogenous antagonist of the NMDA receptor glycine site) on the phasic activity of DA neurons in the VTA (Erhardt and Engberg, 2002). Elevation of kynurenic acid levels resulted in a significant increase in both firing rate and bursting activity of VTA DA neurons. This effect was mimicked by the administration of MK 801, an NMDA receptor antagonist, and reversed by administration of D-cycloserine, a partial agonist at the NMDA receptor glycine site. As such, the study demonstrated that VTA DA neuron firing can be manipulated via the NMDA receptor glycine site.

A further study in 2008 considered how agonist activity at the glycine site may affect VTA DA neuron activity, and investigated the potential relevance of D-serine in behavioural sensitisation to cocaine (Fernandez-Espejo *et al*., 2008), a process critically involving DA transmission from the VTA (Kalivas and Stewart, 1991). There is enhanced glutamate release in the VTA in motor sensitisation to cocaine (Kalivas and Duffy, 1998), accompanied by increased NMDA and AMPA receptor stimulation, and an increase in calcium levels in dopaminergic neurons (Licata and Pierce, 2003). The authors therefore considered it conceivable that D-serine could participate in the sensitisation, given its role in NMDA receptor mediation. Administration of D-serine or sodium benzoate directly into the VTA enhanced the motor sensitisation to cocaine. Conversely, 7-chlorokynurenate (an antagonist at the glycine site of NMDA receptors) administration blocked the development of sensitisation. These findings implicate a functional role for D-serine in mediating NMDA receptor activity on DA neurons in the VTA, and thereby provide indirect evidence that DAO may also be active in the VTA. The demonstration that DAO is expressed in the VTA (Chapter 2) supports this possibility, but a functional role for DAO in the VTA has yet to be directly demonstrated.

4.2 Summary and aims

Taken together, the available data suggest a functional role for the NMDA receptor glycine site in modulating VTA DA neuron activity. As such, altering D-serine availability in the VTA may affect levels of DA in output regions of the different DA pathways, such as the PFC, the projection site of mesocortical DA neurons. This may help to explain changes in extracellular levels of DA metabolites seen in Chapter 3.

This study aimed to measure levels of DA and its metabolites in the mPFC of anaesthetised rats using *in vivo* microdialysis coupled with HPLC, while pharmacologically manipulating levels of D-serine specifically in the VTA. This will allow for the testing of the hypothesis that the increases in extracellular levels of the DA metabolites DOPAC and HVA seen in the previous chapter (following systemic injection of D-serine and a DAO inhibitor) may be mediated, at least in part, by the VTA. Firstly, NMDA was injected into the VTA, to directly activate NMDA receptors therein and compare observed with expected effects. Then, D-serine availability in the VTA was specifically altered via direct local injection of either D-serine or a DAO inhibitor. Injections of the following compounds were performed:

- D-serine
- Sodium benzoate (a DAO inhibitor)
- D-serine + sodium benzoate
- 5-chlorobenzo[d]isoxazol-3-ol (CBIO; a DAO inhibitor)
- 0.9% NaCl (vehicle control)

Measurements of DA and its metabolites were continued for 2 hours post-injection. Levels of the 5-HT metabolite 5-HIAA and the noradrenaline metabolite MHPG were also measured, to assess if any effects observed were specific to the DA system.

4.3 Methods

4.3.1 In vivo microdialysis – local injection

Adult male Sprague-Dawley rats (Harlan, UK) were anaesthetised with chloral hydrate (500mg/kg i.p.) and mounted in a stereotaxic frame in the flat skull position. Anaesthesia was maintained with 20–40mg doses of chloral hydrate throughout, and body temperature monitored and maintained at 36–37°C. Hydration was also closely monitored, and maintained using 4% glucose in 0.18% saline. A hole was made in the skull using a dental drill (Foredom®, Bethel, USA) and a microdialysis probe (crafted in-house) was implanted into the mPFC at the following coordinates relative to bregma: ML +0.6mm; AP +3.2mm; DV -5.0mm (from dura), according to the rat brain atlas of Paxinos and Watson (1998). The probe was secured using a single cranial screw (2mm, Royem Scientific, Luton, UK) and Simplex Rapid™ dental cement (Kemdent®, Swindon, UK). The probe was perfused with artificial CSF (containing 140.0mM NaCl, 3.0mM KCl, 1.2mM $Na_2HPO_4.2H_2O$, 0.27mM $NaH_2PO_4.H_2O$, 1.0mM $MgCl_2.6H_2O$, 2.4mM $CaCl_2$ and 7.2mM glucose) containing 3μM nomifensine at a flow rate of 1μl/min. Samples were collected every 20 minutes in small tubes containing 5μl of 0.1M perchloric acid, and run through the HPLC machine for analysis.

A second hole was then made in the skull, and a guide cannula (26G, 11m length, PlasticsOne®) implanted and cemented above the VTA at the following coordinates relative to bregma: ML + 0.8mm; AP -5.7mm; DV -6.8mm (from dura; best coordinates to hit VTA determined from pilot studies). Once a stable baseline was achieved, an injection needle (33G, extending 1mm below guide, PlasticsOne®) was inserted into the guide cannula and a single injection per animal administered (0.5μl) at a rate of 90nl/min.

The following injections were performed:

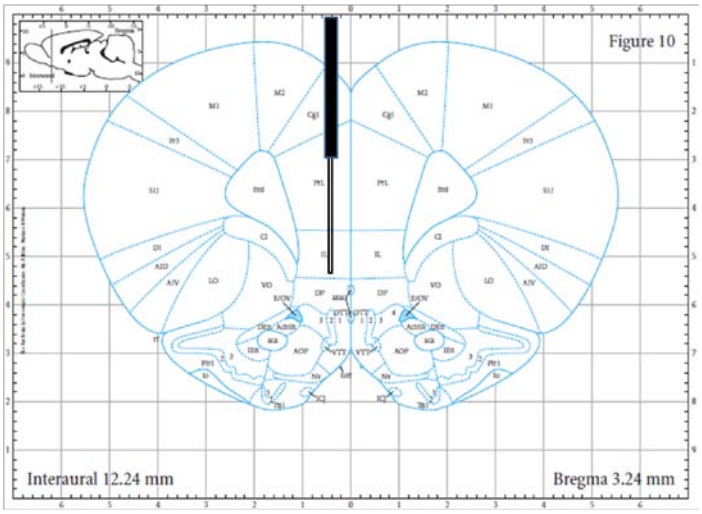
- 1mM NMDA in aCSF (n=1) or aCSF (vehicle; n=1)
- 5mM D-serine in 0.9% NaCl (n=4)
- 200µg/µl sodium benzoate in 0.9% NaCl (n=4)
- 5mM D-serine plus 200µg/µl sodium benzoate in 0.9% NaCl (n=7)
- 0.1µg CBIO (n=1), 1µg CBIO (n=3) or 10µg CBIO (n=1) in 0.9% NaCl
- 0.9% NaCl (vehicle; n=6. NB: The same vehicle group was used for comparison with all D-serine/DAO inhibitor treatment groups, in order to keep the number of animals used to a minimum.)

Sampling continued for 2 hours post-injection, after which a single injection of pontamine sky blue (0.5µl) was given through the injection cannula at the same rate to allow for histological determination of injection site placement. The animals were then euthanased, and their brains harvested. Both probe and injection placements were checked histologically.

4.3.2 Histological determination of probe and injection placement

Figure 32 shows a schematic representation of expected probe and injection site placements. Each individual brain was histologically assessed for placement accuracy, and those not matching this schematic removed from the analysis.

A



B

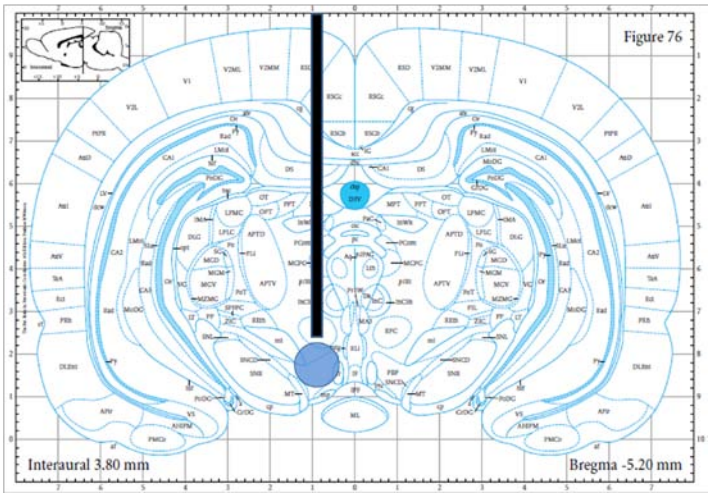


Figure 32. Schematic representation of observed mPFC probe (A) and VTA injection site (B) placement in sections of brain taken from animals used for microdialysis. *The blue circle in (B) represents the typical diffusion of pontamine sky blue observed. Brain atlas images taken from Paxinos and Watson, 2007.*

4.3.3 Drug preparation and dosing

4.3.3.1 D-serine

The dose of D-serine to be administered was determined based on the experimental protocol of Fernandez-Espejo and colleagues (Fernandez-Espejo *et al*., 2008). The behavioural effects of D-serine were only seen in the study when injected into the VTA at 5mM (not at 1mM); this was thus the dose selected. D-serine was freshly dissolved in 0.9% NaCl on the day of each experiment.

4.3.3.2 Sodium benzoate

The dose of sodium benzoate to be administered was also determined based on the experimental protocol of Fernandez-Espejo and colleagues (Fernandez-Espejo *et al*., 2008). Behavioural effects of sodium benzoate were seen at both 100 and 200µg/µl. 200µg/µl was selected as the dose for the current study, in order to try to maximise any effects seen. Sodium benzoate was prepared daily in 0.9% NaCl.

4.3.3.3 D-serine plus sodium benzoate

The doses used for the combination injection were the same as those for the individual injections; 5mM D-serine and 200µg/µl sodium benzoate. The two were dissolved together in 0.9% NaCl on the day of each experiment, and administered as a single bolus.

4.3.3.4 CBIO

At the time of the design of the experiment, no known studies had directly administered CBIO into the rat brain. However, one paper compared the effects of various DAO inhibitors on DAO enzymatic activity *in vitro*, before going on to investigate their effects on formalin-induced tonic pain in rats when injected intrathecally (Gong *et al*., 2011). CBIO was shown to have a half-inhibitory concentration (IC_{50}) value of 0.09µM, whereas sodium benzoate had a value of 15.4µM – values roughly two orders of magnitude different. When injected intrathecally, CBIO was administered in a range of doses from 0.01µg-3µg,

and sodium benzoate was administered in doses of 3–300µg. These dose ranges were again roughly two orders of magnitude different. As such, in this study, CBIO was injected at a dose range approximately two orders of magnitude smaller than the 100µg dose of sodium benzoate used above. A series of pilot experiments were performed, injecting CBIO at doses from 0.1–10µg.

CBIO was freshly dissolved in 0.9% NaCl solution on the day of each experiment. The pH of the solution was adjusted with 1M NaOH as needed to dissolve the compound, and then returned to pH7 using HCl.

4.3.4 Data analysis

Statistical analyses were carried out using SPSS software (version 17.0). A repeated measures ANOVA was performed to assess the effects of time and drug on DA, DOPAC, HVA, 5-HIAA and MHPG dialysate levels (relative to baseline). Simple main effects analysis was then performed if a drug x time interaction effect was seen in the original ANOVA. Time points were included from 20 minutes post-injection onwards.

4.3.5 Protocol optimisation: creating an HPLC Time File

Throughout Chapter 3, all samples analysed using HPLC were run with constant detection settings. Specifically, the range was kept constant at 5nA throughout each run. However, the DA peaks on the chromatograms were very small, and in some cases difficult to measure. Therefore, in order to ensure maximum sensitivity when analysing the peak produced by each of the sample eluates, it was pertinent to set up a 'Time File', allowing for changes in the range of detection at different time points during the run. Given that a smaller range allows for a greater degree of 'zoom' into the chromatogram, settings were altered such that the range was reduced to 0.2nA at the time of elution of

DA, resulting in a peak that was as large as possible on the screen. This allowed for accurate measurement of even subtle changes in peak height, and was the smallest range allowed by the system. Alterations were also made to the range at the time of elution of the other peaks at the start of each experiment, in order to ensure that the peaks were of sufficient size for accurate measurement. The peak heights were calibrated with reference standards daily.

Figure 33 and Figure 34 show two example reference standard traces, one with a constant time file and one with altering range settings, demonstrating the 'zooming' allowed by the time file.

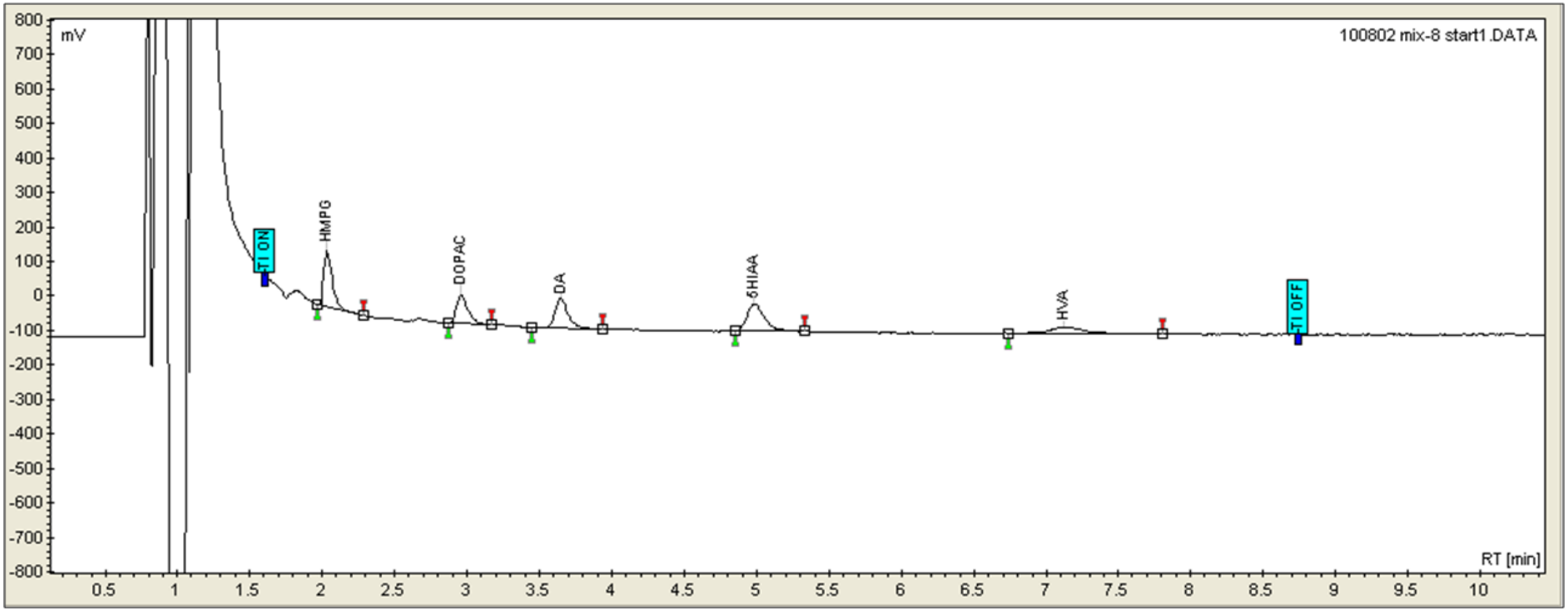


Figure 33. Example HPLC standard trace with a constant range setting of 5nA. *Each peak represents a known quantity (0.25 pmol) of each reference standard.*

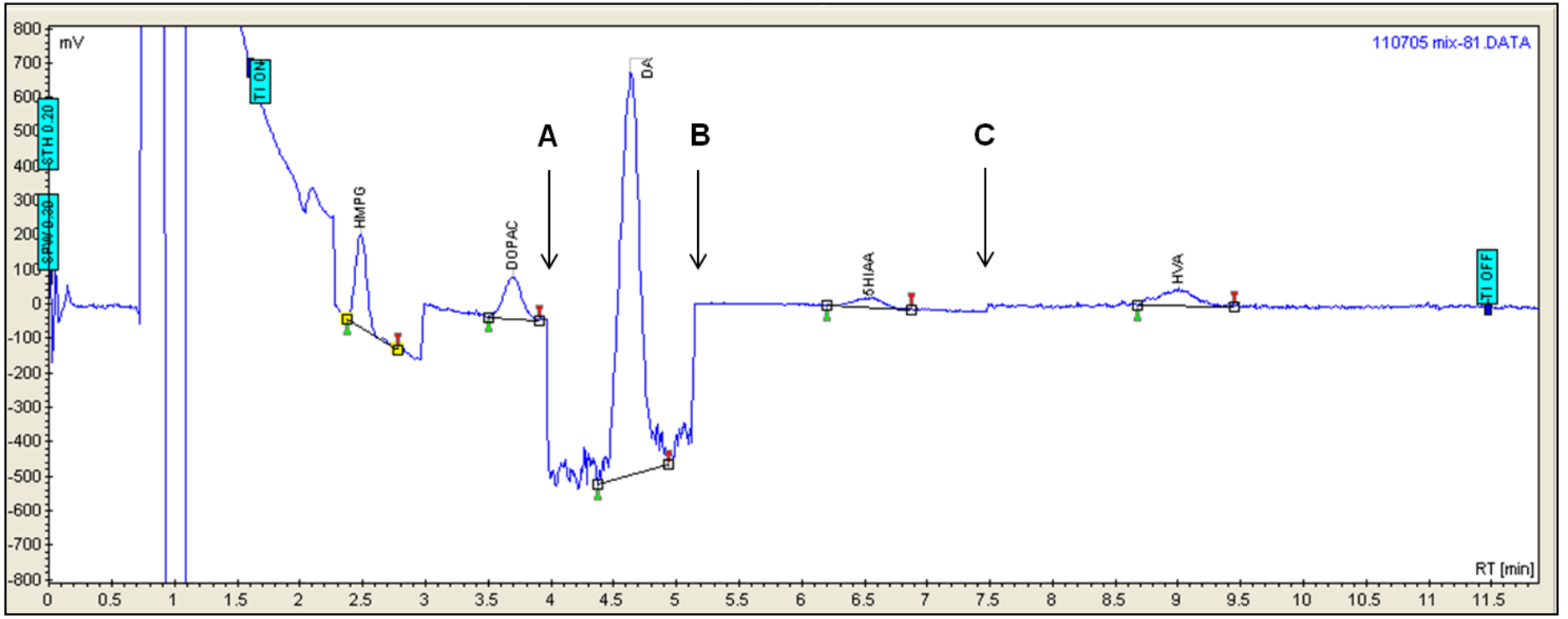


Figure 34. Example HPLC standard trace with variable range settings. *Each peak again represents a known quantity (0.25 pmol) of each reference standard. The run begins at a range setting of 5nA, and changes to 0.2nA at approximately 4 minutes (A), allowing for a degree of 'zoom' into the DA standard peak, and accurate measurement of very subtle changes in height. The range then alters to 10nA just after 5minutes(B), to allow for anticipated large 5-HIAA peaks during sample runs, and then changes back to 5nA at around the 7.5 minute mark (C) to accommodate anticipated HVA peaks.*

4.4 Results

4.4.1 Pilot study: Effect of NMDA injection into the VTA on extracellular DA and its metabolites in the mPFC

The administration of NMDA directly into the VTA caused an immediate increase in extracellular DA levels in the mPFC, which peaked 20 minutes after injection (Figure 35). There was also a slight increase in both DOPAC and HVA levels, peaking 100 minutes after injection (Figure 35). These results verified that NMDA receptor activation in the VTA elevated cortical DA and its metabolites, as would be expected. As such, the pilot study confirmed that the experimental procedure was successful in allowing for injection of a drug directly into the VTA, while concurrently measuring levels of cortical DA and its metabolites.

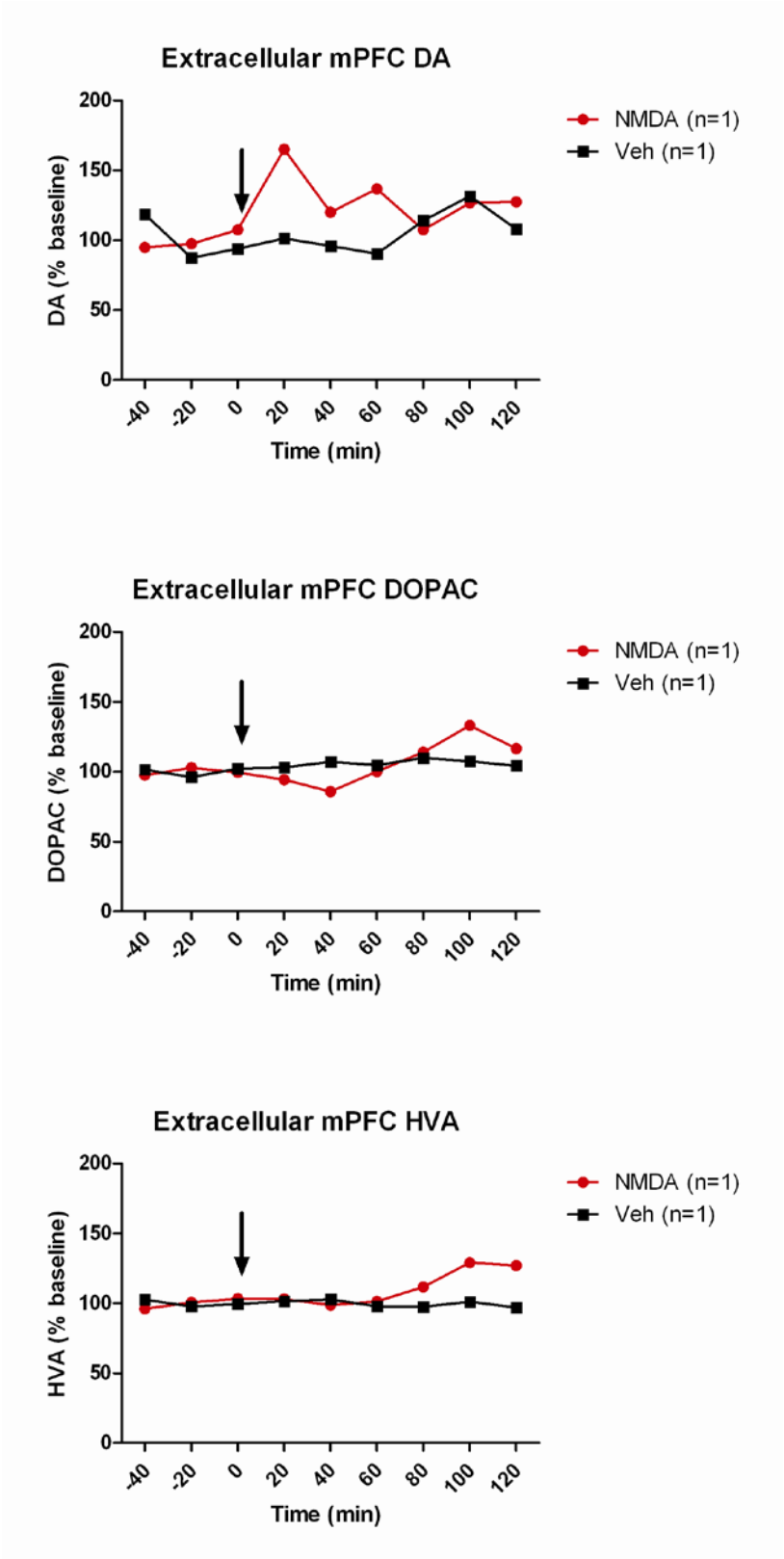


Figure 35. Effect of NMDA injection into the VTA on extracellular DA and its metabolites in the mPFC. *Time point 0 indicates the time at which the baseline was stable and either NMDA or vehicle (aCSF) was administered (arrow).*

4.4.2 Main study: Raw baseline dialysate levels

Table 7 shows the average raw baseline levels of DA, DOPAC, HVA, 5-HIAA and MHPG found in the final three baseline dialysis samples collected in each group before drug administration.

Treatment Group	DA	DOPAC	HVA	5-HIAA	MHPG
D-serine	0.054 ± 0.005 (n=4)	0.681 ± 0.027 (n=4)	1.890 ± 0.259 (n=4)	2.205 ± 0.130 (n=4)	0.104 ± 0.021 (n=3)
Sodium benzoate	0.060 ± 0.010 (n=4)	0.835 ± 0.247 (n=4)	1.779 ± 0.441 (n=4)	2.376 ± 0.286 (n=4)	0.152 ± 0.020 (n=3)
D-serine + sodium benzoate	0.056 ± 0.007 (n=7)	0.485 ± 0.120 (n=7)	1.470 ± 0.344 (n=7)	1.766 ± 0.382 (n=7)	0.092 ± 0.014 (n=7)
CBIO	0.071 (n=2)	0.621 ± 0.170 (n=3)	2.103 ± 0.327 (n=3)	3.298 ± 0.510 (n=3)	0.149 (n=2)
Vehicle (0.9% NaCl)	0.063 ± 0.008 (n=5)	0.538 ± 0.134 (n=6)	1.865 ± 0.251 (n=6)	2.676 ± 0.228 (n=6)	0.133 ± 0.024 (n=6)

Table 7. Raw baseline levels of DA, DOPAC, HVA, 5-HIAA and MHPG in the dialysates from male Sprague-Dawley rats. *Values given are mean ± S.E.M. (pmol) in each treatment group, and are calculated from the final three baseline samples collected from each animal before any drugs were administered.*

4.4.3 Effect of D-serine injection into the VTA on extracellular DA and its metabolites in the mPFC

Injection of D-serine into the VTA had no effect on extracellular levels of DA in the mPFC when compared with vehicle [effect of drug $F(1,7)=0.223$, $p=0.651$; effect of time $F(5,35)=1.081$, $p=0.388$; drug x time interaction $F(5,35)=0.854$, $p=0.521$; Figure 37]. There were, however, clear increases in extracellular DOPAC and HVA levels in the mPFC after D-serine injection, which continued to rise throughout the 120 minutes after injection [DOPAC: effect of drug $F(1,8)=8.582$, $p=0.019$; effect of time $F(5,40)=30.886$, $p<0.001$; drug x time interaction $F(5,40)=7.006$, $p<0.001$; HVA: effect of drug $F(1,8)=22.805$, $p=0.001$; effect of time $F(5,40)=41.256$, $p<0.001$; drug x time interaction $F(5,40)=8.076$, $p<0.001$; Figure 37].

There was no effect of intra-VTA D-serine injection on mPFC levels of 5-HIAA or MHPG when compared with vehicle [5-HIAA: effect of drug $F(1,8)=0.006$, $p=0.939$; effect of time $F(5,40)=1.401$, $p=0.245$; drug x time interaction $F(5,40)=1.006$, $p=0.427$; Figure 37. MHPG: effect of drug $F(1,7)<0.001$, $p=0.996$; effect of time $F(5,35)=0.306$, $p=0.906$; drug x time interaction $F(5,35)=0.211$, $p=0.956$; Figure 37].

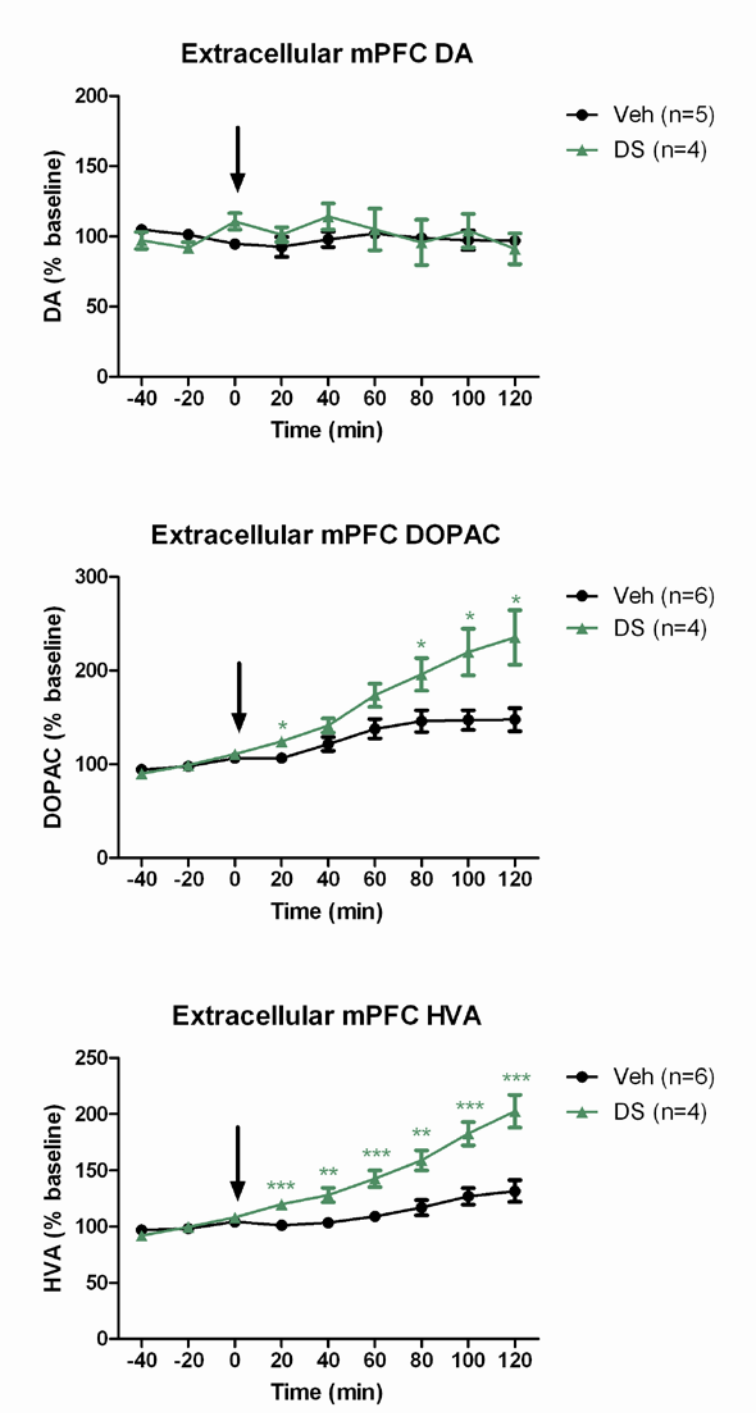


Figure 36. Effect of D-serine injection into the VTA on extracellular DA and its metabolites in the mPFC. *Time point 0 indicates the time at which the baseline was stable and local injection of D-serine, or vehicle, was administered. Points represent mean ± SEM, and are expressed as a percentage of the final three baseline samples. Statistical analysis carried out with repeated measures ANOVA and simple main effects post-hoc testing. *p<0.05, **p<0.01, ***p<0.005.*

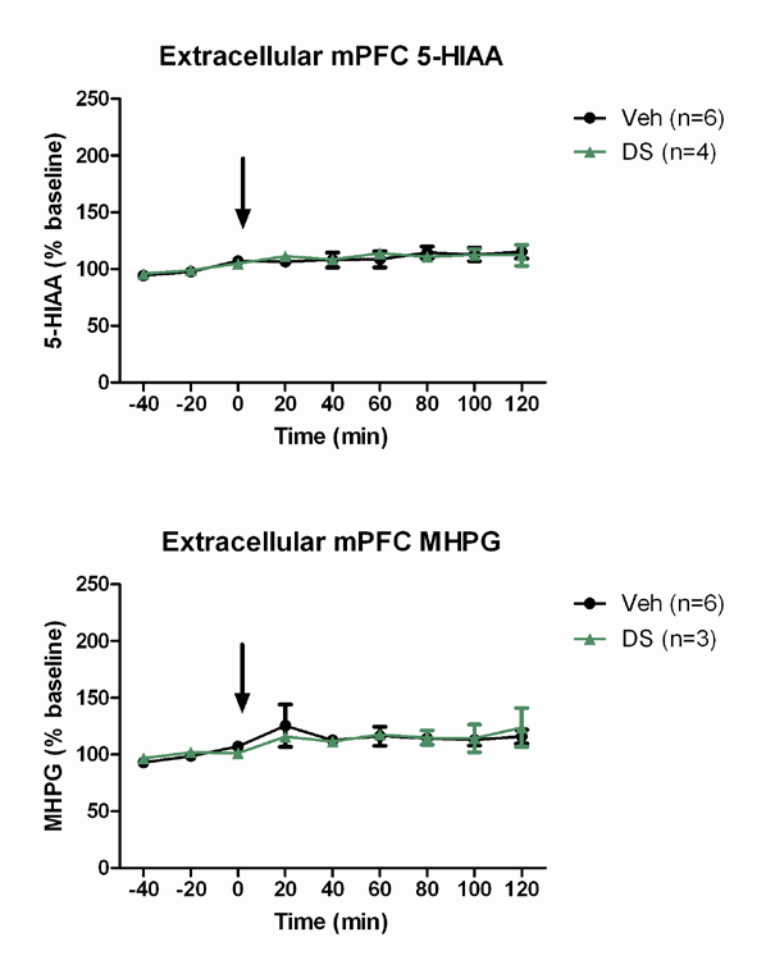


Figure 37. Effect of D-serine injection into the VTA on extracellular 5-HIAA and MHPG in the mPFC. *Time point 0 indicates the time at which the baseline was stable and local injection of D-serine, or vehicle, was administered. Points represent mean ± SEM, and are expressed as a percentage of the final three baseline samples. Statistical analysis carried out with repeated measures ANOVA.*

4.4.4 Effect of sodium benzoate injection into the VTA on extracellular DA and its metabolites in the mPFC

There were robust increases in extracellular levels of DA, DOPAC and HVA in the mPFC following intra-VTA injection of sodium benzoate when compared with vehicle (Figure 38), but no clear changes in levels of 5-HIAA or MHPG (Figure 40).

Overall, levels of DA were increased by sodium benzoate injection but not by vehicle [effect of drug: $F(1,7)=14.559$, $p=0.007$], and peaked at 40 minutes post-injection [main effect of time: $F(5,35)=5.413$, $p=0.001$; drug x time interaction [$F(5,35)=5.456$, $p=0.001$; Figure 38]. There was a similar effect on levels of DOPAC and HVA, which peaked at 100 and 120 minutes post-injection, respectively [DOPAC: effect of drug $F(1,8)=8.643$, $p=0.019$; effect of time $F(5,40)=48.919$, $p<0.001$; drug x time interaction $F(5,40)=16.775$, $p<0.001$. HVA: effect of drug $F(1,8)=11.931$, $p=0.009$; effect of time $F(5,40)=37.848$, $p<0.001$; drug x time interaction $F(5,40)=8.625$, $p<0.001$; Figure 38].

Intra-VTA injection of sodium benzoate had no clear effect on extracellular levels of 5-HIAA [effect of drug $F(1,8)=2.483$, $p=0.154$; Figure 40] or MHPG [effect of drug $F(1,7)=0.297$, $p=0.603$; effect of time $F(5,35)=0.645$, $p=0.667$; drug x time interaction $F(5,35)=1.106$, $p=0.375$; Figure 40], although a subtle increase in 5-HIAA over time was detected [effect of time $F(5,40)=13.423$, $p<0.001$; drug x time interaction $F(5,40)=3.553$, $p=0.009$].

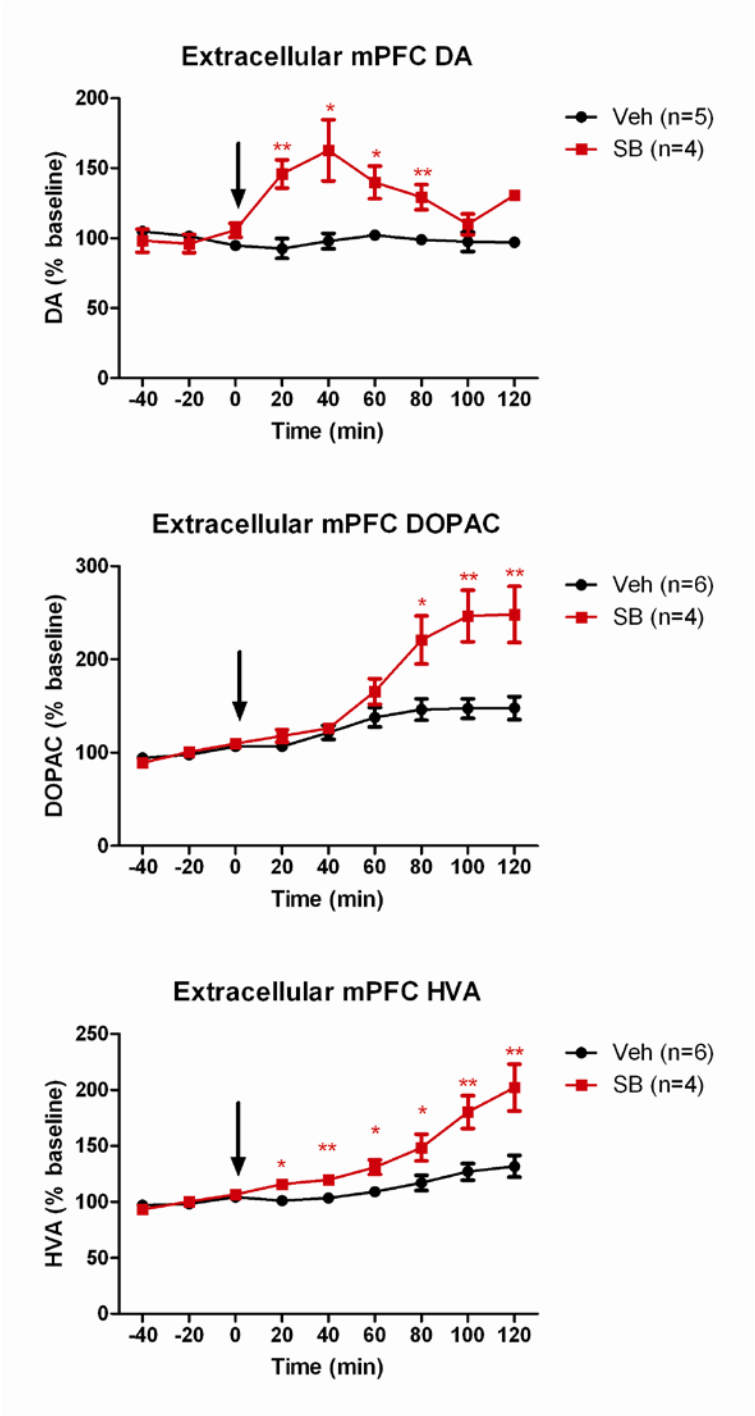


Figure 38.Effect of sodium benzoate injection into the VTA on extracellular DA and its metabolites in the mPFC. *Time point 0 indicates the time at which the baseline was stable and local injection of sodium benzoate, or vehicle, was administered. Points represent mean ± SEM, and are expressed as a percentage of the final three baseline samples. Statistical analysis carried out with repeated measures ANOVA and simple main effects post-hoc testing. *p<0.05, **p<0.01, ***p<0.005.*

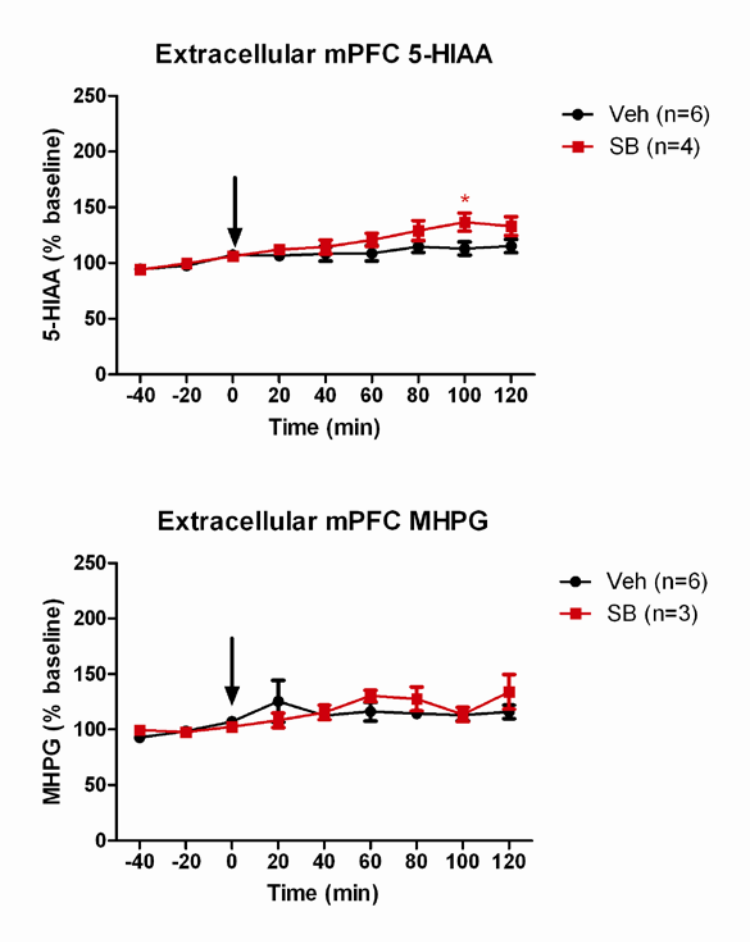


Figure 39. Effect of sodium benzoate injection into the VTA on extracellular 5-HIAA and MHPG in the mPFC. *Time point 0 indicates the time at which the baseline was stable and local injection of sodium benzoate, or vehicle, was administered. Points represent mean ± SEM, and are expressed as a percentage of the final three baseline samples. Statistical analysis carried out with repeated measures ANOVA and simple main effects post-hoc testing. *p<0.05.*

4.4.5 Effect of concurrent D-serine and sodium benzoate injection into the VTA on extracellular DA and its metabolites in the mPFC

There was a clear increase in extracellular levels of DA in the mPFC following intra-VTA injection of D-serine and sodium benzoate in combination, when compared with vehicle [effect of drug $F(1,10)=5.504$, $p=0.041$], which peaked 40 minutes after injection [effect of time $F(5,50)=3.252$, $p=0.013$; drug x time interaction $F(5,50)=3.678$, $p=0.007$; Figure 40]. This time by drug interaction was driven by an increase in DA seen in the D-serine and sodium benzoate treatment group that was not seen in the vehicle group.

Extracellular levels of DOPAC in the mPFC were not significantly affected by injection of D-serine and sodium benzoate, when compared with vehicle [effect of drug $F(1,11)=0.714$, $p=0.416$; Figure 40], Despite this, there was a significant effect of time on levels of DOPAC [$F(5,55)=13.062$, $p<0.001$], and a significant drug x time interaction [$F(5,55)=3.117$, $p=0.015$]. However, no individual time points were significant. Individual analysis of the effect of time on each treatment group revealed a significant effect on each, describing the increase in levels of DOPAC seen in both the SB+DS and vehicle group [SB+DS: $F(5,30)=8.838$, $p<0.001$; veh: $F(5,25)=13.309$, $p<0.001$].

There was no effect of combined D-serine and sodium benzoate injection into the VTA on mPFC extracellular levels of HVA, when compared with vehicle [effect of drug $F(1,11)=1.764$, $p=0.211$; Figure 40]. There was, however, a significant main effect of time [$F(5,55)=16.888$, $p<0.001$], but no significant drug x time interaction [$F(5,55)=1.081$, $p=0.381$] reflecting an increase in HVA over time in both treatment groups (Figure 40).

Intra-VTA injection of D-serine and sodium benzoate in combination also had no effect on mPFC levels of 5-HIAA [effect of drug $F(1,11)=0.451$, $p=0.516$; drug x time interaction $F(5,55)=0.742$, $p=0.596$; Figure 42]. There was, however, a main effect of time of 5-HIAA levels [$F(5,55)=3.391$, $p=0.010$], although individual analysis of the two treatment groups revealed no significant effect in either, despite a trend towards significance in both

[SB+DS: F(5,30)=2.319, p=0.068; veh: F(5,25)=2.554, p=0.053]. D-serine and sodium benzoate injection had no significant effect on extracellular levels of MHPG in the mPFC, when compared with vehicle injection [effect of drug F(1,11)=0.162, p=0.695; effect of time F(5,55)=0.206, p=0.959; drug x time interaction F(5,55)=0.461, p=0.803; Figure 42].

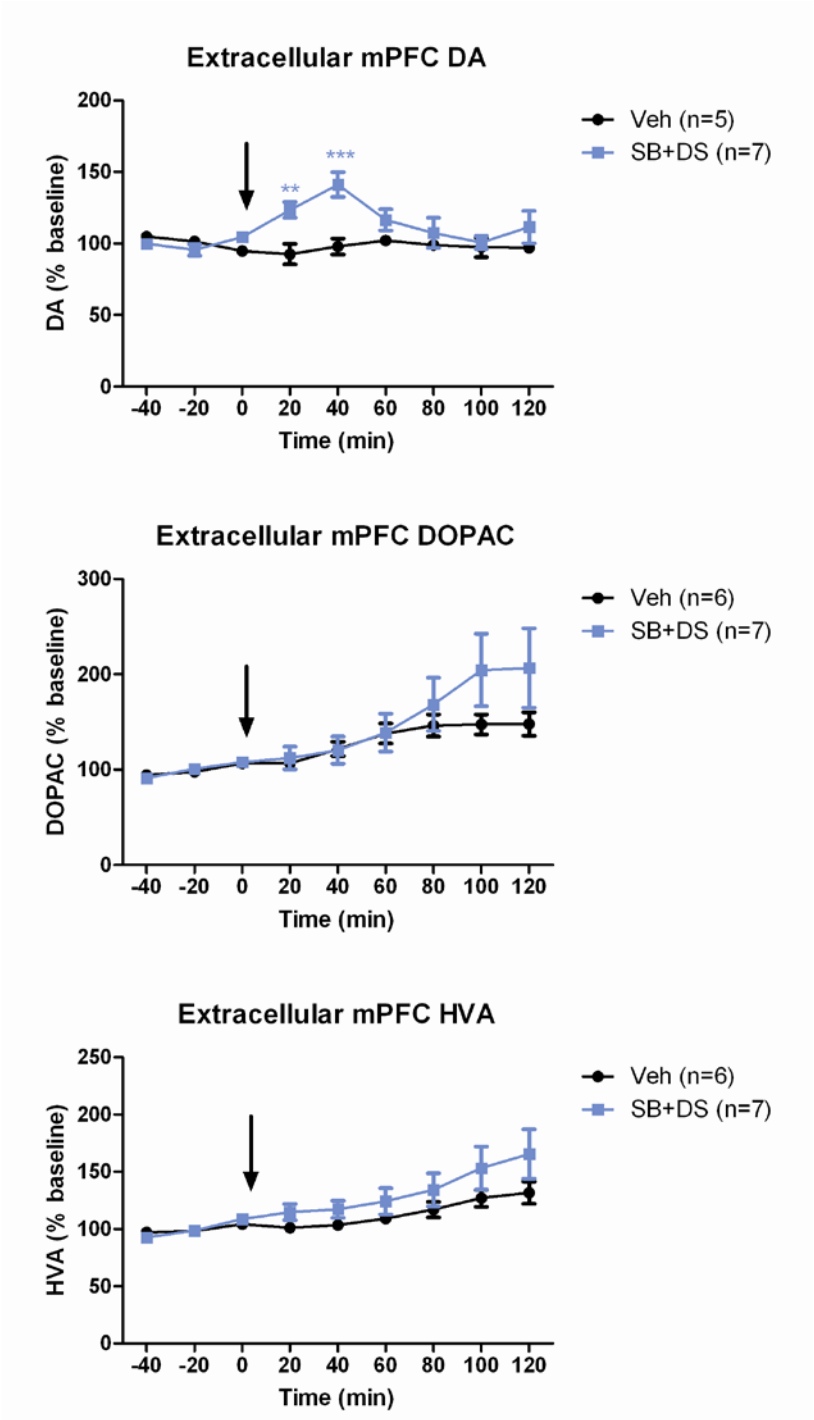


Figure 40. Effect of concurrent D-serine and sodium benzoate injection into the VTA on extracellular DA and its metabolites in the mPFC. *Time point 0 indicates the time at which the baseline was stable and local injection of D-serine and sodium benzoate, or vehicle, was administered. Points represent mean ± SEM, and are expressed as a percentage of the final three baseline samples. Statistical analysis carried out with repeated measures ANOVA and simple main effects post-hoc testing. *p<0.05, **p<0.01, ***p<0.005.*

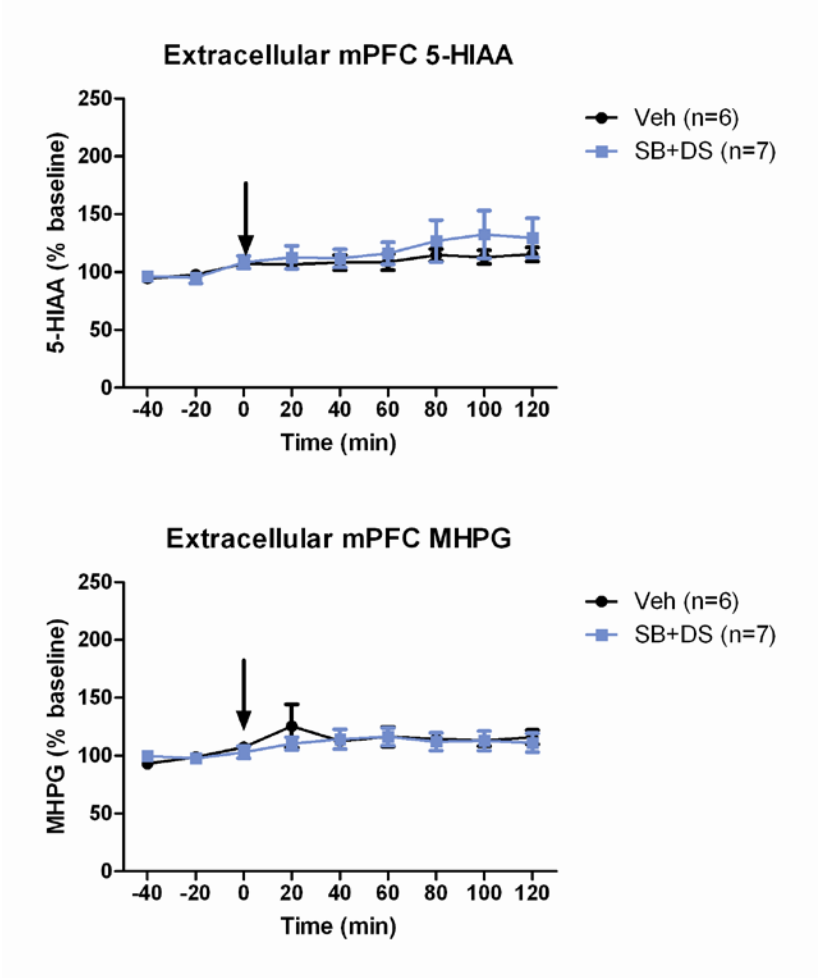


Figure 41. Effect of concurrent D-serine and sodium benzoate injection into the VTA on extracellular 5-HIAA and MHPG in the mPFC. *Time point 0 indicates the time at which the baseline was stable and local injection of D-serine and sodium benzoate, or vehicle, was administered. Points represent mean ± SEM, and are expressed as a percentage of the final three baseline samples. Statistical analysis carried out with repeated measures ANOVA.*

4.4.6 Effect of CBIO injection into the VTA on extracellular DA and its metabolites in the mPFC

First, pilot injections of 0.1µg, 1µg and 10µg CBIO were administered into the VTA. When plotted together, these three doses did not appear to differ in their effects on extracellular levels of DA, DOPAC, HVA, 5-HIAA and MHPG in the mPFC (Figure 42, Figure 43). As such, the intermediate dose of 1µg was selected, and two more experiments at this dose were performed. These results were plotted with the vehicle traces from the previous experiments (Figure 44, Figure 46).

CBIO injection into the VTA had no effect on extracellular levels of DA in the mPFC when compared with vehicle injection [effect of drug $F(1,5)=0.229$, $p=0.652$; effect of time $F(5,25)=0.727$, $p=0.609$; drug x time interaction $F(5,25)=1.877$, $p=0.134$; Figure 44]. There was also no effect on levels of DOPAC [effect of drug $F(1,7)=0.873$, $p=0.381$; Figure 44], although analysis of the effects of time revealed a significant change [$F(5,35)=16.346$, $p<0.001$], despite no drug x time interaction [$F(5,35)=0.573$, $p=0.720$]. Both the vehicle and CBIO traces clearly show an increase in DOPAC over time (Figure 44).

Similarly, despite an effect of time on HVA levels following CBIO injection [$F(5,35)=17.455$, $p<0.001$; Figure 44], there was no significant effect of drug [$F(1,7)=0.236$, $p=0.642$], or drug x time interaction [$F(5,35)=0.284$, $p=0.919$]. Again this is indicative of an increase in levels of HVA over time in both the CBIO and vehicle groups, as can be seen in Figure 44.

Injection of CBIO in the VTA had no significant effect on levels of 5-HIAA in the mPFC when compared with vehicle [effect of drug $F(1,7)=1.163$, $p=0.317$; Figure 46]. There was also no significant effect of time [$F(5,35)=1.239$, $p=0.312$], although there was a drug x time interaction [$F(5,35)=3.228$, $p=0.017$]. Further analysis revealed no significant differences between the two groups at any individual time point.

Finally, there was no effect of intra-VTA injection of CBIO on extracellular levels of MHPG in the mPFC when compared with vehicle injection [effect of drug $F(1,6)=1.027$, $p=0.350$; effect of time $F(5,30)=0.119$, $p=0.987$; drug x time interaction $F(5,30)=0.170$, $p=0.972$; Figure 46].

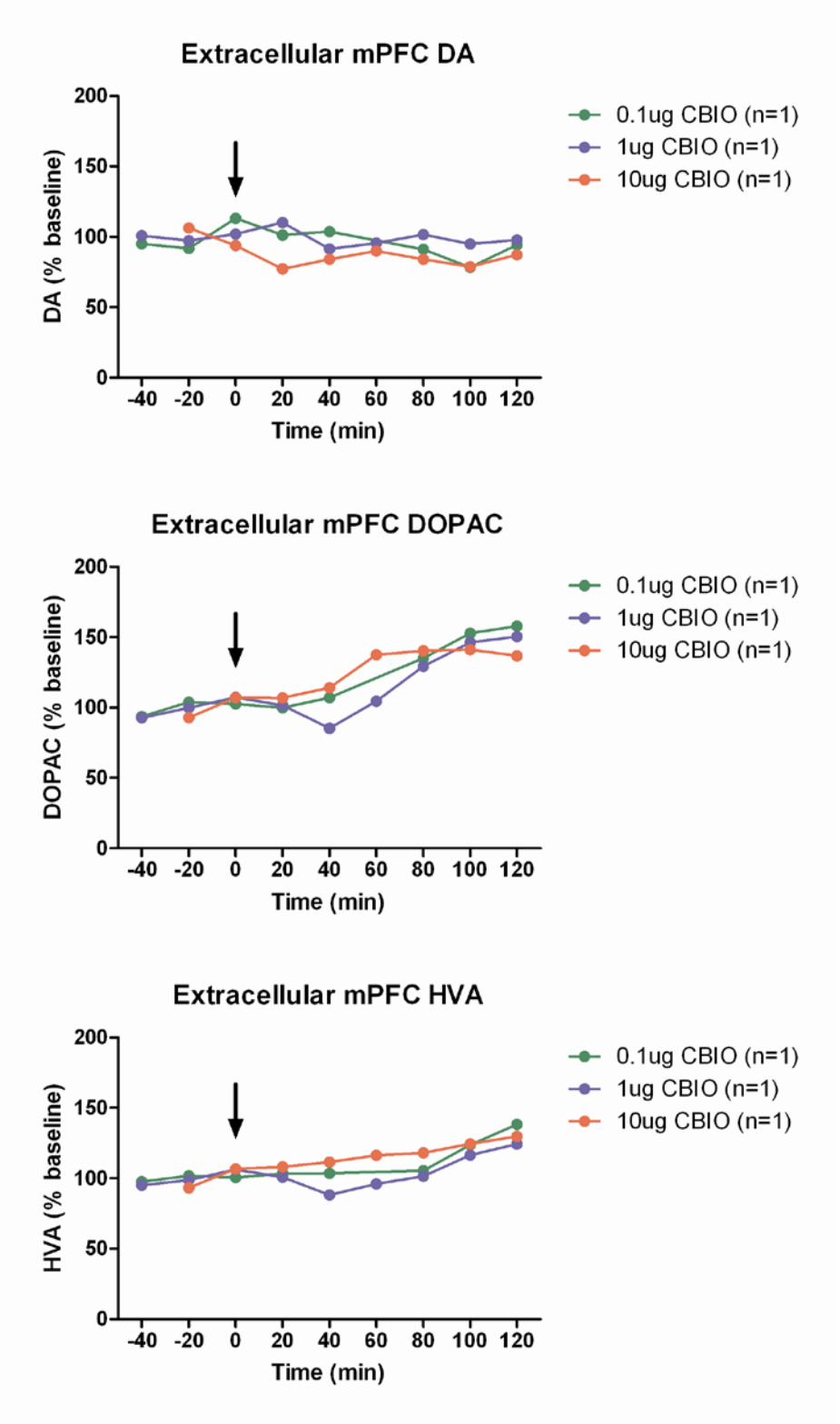


Figure 42. Effect of 0.1–10µg CBIO injection into the VTA on extracellular DA and its metabolites in the mPFC. *Time point 0 indicates the time at which the baseline was stable and a single local injection of CBIO (0.1, 1, or 10µg) was administered.*

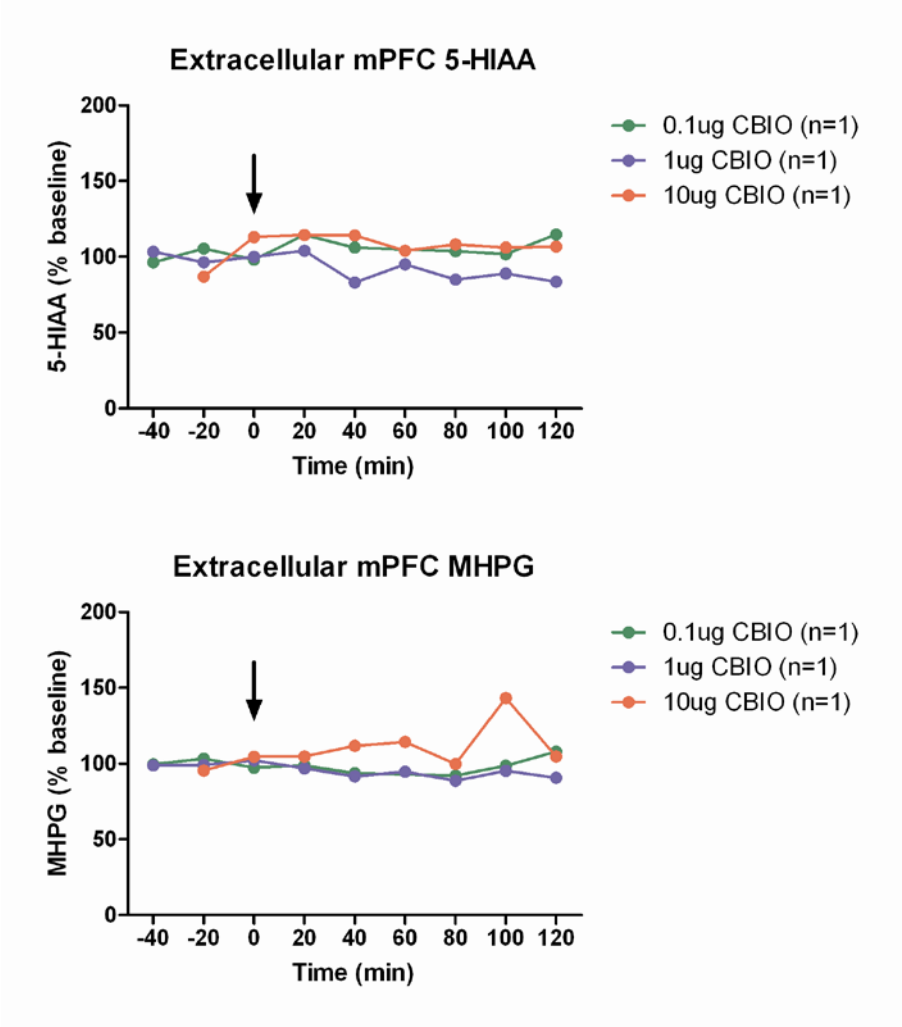


Figure 43. Effect of 0.1–10µg CBIO injection into the VTA on extracellular 5-HIAA and MHPG in the mPFC. *Time point 0 indicates the time at which the baseline was stable and a single local injection of CBIO (0.1, 1, or 10µg) was administered.*

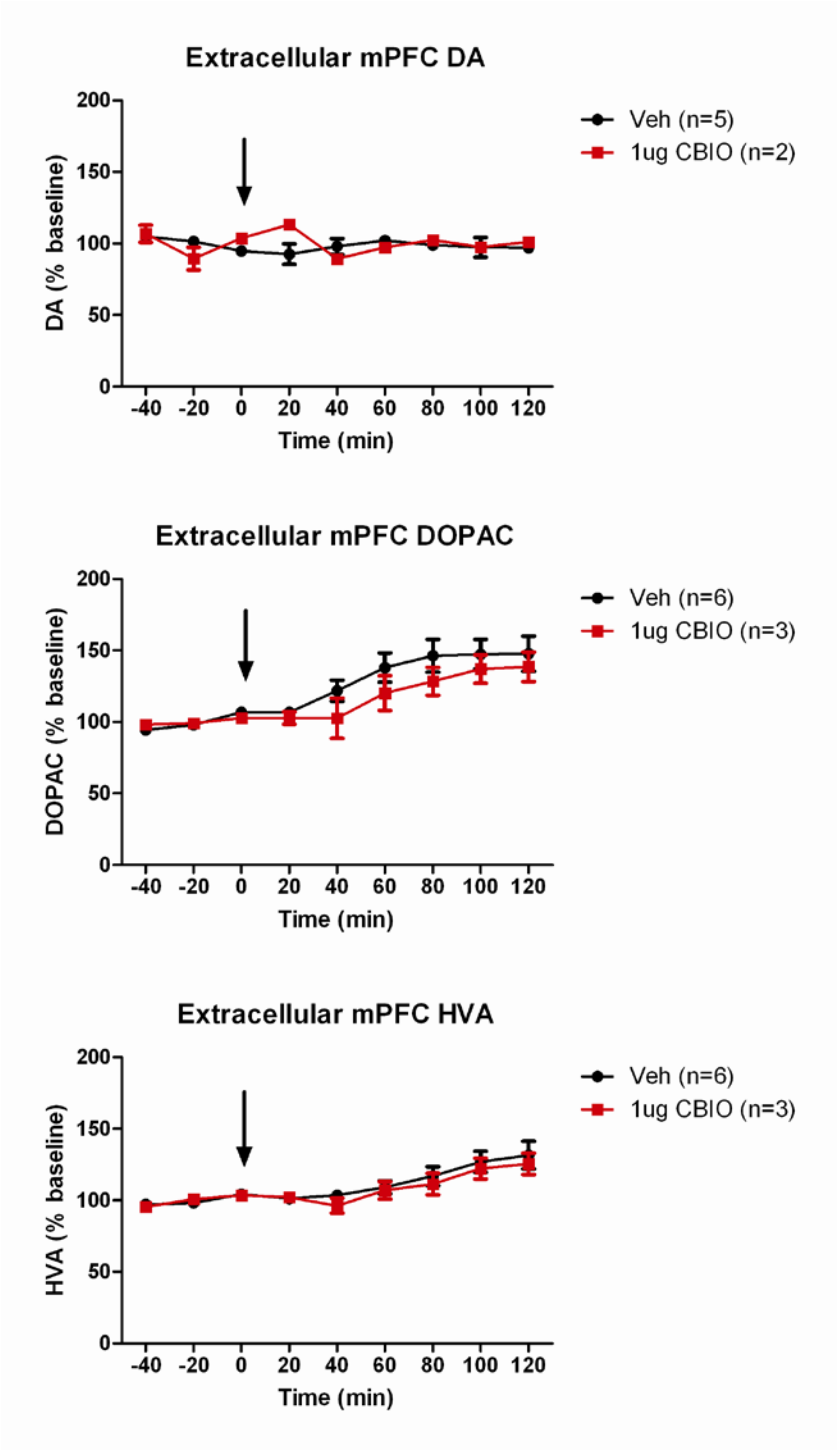


Figure 44. Effect of 1µg CBIO injection into the VTA on extracellular DA and its metabolites in the mPFC. *Time point 0 indicates the time at which the baseline was stable and a single local injection of CBIO was administered. Points represent mean ± SEM, and are expressed as a percentage of the final three baseline samples. Statistical analysis carried out with repeated measures ANOVA.*

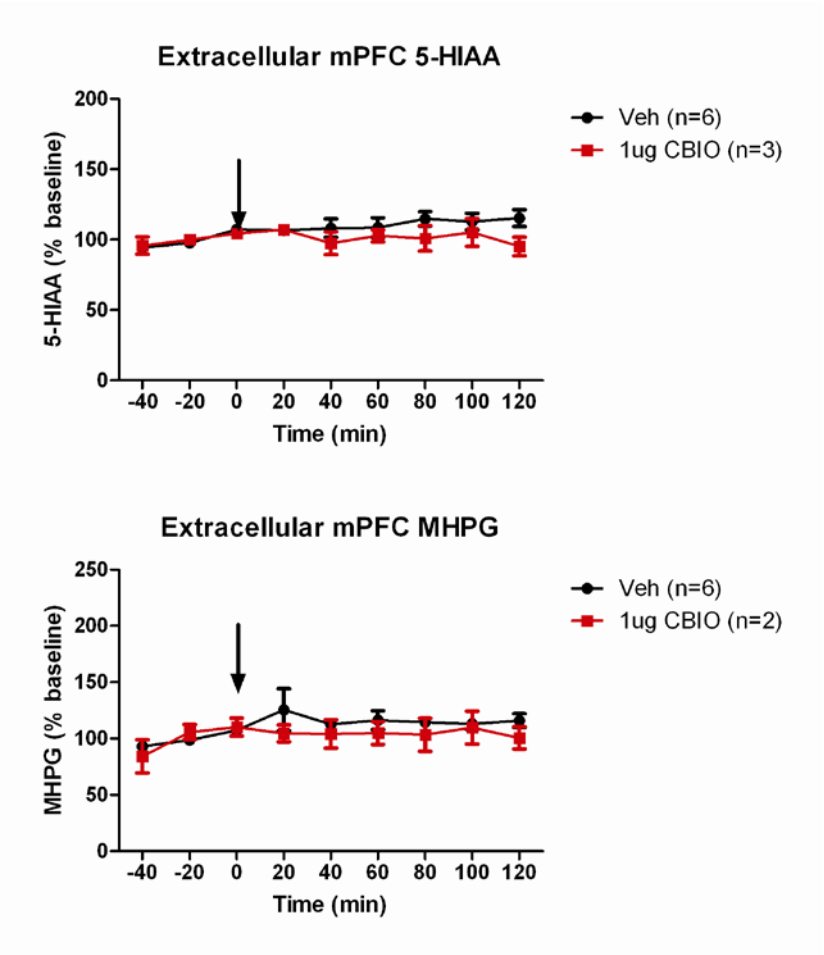


Figure 45. Effect of 1µg CBIO injection into the VTA on extracellular 5-HIAA and MHPG in the mPFC. *Time point 0 indicates the time at which the baseline was stable and a single local injection of CBIO was administered. Points represent mean ± SEM, and are expressed as a percentage of the final three baseline samples. Statistical analysis carried out with repeated measures ANOVA.*

4.5 Discussion

Experiments in this chapter successfully established a protocol in which *in vivo* microdialysis coupled with HPLC was used to measure extracellular levels of DA, DOPAC, HVA, 5-HIAA and MHPG in the mPFC of the anaesthetised rat, while concurrently performing intra-VTA injections of exogenous D-serine and DAO inhibitors.

The results can be summarised as follows:

1. Intra-VTA injection of D-serine caused an increase in extracellular levels of DOPAC and HVA in the mPFC when compared with vehicle, but had no effect on levels of DA, 5-HIAA or MHPG (Figure 37, Figure 38).

2. Intra-VTA injection of sodium benzoate caused an increase in mPFC levels of DA, DOPAC and HVA when compared with vehicle, but had no significant effect on levels of 5-HIAA or MHPG (Figure 39, Figure 40).

3. Intra-VTA injection of both D-serine and sodium benzoate together resulted in an increase in levels of DA in the mPFC when compared with vehicle, but had no significant effect on levels of DOPAC, HVA, 5-HIAA or MHPG (Figure 41, Figure 42).

4. Intra-VTA injection of CBIO had no effect on mPFC levels of DA, DOPAC, HVA, 5-HIAA or MHPG, when compared with vehicle (Figure 45, Figure 46).

Previous experiments have shown that administering D-serine or sodium benzoate into the VTA affects motor sensitisation to cocaine, which is known to be reliant on DA transmission from the VTA (Fernandez-Espejo *et al*., 2008). The current experiments are consistent with this; following intra-VTA injection of D-serine or sodium benzoate, there was an increase in extracellular levels of DOPAC and HVA in the mPFC, which is the

projection site of mesocortical DA neurons originating in the VTA. As such, DA transmission from the VTA appears to have been increased. In Chapter 3, systemic injection of D-serine and a DAO inhibitor resulted not only in an increase in levels of DOPAC and HVA in the mPFC, but also levels of 5-HIAA and MHPG. This was consistent with a study by Bannai and colleagues, who demonstrated that oral administration of D-serine to rats resulted in transient increases in both DA and 5-HT in the PFC (Bannai *et al*., 2011). The authors suggested that the global increase in brain levels of D-serine as a result of systemic injection was having an augmenting effect on NMDA receptor function throughout the brain, including perhaps those receptors located on 5-HT neurons in the raphe nuclei and those on noradrenergic neurons in the locus coeruleus (both of which project to the PFC), in addition to those on DA neurons in the VTA. However, in this chapter, no effect was seen on levels of 5-HIAA and MHPG in the mPFC after injection of D-serine and DAO inhibitors directly into the VTA. As such, this suggests that the DA system is being specifically targeted by the intra-VTA injections in this study, and that the 5-HT and noradrenergic systems are not being directly affected.

When D-serine was injected into the VTA, there was an increase in mPFC extracellular levels of DOPAC and HVA, but in the absence of any detectable changes in DA levels (Figure 37). There are several possible explanations for this finding. First, it could be that DA release is increased after D-serine, but then is rapidly taken up and metabolised before it is able to diffuse across the microdialysis probe membrane; in other words, the time resolution of the detection technique may be insufficient to detect transient changes. Synaptic DA can be metabolised in one of two ways; first, via degradation to DOPAC by monoamine oxidase (MAO) and aldehyde dehydrogenase, followed by further metabolism to HVA by catechol-O-methyltransferase (COMT), or second, by initial degradation by COMT to 3-methoxytyramine (3-MT), then further metabolism by MAO and aldehyde dehydrogenase to HVA. However, both these pathways require initial re-uptake of DA

from the synaptic cleft by either the DA transporter DAT, or the noradrenaline transporter NET, with the latter thought to be the major transporter for DA uptake in the PFC (Moron *et al*., 2002), However, it seems unlikely that the lack of detected change in DA levels following injection is due to DA reuptake and metabolism, since the perfusing aCSF contained nomifensine, an inhibitor of both DAT and NET (Bymaster *et al*., 2002).

A further possible explanation involves the theory of NMDA receptor internalisation (Nong *et al*., 2003). Stimulation of the glycine site of the NMDA receptor by glycine or D-serine induces priming of the receptors for clathrin-dependent endocytosis. Binding of both a glycine site agonist and glutamate site agonist is required for internalisation, but the initiation of signalling through the NMDA receptor complex by binding at the glycine site precedes this internalisation (Nong *et al*., 2003). It is possible that the elevated levels of D-serine as a result of its local injection are priming the NMDA receptor on DA neurons for internalisation, such that physiological stimulation by glutamate then causes the receptors to internalise. This would compensate for the increase in NMDA receptor-mediated neuron stimulation that may otherwise occur. This may explain the increase in levels of DOPAC and HVA observed; perhaps the signal transduced through the NMDA receptor complex as a result of D-serine binding initiates an increase in DA synthesis. However, since the receptors then rapidly internalise upon binding of glutamate, any increase in DA firing that may occur as a result of increased NMDA receptor activation may be very short-lived, and may not produce a detectable increase in DA levels in the mPFC. However, since the majority of DOPAC and HVA formed in the brain are as a result of the breakdown of newly synthesised DA before it is released (Zetterström *et al*., 1988), DOPAC and HVA would increase. Electrophysiological measurement of the firing rate of DA neurons after intra-VTA administration of D-serine would therefore be key in further investigating this idea. Indeed, initial studies using electrophysiology suggest an increase in burst firing in some VTA DA neurons following systemic administration of

D-serine (Dr Judith Schweimer, personal communication). A further interesting follow-up experiment would be to carry out additional intra-VTA injections of different doses of D-serine in combination with a measurement of receptor internalisation, in order to investigate a potential threshold for maximum D-serine levels in the VTA before receptor internalisation is primed.

Clearly, an intra-VTA injection of D-serine would appear to be having an activating effect on the mesocortical DA neurons in some form, though the exact explanation is uncertain. It is important to note that exogenous D-serine injected into the VTA is still susceptible to degradation by endogenous DAO, so it may be possible that the D-serine injected is being subject to considerable and rapid metabolism. The time resolution of the microdialysis technique may therefore not allow for the detection of any short-lived changes as a result of transiently increased D-serine levels. However, DAO inhibition provides an alternative route by which to elevate endogenous levels of D-serine, which may allow for a more sustained increase.

Interestingly, injection of the DAO inhibitor sodium benzoate into the VTA also resulted in increased levels of DOPAC and HVA in the mPFC, in a pattern very similar to that observed following injection of D-serine (Figure 37, Figure 39). However, there was also an increase in extracellular levels of DA in the mPFC, which became significant within the first 20 minutes after injection, and peaked 40 minutes after injection (Figure 38). This increase in DA, in addition to increases in levels of the metabolites, provides further support for the hypothesis that greater D-serine availability in the VTA (as a result of DAO inhibition) results in enhanced NMDA receptor activation on mesocortical DA neurons, and a consequent release of DA in the mPFC. Considering the theory of receptor internalisation outlined above, it is possible that the elevation in levels of D-serine produced by DAO inhibition is smaller than when D-serine itself is injected, and is insufficient to evoke NMDA receptor internalisation. This would allow for a long-lasting

increase in firing of the DA neurons, and a detectable increase in extracellular DA levels in the mPFC. It is also possible that the increase in D-serine levels produced by the inhibition of DAO persists over a greater time frame than when D-serine itself is injected, again resulting in a prolonged effect on DA neuron firing and a detectable increase in extracellular levels of DA.

Concurrent injection of both sodium benzoate and D-serine into the VTA also resulted in an increase in extracellular levels of DA in the mPFC when compared with vehicle (Figure 40), in a pattern similar to when sodium benzoate was administered alone. DOPAC and HVA levels followed a similar pattern of increase to those observed when the two substances were administered independently (Figure 37, Figure 39, Figure 41), although in this case, the effect did not reach statistical significance at individual time points. For DOPAC, however, there was a significant interaction between time and drug, suggesting that the failure to reach significance at individual time points may be due to variation within the data (as evidenced by the large error bars). Nevertheless, it appears that injecting sodium benzoate and D-serine together did not have an additive, or indeed synergistic, effect on levels of DA and its metabolites in the mPFC. However, these results may also be considered potentially cohesive with the receptor internalisation theory. Concurrent administration of D-serine and sodium benzoate may have had a supra-optimal effect on the system, increasing levels of D-serine in the VTA beyond the physiological range. As such, the system may have been unable to perform the usual adaptations (i.e. controlled NMDA receptor internalisation in order to adapt to the high levels), resulting in increased NMDA receptor activation, a sustained increase in DA neuron firing and elevated extracellular DA levels in the mPFC. This would account for the similarity in form of the elevation pattern of DA and its metabolites when sodium benzoate was administered alone compared with when sodium benzoate and D-serine were given together. However, the more subtle elevation in D-serine levels produced by DAO

inhibition alone may have had a more specific activating effect on the system than the gross increase produced by the injection of both the DAO inhibitor and exogenous D-serine together. This may account for the greater increase in levels of DA and its metabolites in the PFC seen in this case, compared with the combined injection.

All the experiments above used sodium benzoate as the DAO inhibitor. Sodium benzoate was originally selected for this study since it has been described as a 'prototypical competitive DAO inhibitor' (Vanoni *et al*., 1997; Gong *et al*., 2011), and also on the basis of its commercial availability and solubility. However, it should be noted that the current study cannot exclude a contribution of off-target effects of sodium benzoate (although we are unaware of any detailed pharmacological characterisation of the drug that provides positive evidence of such effects). Other DAO inhibitors also exist, some with greater potency at DAO than sodium benzoate; for example 5-chlorobenzo[d]isoxazol-3-ol (CBIO), 4H-thieno[3,2-b]pyrrole-5-carboxylic acid (Compound 8) and 5-methylpyrazole-3-carboxylic acid (AS057278) (Gong *et al*., 2011). During the course of this thesis, CBIO became available and was sufficiently soluble for intra-VTA injection. Pilot injections of CBIO into the VTA were carried out, in order to investigate its effects on the DA system. However, data in Figure 44 and Figure 46 indicates that injection of 1µg CBIO directly into the VTA had no detectable effect on extracellular levels of DA, DOPAC, HVA, 5-HIAA or MHPG in the rat mPFC, when compared with vehicle. Given that levels of DOPAC and HVA were unequivocally elevated following injection of D-serine and sodium benzoate alone, and that DA itself was elevated following injection with sodium benzoate and both D-serine and sodium benzoate together, it is surprising to see no effect on DA or its metabolites following injection of CBIO.

It is important to take into account the fact that no known studies to date have injected CBIO directly into the brain. While the doses used in this study were carefully calculated, there is no guarantee that they were suitable, or sufficient, to inhibit DAO. Although pilot studies were carried out with a dose range over 2 orders of magnitude, a wider range of doses is required in order to confirm the lack of effect. Furthermore, the pharmacological profile of CBIO is also poorly documented, and there is no guarantee that it is free from off-target effects. Overall, it is difficult to interpret these apparently negative pilot results, and a more thorough investigation of CBIO (or one of the other novel DAO inhibitors being developed) would be required in order to draw any conclusions.

Overall, it is important to consider the results of this chapter in the context of other studies that have investigated the effects of compounds that are relevant to the NMDA receptor glycine site (Fernandez-Espejo *et al*., 2008; Smith *et al*., 2009; Adage *et al*., 2008). Studies to date have been performed in systems that are not functioning under their baseline conditions, and have suggested that a degree of activation or stimulation is required before the full effects of DAO inhibition or D-serine administration occur. Fernandez-Espejo and colleagues reported that injection of D-serine and sodium benzoate into the VTA were able to enhance expression of behavioural sensitisation to cocaine, a process believed to involve the VTA, but had no effect on behaviour when administered alone, in the absence of any cocaine (Fernandez-Espejo *et al*., 2008). In a microdialysis study, Smith and colleagues (2009) found that systemic administration of D-serine in rats decreased amphetamine-evoked DA release in the nucleus accumbens shell when compared with vehicle, but did not affect basal DA levels (Smith *et al*., 2009). Also in this study, D-serine significantly attenuated amphetamine-induced psychomotor activity, but had no effect on spontaneous activity (Smith *et al*., 2009). In a further paper by Adage and co-workers (2008), chronic administration of the DAO inhibitor AS057278

was shown to significantly normalise PCP-induced hyperlocomotion, despite having no effect on general locomotion (Adage *et al*., 2008).

All the above studies show effects of D-serine administration or DAO inhibition in the context of a prior dopaminergic or glutamatergic intervention. Translating this to the current experiments, it may be that before the full effects of the intra-VTA manipulations of D-serine can be seen, blockade of NMDA receptors may be required. Although the effects observed in the resting state remain equivocal, the effects in a brain with hypofunctioning NMDA receptors may be more robust. Animal models may be useful in this regard; it would be interesting to investigate the effects of intra-VTA injections of D-serine and/or DAO inhibitors in animals that have been administered an NMDA receptor antagonist, such as PCP or MK801. Furthermore, genetically altered animals, such as those with a VTA-specific inactivation of NMDA receptors on DA neurons (Zweifel *et al*., 2011; Wang *et al*., 2011), could be used in order to test the hypothesis that the effects of intra-VTA injections of D-serine or DAO inhibitors are specific to activation of NMDA receptors. In those animals in which NMDA receptors on DA neurons are inactivated, no effects on DA/DOPAC/HVA levels in the mPFC would be expected as a result of administration of D-serine or DAO inhibitors. Furthermore, DAO knockout mice (such as those described in Chapter 5) may also be very useful in assessing the specificity of the DAO inhibitors; injection of a DAO inhibitor into the VTA of a DAO knockout mouse should have no effect on D-serine levels, NMDA receptor function or DA neuron activation.

In summary, this chapter has shown that injecting D-serine and/or a DAO inhibitor into the VTA has an activating effect on the mesocortical DA system. This provides support for the hypothesis that both D-serine and DAO have a functional role in NMDA receptor modulation in the VTA, which can, in turn, affect levels of DA and its metabolites in the mPFC. This may be a contributing factor to the effects of systemic DAO inhibition and D-serine administration on cortical DA metabolites seen in Chapter 3.

Chapter 5:

The expression of dopamine-related genes in the PFC of DAO knockout mice

5.1 Background

During the course of this thesis, a new strain of DAO knockout mice became available, providing an ideal model with which to examine the role of constitutive DAO activity. In Chapter 2, the expression of both DAO mRNA and protein was observed in the VTA, which was particularly interesting given its role as the source of mesocortical and mesolimbic DA neurons. In Chapters 3 and 4, DAO inhibition and D-serine administration, both systemically and locally in the VTA, resulted in increases in DA and/or its metabolites in the PFC of the rat *in vivo*. This suggested that DAO may have a role in modulating cortical DA, possibly via its activity in the VTA. These mice, therefore, provided the opportunity to compare the potential effects of the constitutive presence or absence of DAO activity on cortical DA, and to assess its potential role in cortical DA modulation.

Over several decades, the naturally occurring ddY/DAO- mutant mouse (Konno and Yasumura, 1983; Sasaki *et al*., 1992) has been studied, which lacks DAO activity due to a single glycine-arginine substitution at position 181 (Gly181Arg; Sasaki *et al*., 1992). These mice show elevated levels of DAO substrates in most brain regions (Morikawa *et al*., 2001), and as such, have been very useful tools for investigating the effects of constitutively elevated D-amino acid levels in the brain on NMDA receptor-mediated processes. Indeed, several studies have studied the effects of drugs acting at the NMDA receptor in ddY/DAO$^{-/-}$ mice, and compared behavioural measures with wild-types.

One study investigated the effects of acute challenge with MK801 (an NMDA receptor antagonist), and found that whereas MK801 induced severe stereotyped behaviours in wild-types, the ddY/DAO$^{-/-}$ mutants showed significant attenuation of the stereotypy, in addition to a reduced cumulative ataxia score (Hashimoto *et al*., 2005a). The authors postulated that enhanced D-serine levels in the ddY/DAO$^{-/-}$ mice antagonised the behavioural effects of the MK801, suggesting that elevated D-serine levels could be

relevant in reversing NMDA receptor hypofunction. In a further study, phencyclidine (PCP, another NMDA receptor antagonist) was administered in the context of habituated locomotor activity, and whereas wild-type mice became hyperlocomotive, the ddY/DAO$^{-/-}$ mice did not (Almond *et al*., 2006). Again, this was suggestive of increased NMDA receptor function in the ddY/DAO$^{-/-}$ mice, compensating for the effects of MK801. Interestingly, the authors also investigated levels of cerebellar cGMP in the ddY/DAO$^{-/-}$ mice compared with wild-types, both under basal and harmaline-stimulated conditions. Cerebellar cGMP is an *in vivo* indicator of NMDA receptor-mediated activity (Wood *et al*., 1994) and harmaline is an NMDA receptor agonist (Du *et al*., 1997); as such, harmaline administration would be expected to cause an increase in cerebellar cGMP. Under basal conditions, there was no difference between the ddY/DAO$^{-/-}$ mice and wild-types in levels of cGMP, but when stimulated with harmaline, there was a significantly greater increase in cGMP in the mice lacking functional DAO than in the wild-types (Almond *et al*., 2006). This suggested that although basal NMDA receptor function was not different between the two groups, the mutant mice displayed enhanced NMDA receptor function when stimulated. Indeed, the mutant mice have also been shown to have enhanced NMDA receptor-mediated excitatory postsynaptic currents in spinal cord dorsal horn neurons (Wake *et al*., 2001).

Taken together, these studies suggest that constitutive absence of DAO activity in the ddY/DAO$^{-/-}$ mouse results in increased NMDA receptor function, putatively via increased D-serine levels in the brain. However, no known studies have investigated possible effects of DAO knockout on the DA system. In Chapters 3 and 4 of this thesis, DAO inhibition and D-serine administration *in vivo* (both systemic and intracranial) were shown to increase extracellular levels of DA and/or its metabolites in the mPFC. Based on these results, it is interesting to consider that mice constitutively lacking DAO activity may also have altered levels of DA in the PFC.

5.2 Summary and aims

No known studies had investigated the effects of DAO knockout on the DA system, and a new DAO knockout mouse was available. The aim of this chapter was therefore to perform some initial pilot studies investigating the effects of constitutive DAO knockout on the DA system. The expression of DA-related genes in the PFC of DAO knockout mice was measured, and compared with expression in heterozygote and wild-type mice. First, the mice were genotyped and the expression of DAO confirmed at the protein level using Western blot analysis. Then, quantitative real-time PCR (qRT–PCR) was used to investigate the expression of various DA-related genes in the PFC of the mice. Specifically, expression of COMT (Comt, a metabolising enzyme of DA), the D1 receptor (Drd1a) and the D2 receptor (Drd2) were quantified and compared.

5.3 Methods

5.3.1 DAO knockout mice

DAO mutant mice were obtained as a gift from Pfizer. The mutations were generated in embryonic stem cells from 129Sv/Ev mice (Taconic). Briefly, 1957 base pairs encompassing exons 7–8 of Dao1 were deleted by standard homologous recombination (TG-ES01-01 ESM07, Eurogentec), and the stem cells then inserted back into the embryo. Colonies were maintained as heterozygotes, and knockout and wild-type populations derived from heterozygote (x heterozygote) matings.

5.3.1.1 Prior characterisation

The mice were examined at Pfizer before arriving in Oxford. There was a statistically significant increase in tissue D-serine levels in the cerebellum of knockouts versus wild-types. There was also a trend towards significance in tissue D-serine levels in the hippocampus, but no difference in D-serine levels in the striatum or cortex. These findings were consistent with studies of the traditional ddY/DAO- knockout animals, which also show increases in D-serine largely confined to the cerebellum (Hashimoto *et al*., 1993b). The knockout mice also displayed a significant increase in cerebellar cGMP levels in response to harmaline [an NMDA receptor agonist; (Du *et al*., 1997)] when compared with wild-type ($p<0.05$), but no difference in cGMP response to vehicle treatment. This suggested that these mice show no increase in tonic NMDA receptor-mediated neurotransmission, but that they show enhanced NMDA receptor function under conditions of stimulation.

Mice were firstly genotyped using PCR by Dr Judith Schweimer. The following procedures were then run blind to genotype.

5.3.2 Western blotting

This work was carried out in collaboration with Dr Phil Burnet and Simone Paulson.

Mice (4 months old; both male and female) were given a lethal injection of pentobarbitone, decapitated and their brains removed. Half the cerebellum was removed and this, along with the rest of the brain, was snap-frozen in isopentane. Protein was extracted from the cerebellum halves using standard procedures, and the concentration measured using a Bradford assay. Extracts were diluted in RIPA buffer to a final concentration of 5µg protein in 12µl.

A 12% SDS/polyacrylamide resolving gel was made up in a total volume of 5ml, containing the following: 1.6ml dH_20, 2ml 30% acrylamide mix, 1.3ml 1.5M Tris (pH 8.8), 50µl 10% sodium dodecyl sulphate (SDS), 50µl 10% ammonium persulphate, 2µl TEMED. This was left to set. Once set, a 5% SDS/polyacrylamide stacking gel was added (and combs inserted), made up in a volume of 2ml as follows: 1.4ml dH_20, 330µl 30 % acrylamide mix, 250µl 1.0M Tris (pH 6.8), 20µl 10% SDS, 20µl 10% ammonium persulphate, 2µl TEMED.

A 5X loading buffer was prepared in a 20ml mix containing the following: 1.6g 0.5M Tris HCL, 2g 10% SDS, 10ml 50% glycerol, 20µg 0.1% Bromophenol blue, 1ml 5% β-mercaptoethanol. The protein samples prepared above were then mixed with loading buffer at a ratio of 4:1 and boiled for 10 minutes before being quenched on ice for 2 minutes. The mixture was then centrifuged and loaded onto the gel stack along with BIORAD Kaleidoscope prestained standards (Hercules, CA). The gel was run for approximately 2 hours at 100mV in a tank containing 5X running buffer, made up in 2 litre stock (containing 30.2g Tris base, 188g glycine, 100ml 10% SDS).

A 4 litre 20X stock of transfer buffer was made, containing 290g glycine, 580g Tris base and 37g SDS diluted in dH_20. 50ml of this was then diluted to 1x by adding 750ml dH_20

and 200ml methanol. A polyvinyl difluoride membrane (Immobilon®-P Transfer Membrane, Millipore) was soaked in methanol for 5 minutes, dH_20 for a further 2 minutes and finally briefly in transfer buffer. Two sponges and two pieces of filter paper were also soaked in transfer buffer. The gels were removed from the electrophoresis tank, the stacking gel discarded and the resolving gel adhered to the filter paper. The gel was then stacked into a 'transfer sandwich', as per Figure 46 below, and the transfer run overnight at 25V in a tank containing 1x transfer buffer.

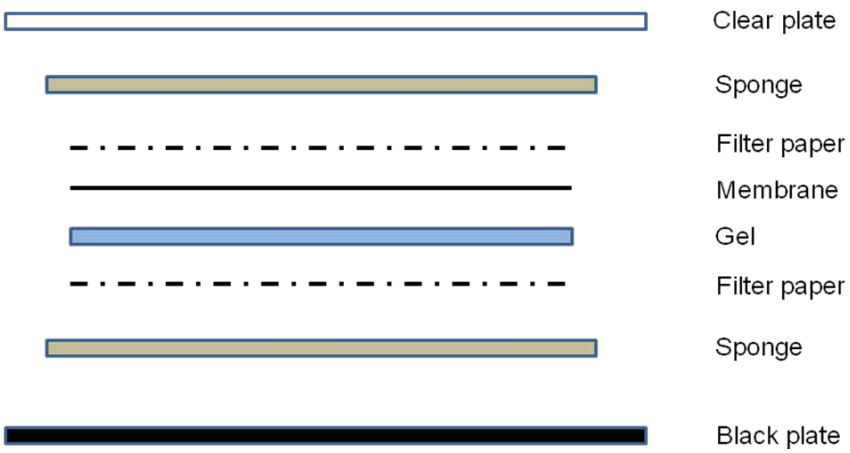


Figure 46. 'Transfer sandwich' used for transfer of protein from SDS/polyacrylamide gel to a polyvinyl difluoride membrane.

The membrane was removed from the transfer stack and blocked for 1 hour in PBST (1X PBS with 0.1% Tween 20) containing 2% non-fat milk (Marvel) with constant agitation. The membrane then underwent a 1-hour exposure to either a rabbit polyclonal DAO antibody (GSK1845, as used in Chapter 2), diluted in PBST containing 1% BSA (bovine serum albumin) to a concentration of 1:1000, or a mouse β-actin antibody (AbCam ab6276) at a concentration of 1:50,000 (to be used as a housekeeping gene). This was followed by three 15-minute washes in PBST. The membrane was then exposed to a

secondary horseradish peroxidise-conjugated antibody for 30 minutes; either goat anti-rabbit antibody (BioRad 172-1019) at a concentration of 1:10,000, or goat anti-mouse (BioRad 172-1011) at 1:5000, both diluted in PBST containing 2% milk. After a further three 15-minute washes in PBST, the membrane was visualised using the 'ECL Plus' kit (GE Healthcare, Amersham, UK) and exposed to X-ray film (Kodak). Films were magnified and inspected using a lightbox and camera, and MCID Elite software was used to measure the density of the bands (by Dr Phil Burnet and Simone Paulson). The DAO signal was normalised to the β-actin signal, and the samples unblinded and grouped by genotype. Data were analysed using a one-way ANOVA in SPSS (version 17.0).

5.3.3 qRT–PCR

Sections were taken from the snap-frozen PFC of the mice sacrificed above (by Li Chen). RNA was extracted from sections from each animal before the corresponding cDNA was produced using standard reverse transcriptase methods (by Mary Walker/Dr Phil Burnet). The concentrations of all cDNA samples were then measured, and each was diluted to 100ng/µl. 9µl of each sample was added to a central cDNA pool (with which standard curves were to be run). Serial dilutions were then made of this central pool, from 100ng/µl to 0.39ng/µl, by diluting with nuclease-free water.

384-well PCR plates were loaded with 3µl triplicates of the standard serial dilution. To each well was then added 9µl of 'master mix', containing 2.4µl nuclease-free water, 6µl Taqman® Mastermix (Universal PCR Master Mix, No AmpErase® UNG, Applied Biosystems™) and 0.6µl of either a mouse Comt, Drd1a or Drd2 quantitative PCR probe (Mm00514377_m1, Mm01353211_m1, or Mm00438545_m1, respectively; Taqman® Gene Expression Assays, Applied Biosystems™). The plate was then sealed and centrifuged, and analysed using a 7900HT Fast Real-Time PCR System (Applied Biosystems™) with SDS 2.2.2 Software. qRT–PCR conditions consisted of an initial 10

minute period at 95°C, the 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Standard curves for each of Drd1a, Drd2 and Comt were plotted and inspected for an approximate mid-point, and the cDNA concentration at that approximate point noted. This was then selected as appropriate for the concentration of the individual cDNA samples to be run, as it was well within the detectable range. This was 20ng per well for each gene.

5µl of each cDNA sample was then individually diluted with 70µl of nuclease-free water to create a cDNA concentration of 6.67ng/µl (such that 3µl contained 20ng cDNA). 384-well plates were then loaded with either 3µl of a standard dilution or 3µl of an individual cDNA sample (in triplicate), before 9µl of mastermix was added to each (as above). Probes for the standard housekeeping genes transferrin receptor (Tfrc) and β-2-microglobulin (B2m) were also run, in order to provide a control reference point (Mm00441941_m1 and Mm00437762_m1, respectively; Taqman® Gene Expression Assays, Applied Biosystems™). A standard curve and full set of cDNA samples in triplicate for each probe were present on a single plate. The plates were sealed and centrifuged, before being run through the qRT–PCR system and analysed as above. Standard curves for each probe were plotted, and then individual cDNA samples quantified using comparison with the standard curve. The values for the two housekeeping genes for each sample were averaged, and all other measurements for the same sample normalised to this housekeeping value.

For analysis, samples were unblinded and grouped into wild-types (n=6), heterozygotes (n=8) and DAO knockouts (n=5). Each group was analysed using a Grubbs Test for outliers, and resulted in one outlier being removed from the DAO knockout group in the analysis of the Drd2 gene. Groups were then compared statistically using a univariate ANOVA and Scheffe post-hoc testing (where the ANOVA was significant) in SPSS (version 17.0). In addition, wild-types and heterozygotes were then compiled into a single

group and compared with knockouts, in order to compare the effects of no DAO activity at all with *some* DAO activity. The statistical analysis was repeated.

5.4 Results

5.4.1 Western blot

Western blot analysis assessed the expression of DAO protein in each mouse. DAO was detected as a single band of 37KDa. No DAO protein was detected in knockout mice (Figure 48). When DAO wild-types were compared with heterozygotes, heterozygotes were shown to express significantly less DAO when signal measurements were quantified using a lightbox and MCID software and normalised to *β*-actin (WT: 0.77±0.06, Het: 0.45±0.06, $p<0.05$; Figure 48).

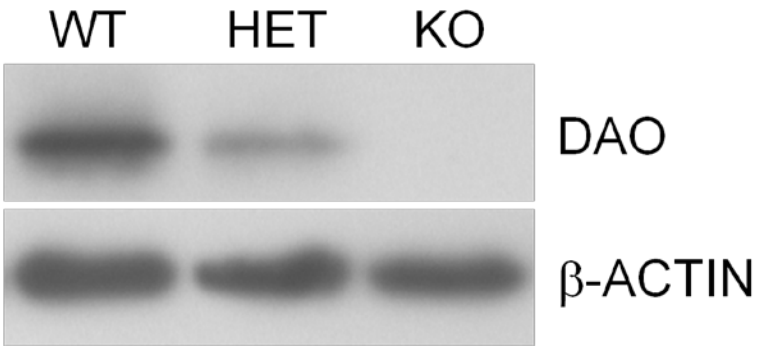


Figure 47. Autoradiographs of DAO and β-actin immunoreactivity in cerebellar protein extracts from DAO wild-type, heterozygote and knockout mice. *No band is seen in the knockout lane.*

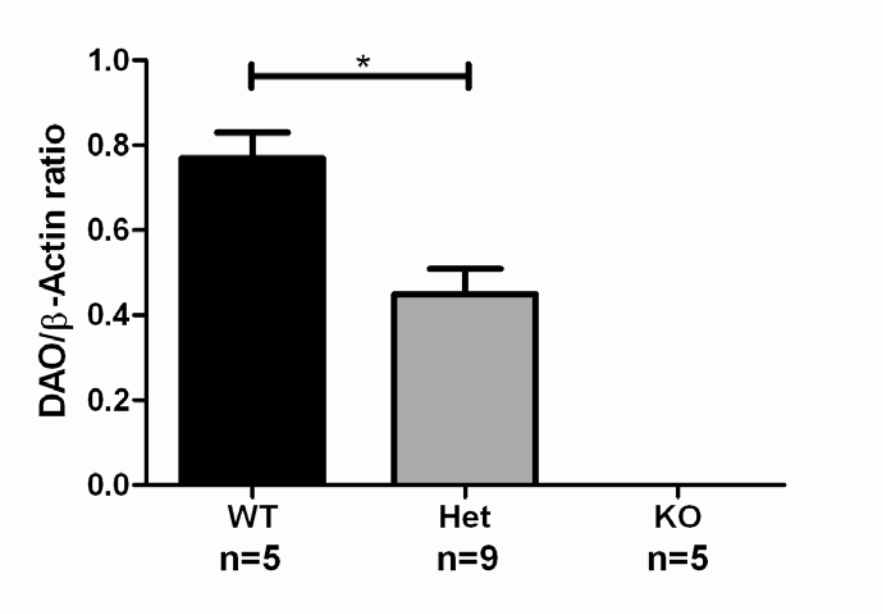


Figure 48.Quantification of DAO signal in Western blots of cerebellar protein taken from DAO wild-type, heterozygote and knockout mice, normalised to β-actin. *Plot shows mean with S.E.M., p<0.05.*

5.4.2 qRT–PCR

qRT–PCR allowed for the successful amplification and detection of mRNA from the standard dilution series and PFC samples. Figure 49 below shows an example amplification plot and standard curve from qRT–PCR of the housekeeping gene B2m.

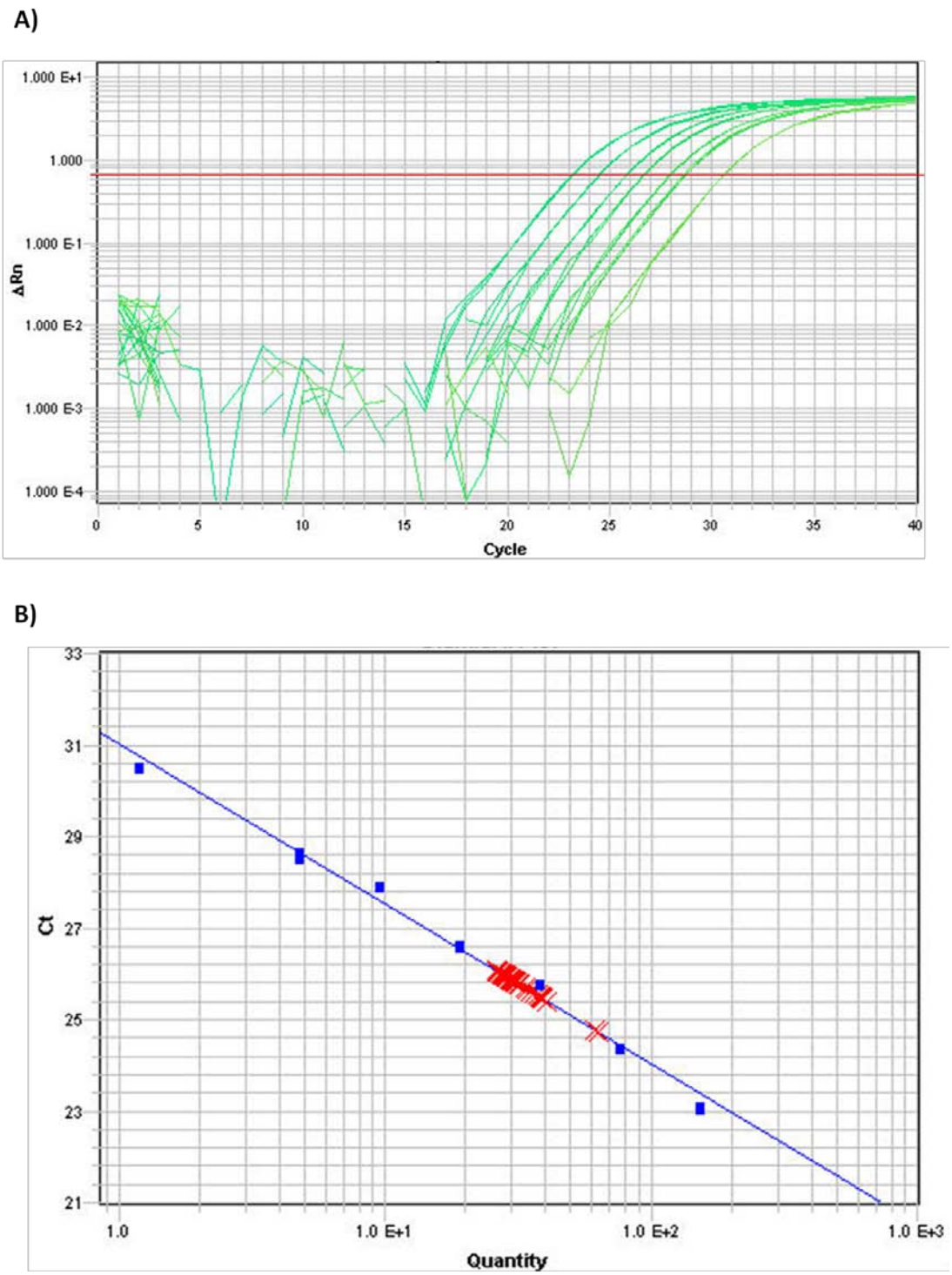


Figure 49. Amplification plot (A) and standard curve (B) following qRT-PCR of B2m in the PFC. *(A) Amplification plot of B2m in a standard dilution series. Red horizontal line indicates the threshold at which the standards were quantified. (B) Standard curve plotted with data taken from the amplification plot in (A). Blue squares indicate standard dilution series, and red crosses represent measurement of unknown samples in comparison with the curve.*

There was a significant effect of genotype on expression of Comt when the three animal groups were analysed individually [F(2,16)=4.775, p=0.024; Figure 50]. Scheffe post-hoc analysis revealed a significant increase in the expression of Comt in the knockout mice when compared with heterozygotes (p=0.028), but no difference between wild-type and knockout animals (p=0.514) or wild-type and heterozygote animals (p=0.210). When wild-types and heterozygotes were grouped together, there was a significant increase in Comt expression in the knockout animals when compared with this group [F(1,17)=5.328, p=0.034; Figure 51].

There was no difference in the expression of Drd1a in the PFC between wild-type, heterozygote and knockout mice (Figure 52). Statistical analysis of Drd1a expression across the three groups revealed no main effect of genotype [F(2,16)=1.660, p=0.221]. When the wild-types and heterozygotes were collected into a single group, there was no difference between this group and the knockouts, although there was a trend towards a significant increase in the knockouts [F(1,17)=3.478, p=0.08; Figure 53].

There was a trend towards a main effect of genotype on Drd2 expression when the three groups were analysed individually [F(2,15)=2.785, p=0.094; Figure 54]. Grouping of the wild-types and heterozygotes revealed a significant difference between these animals and the knockouts, with the knockouts showing greater expression of Drd2 [F(1,16)=5.837, p=0.028; Figure 55].

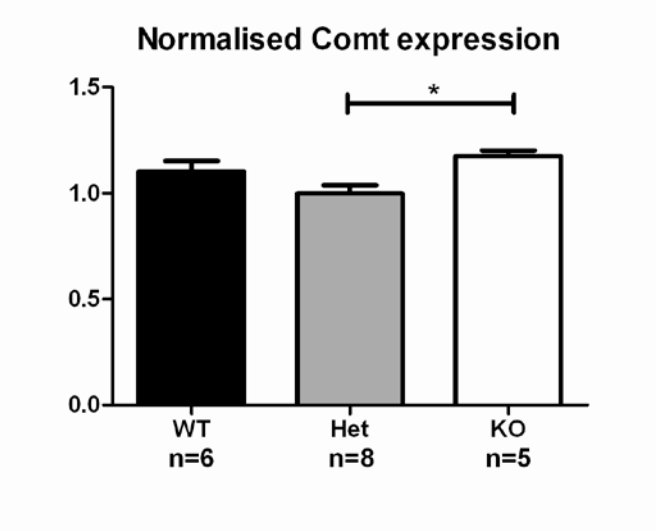


Figure 50. Normalised expression of Comt in the PFC of DAO wild-type, heterozygote and knockout mice, measured using qRT–PCR. *Plot shows mean with S.E.M.; *p<0.05.*

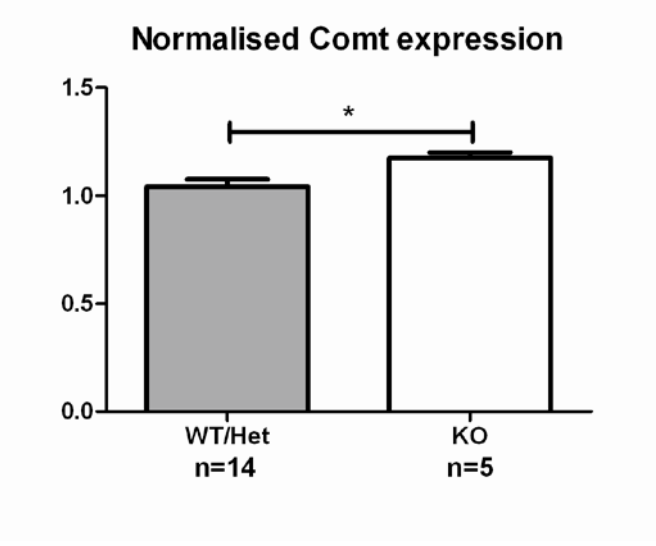


Figure 51. Normalised expression of Comt in the PFC of DAO wild-type, heterozygote and knockout mice, measured using qRT–PCR. *Wild-type and heterozygote animals are grouped together. Plot shows mean with S.E.M.; *p<0.05.*

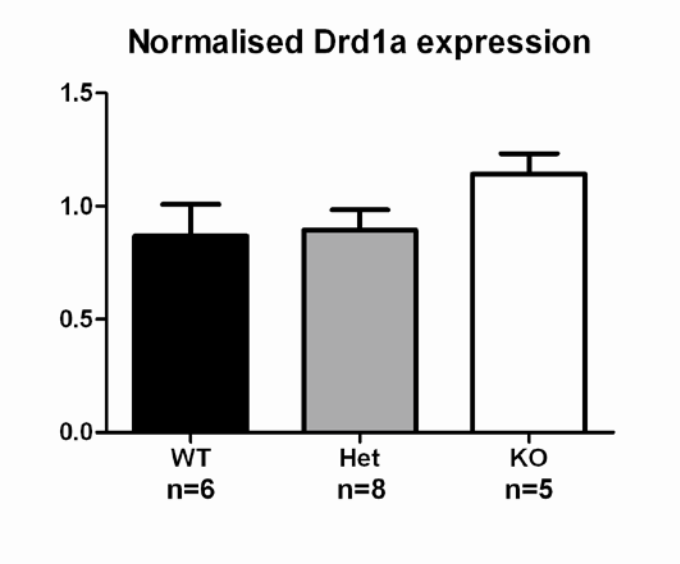


Figure 52. Normalised expression of Drd1a in the PFC of DAO wild-type, heterozygote and knockout mice, measured using qRT–PCR. *Plot shows mean with S.E.M..*

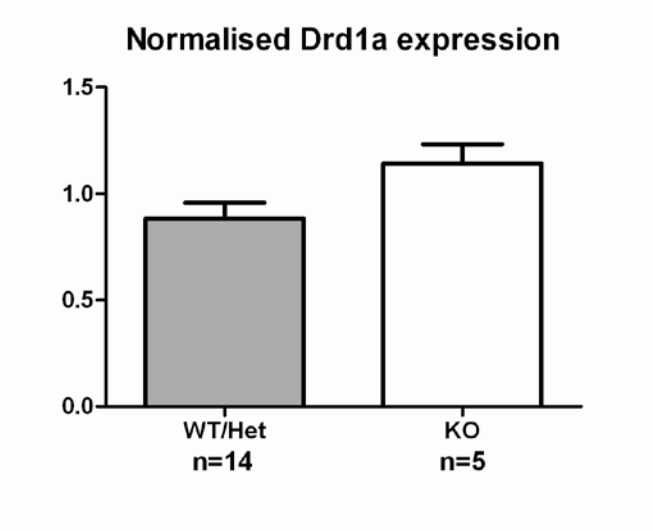


Figure 53. Normalised expression of Drd1a in the PFC of DAO wild-type, heterozygote and knockout mice, measured using qRT–PCR. *Wild-type and heterozygote animals are grouped together. Plot shows mean with S.E.M..*

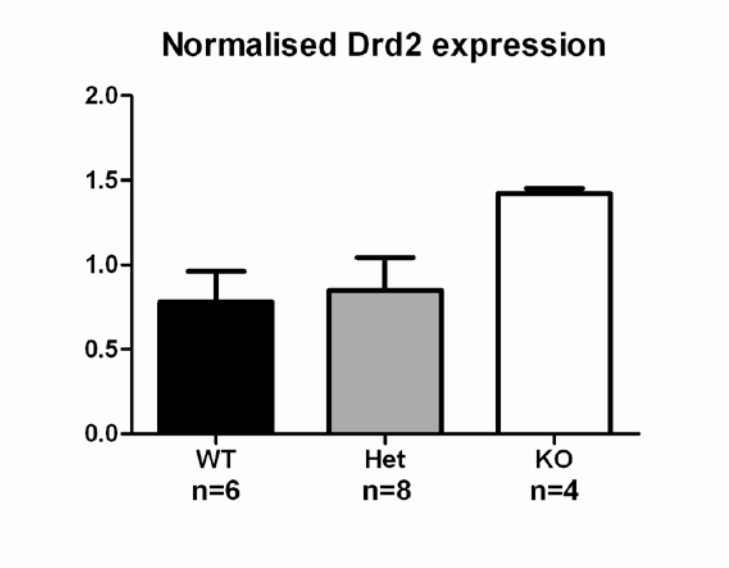


Figure 54. Normalised expression of Drd2 in the PFC of DAO wild-type, heterozygote and knockout mice, measured using qRT–PCR. *Plot shows mean with S.E.M..*

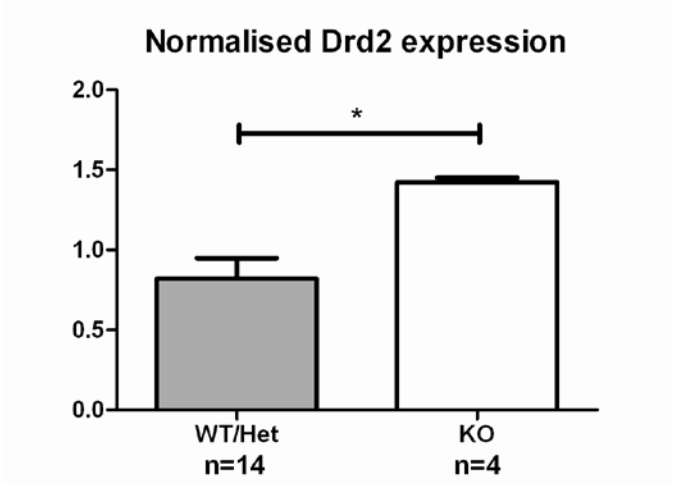


Figure 55. Normalised expression of Drd2 in the PFC of DAO wild-type, heterozygote and knockout mice, measured using qRT–PCR. *Wild-type and heterozygote animals are grouped together. Plot shows mean with S.E.M.; *p<0.05.*

5.5 Discussion

In this chapter, western blotting confirmed that the DAO knockout mice did not express any DAO protein (Figure 48). DAO heterozygote mice were shown to express significantly less DAO protein than wild-types, suggesting a gene-dosage effect on protein levels (Figure 48). Pilot studies then suggested that expression of the DA-related genes Comt and Drd2 were elevated in the PFC of DAO knockout mice when compared with a combined group of wild-type and heterozygous mice. Furthermore, there was a trend towards an increase in the expression of Drd1a ($p=0.08$). Together, these results imply that there may be alterations in DA transmission in the PFC of the knockout mice when compared with wild-types and heterozygotes.

Comt is a metabolising enzyme of DA and important for modulating DA levels in the PFC (Tunbridge *et al*., 2004). In Chapters 3 and 4, acute inhibition of DAO produced an increase in levels of DA and/or its metabolites in the PFC, and so it is attractive to assume that DA levels may also be increased in the PFC of the knockout mice. If so, the observed increase in expression of Comt mRNA may represent a homeostatic response in postsynaptic neurons, in order to maintain control of levels of DA in the PFC through increased DA metabolism following uptake from the synaptic cleft. However, the expression of Drd2 mRNA, and putatively Drd1a, was also increased in the knockout mice. Upregulation of receptor expression usually occurs in response to reduced levels of a neurotransmitter, and so these results suggest that DA levels may, instead, be decreased. It is also possible that the increase in expression of Comt, Drd2 and potentially Drd1a observed in the knockout mice is not reflective of a detectable change in DA levels in the PFC, but that it merely represents a ‘molecular reshuffling’ in response to a lack of DAO. Furthermore, the differences in expression, despite being statistically significant, are of small magnitude (especially in the case of COMT), and so may not be physiologically relevant.

DAO expression is globally abolished in the knockout mice, and so it is not possible to attribute any putative change in DA function to any specific location or pathway, regardless of the nature of the change. For example, effects may be mediated via an increase in D-serine levels in the cerebellum, given that DAO is most robustly expressed there in the healthy adult brain. Evidence suggests that the cerebellum can modulate DA function in the PFC, and that disrupted Purkinje cell output from the cerebellum may result in abnormal DA transmission in the PFC (Mittleman *et al*., 2008). In Chapter 2 of this thesis, DAO was shown to be present in glia surrounding Purkinje cells, and was suggested to be serving to minimise D-serine levels in the locality. It is therefore possible that elevated D-serine levels in the cerebellum as a result of constitutive DAO knockout may result in disrupted Purkinje cell output, and consequently affect levels of DA in the PFC of these animals. The cerebellar-PFC connection is also partially reliant on NMDA receptor function in the VTA (Rogers *et al*., 2011), and since DA output from the VTA is modulated by NMDA receptors (see e.g. Bennett and Gronier, 2007), a lack of DAO activity in the VTA in the DAO knockout mice may also contribute to a potential disruption in this system.

This initial study of DA-related genes in DAO knockout mice had several limitations. First, the number of animals in each of the study groups was small, especially in the knockout group (where n=4 or n=5). Increasing the number of animals in each group would allow for more robust comparison of gene expression. Second, this study has only assessed the mRNA expression of Drd1a, Drd2 and Comt in the PFC. Measures beyond the expression of mRNA would also be important in developing these initial investigations; for example quantification of protein expression, or enzyme activity (in the case of Comt), to assess whether changes in mRNA levels are reflective of a physiological relevant increase. qRT-PCR could also be used to probe the expression of other DA-related genes

in the PFC, such as other DA receptors (e.g. Drd4) and the DA transporter DAT. The expression of genes relevant to the NMDA receptor could also be measured, to evaluate the potential effects of the knockout on NMDA receptor function. For example, expression of the D-serine transporter Asc-1 could be measured, in order to assess potential upregulation of the transporter in order to maintain homeostasis with putative increased levels of D-serine. Similarly, the expression of serine racemase may be important, as the enzyme may be downregulated in response to a lack of DAO, reducing synthesis of D-serine and helping to maintain physiologically relevant D-serine levels. The expression of the NR1 subunit of the NMDA receptor (upon which the glycine site is located) could also be considered, to assess potential upregulation of this subunit in response to increased D-serine availability. Together, these studies may allow clearer links to be made between changes in DA transmission in the PFC of the knockout animals with altered glutamatergic signalling.

Finally, in order to more fully and directly investigate the effects of DAO knockout on DA function in the PFC, levels of DA itself need to be measured, perhaps by measuring tissue DA levels in postmortem brains, or measuring DA in the PFC using *in vivo* microdialysis or voltammetry. It would also be interesting to consider the effects of the DAO knockout on NMDA receptor-mediated DA neuron firing in the VTA. Indeed, early *in vivo* electrophysiology results suggest that DA neurons in the VTA of some DAO knockout animals show increased burst firing when compared with heterozygotes and wild-types (Dr Judith Schweimer, personal communication). Given that burst firing is NMDA receptor-dependent (see Overton and Clark, 1997), this is suggestive of an increase in NMDA receptor function in these animals.

Taken together, the results of this chapter are consistent with an alteration in DA levels in the PFC of mice lacking DAO activity when compared with wild-types and heterozygotes. This supports the hypothesis that DAO and D-serine play a role in modulating the mesocortical DA system *in vivo*. However, this chapter represents a pilot study, and further investigation is needed to characterise the changes reported here, and to establish their origins and functional implications.

Chapter 6:

General discussion

DAO is the metabolising enzyme of D-serine, an endogenous agonist at the glycine site of the NMDA receptor. As such, DAO has the potential to indirectly modulate NMDA receptor function. NMDA receptors modulate the firing of VTA DA neurons (e.g. Chergui *et al*., 1993) and as such, DAO may play an indirect, yet physiologically important, role in the control of DA output along the mesocortical and mesolimbic pathways in the brain.

DAO has been identified as a candidate gene for schizophrenia (Chumakov *et al*., 2002), and its expression and activity have been shown to be increased in patients with the disorder (Kapoor *et al*., 2006; Verrall *et al*., 2007; Burnet *et al*., 2008; Madeira *et al*., 2008), while decreased D-serine levels have been observed in the serum and cerebrospinal fluid (Hashimoto *et al*., 2003; Hashimoto *et al*., 2005b). DAO inhibition, and its potential to elevate D-serine levels, may therefore be therapeutically relevant.

The full expression pattern of DAO in the brain remains unclear, and the extent of its physiological importance is unknown. This work therefore had three major aims:

1. to further investigate the regional and cellular expression of DAO in the rat brain, with a focus on the VTA

2. to investigate potential links between DAO activity, D-amino acid levels, NMDA receptor activation and the mesocortical DA system in the rat brain

3. to investigate the effects of genetically abolishing DAO activity on the expression of DA genes in the PFC of mice.

Each of these aims will be considered in turn.

6.1 The regional and cellular expression of DAO in the rat brain

In Chapter 2, investigations were carried out into the regional and cellular expression of DAO in the rat brain. Specifically, the question of whether or not DAO is present in the VTA was addressed, as well as the question of the cell type in which is it expressed, both there and in the cerebellum.

ISHH and immunofluorescence studies provided clear evidence that both DAO mRNA and protein were expressed in the adult rat VTA. However, the expression pattern was complex: DAO immunoreactivity was observed in both neurons and glia, and of the neurons, some were TH-positive and some were TH-negative. TH immunoreactivity is a marker of dopaminergic cells, and as such, it seems that DAO is expressed in, although not confined to, dopaminergic neurons in the VTA, as well as in glia. This is the first known study to date to confirm the expression of DAO in the rat VTA, and to further explore its cellular localisation in this region. It is also one of few studies to report DAO expression in neurons, with only two other studies reporting a neuronal localisation for DAO (Moreno *et al*., 1999; Verrall *et al*., 2007).

In the adult rat cerebellum, both DAO mRNA and protein were expressed predominantly in glia, as assessed by ISHH and immunofluorescence studies. The protein appeared not to be expressed in Purkinje cells, but rather in glial processes enveloping Purkinje cell bodies and extending into the molecular layer, which were identified as the putative processes of Bergmann glia via labelling with a GFAP antibody. This is in accordance with numerous other studies; DAO expression has been repeatedly reported in Bergmann glia (Weimar and Neims, 1977b; Weimar and Neims, 1977a; Horiike *et al*., 1987; Verrall *et al*., 2007; Ono *et al*., 2009; Moreno *et al*., 1999). The results of these studies therefore support the notion that DAO is a glial enzyme in the cerebellum.

The results of this chapter suggested that the traditional view of DAO as a hindbrain

enzyme confined to glia is outdated. Its expression extends beyond the hindbrain, and into neurons as well as glia. This may have important physiological consequences, and opens several new avenues for investigation into the functional relevance of DAO expression throughout the brain.

The expression of DAO in the VTA is particularly interesting in the context of the DA system. The VTA is the source of the mesocortical and mesolimbic DA pathways, and dopaminergic output from the VTA is regulated by NMDA receptors (see e.g. Bennett and Gronier, 2007). It is therefore conceivable that DAO, and its regulation of D-amino acid levels, plays a pivotal role in the modulation of these NMDA receptors, thus indirectly mediating mesocortical and mesolimbic DA function. DAO expression and activity may also be physiologically relevant in other brain areas where NMDA receptor function is critical; for example, in the generation of long-term potentiation in the hippocampus.

Interestingly, in the VTA, DAO was shown to be expressed in both neurons and glia, and of the neurons, some were TH-positive and some were TH-negative. This suggests that the enzyme is expressed in dopaminergic neurons in this brain region, but also in non-dopaminergic neurons. Further studies in this brain region would therefore include the identification of these non-dopaminergic neurons. Around 30% of VTA neurons are GABA-ergic (Dobi *et al*., 2010), and so it would be interesting to carry out further immunofluorescence studies in this region with concurrent DAO and GAD67 (a marker of GABA-ergic neurons) labelling. Furthermore, the VTA contains a population of neurons that are glutamatergic (Yamaguchi *et al*., 2007), and so double-labelling could be carried out with an antibody for VGLUT2 (the vesicular glutamate transporter). These studies would also allow for further investigation into the recent proposals that some VTA neurons release both DA and glutamate (e.g. Hnasko *et al*., 2010).

This study provided evidence that the cellular expression of DAO varies between brain regions, and that whereas DAO appeared to be expressed in both neurons and glia in the VTA, it was confined to glia in the cerebellum. This begs the question: what are the implications of the expression of DAO in different cell types? Traditionally, DAO was thought to be confined to peroxisomes of glia, metabolising D-serine that had been transported back into the glia following its release and action at NMDA receptors. However, a recent study has provided evidence that DAO interacts with various proteins in the presynaptic zone, including Bassoon, which inhibits the enzymatic activity of DAO (Popiolek *et al*., 2011). This interaction may be important in the regulation of D-serine levels. Bassoon expression, however, has not been detected in glia, which suggests that these interactions only occur in neurons, and that the regulation of DAO by Bassoon is specific to neurons. In brain regions where DAO is glial, its activity may be regulated in another way. This suggests that DAO may have varying functions in different brain regions, being expressed in different compartments of different cell types, and being regulated in different ways in each.

Although this thesis has considered the expression of DAO at the level of both mRNA and protein, the *activity* of DAO in the VTA, and indeed in other brain regions, has not been unequivocally confirmed. This may be a very important factor: for example, in the cortex, DAO mRNA is abundant and DAO protein is detectable (Sacchi *et al*., 2008; Verrall *et al*., 2007; Kapoor *et al*., 2006), but its activity is only a fraction of that in the cerebellum (Neims *et al*., 1966; Madeira *et al*., 2008; Weimar and Neims, 1977b). The presence of DAO in the VTA is not necessarily evident of its function therein, and activity should, itself, be confirmed.

6.2 The potential links between DAO activity, D-amino acid levels, NMDA receptor activation and mesocortical DA system function in the rat brain

In Chapter 3, the study focus moved to the potential link between DAO activity and the DA system in the adult brain. In schizophrenia, DAO expression and activity in the brain are increased (Kapoor *et al*., 2006; Verrall *et al*., 2007; Burnet *et al*., 2008; Madeira *et al*., 2008), and D-serine levels in the serum and cerebrospinal fluid are decreased (Hashimoto *et al*., 2003; Hashimoto *et al*., 2005b). Some clinical trials with D-serine have shown a moderate but favourable effect in treating cognitive and negative symptoms (Coyle *et al.*, 2003; Javitt *et al.*, 2007; Kantrowitz *et al.*, 2010). These symptoms are thought to originate from the PFC, with hypofunction of the mesocortical dopaminergic pathway being a major contributor (Davis *et al*., 1991). As such, it is possible that treatment with D-serine in schizophrenia may improve negative and cognitive symptoms by increasing DA levels in the PFC. Therefore, in Chapter 3, studies using *in vivo* microdialysis were carried out to investigate the effects of pharmacologically manipulating D-serine levels on extracellular DA in the PFC.

In the anaesthetised rat, significant elevations in extracellular levels of DOPAC and HVA (DA metabolites) were observed in the PFC following systemic injection of exogenous D-serine together with sodium benzoate (a DAO inhibitor). However, levels of DA itself did not change. Several explanations exist for this observation. First, it may be that whereas extracellular levels of DA in the PFC were not elevated, the turnover of DA in the mesocortical neurons was increased. The majority of DOPAC and HVA formed in the brain are as a result of the breakdown of newly synthesised intracellular DA (Zetterström *et al*., 1988), and so it is possible that the observed increase in the DA metabolites is representative of an increase in the synthesis of DA, which is not necessarily being released. Second, it may be that stimulation of DA release is required before the effects of D-serine elevation become appreciable. Indeed, in a study of the effects of D-serine

administration on DA levels in the nucleus accumbens shell, D-serine alone had no effect, but when an amphetamine challenge was given, the peak in DA levels observed was reduced in animals treated with D-serine (Smith *et al*., 2009). Third, temporal aspects may play a role. In a report published after completion of this study, Bannai and colleagues reported that oral D-serine administration in rats yielded a transient (0-10 mins), yet significant, DA increase in the PFC, which then rapidly returned to baseline (Bannai *et al*., 2011). Any such transient increases in DA may not have been detected in the present study.

D-serine was putatively elevated throughout the brain as a result of the systemic injections, and so the effects on DA may be caused by several possible processes. Elevated D-serine levels may directly affect DA terminals in the PFC, for example by increasing stimulation of presynaptic NMDA receptors, which may have a knock-on effect on DA metabolism. Another possibility is that the primary effect of the D-serine elevation lies within the cerebellum. Finally, it is possible that elevated D-serine levels in the VTA as a result of the systemic injections is resulting in increased NMDA receptor activation on mesocortical DA neurons, culminating in an increase in their activity.

This study provided some evidence that the systemic administration of D-serine and a DAO inhibitor affected levels of DA metabolites in the PFC. This supports the theory that clinical trials with D-serine may have their positive effects on negative and cognitive symptoms of schizophrenia via effects on mesocortical DA. The hypothesis that elevated D-serine levels in the VTA may result in increased activation of mesocortical DA neurons was selected as a route for further investigation. Experiments were performed in Chapter 4 to specifically probe the role of D-serine and DAO in the VTA on levels of DA and its metabolites in the PFC. *In vivo* microdialysis was again used to measure levels of DA and its metabolites in the mPFC of anaesthetised rats, while D-serine and DAO inhibitors were administered directly into the VTA.

Injection of D-serine directly into the VTA resulted in a significant increase in levels of DOPAC and HVA in the PFC, but had no effect on levels of DA. Similarly, when sodium benzoate was injected, there was a significant increase in levels of DOPAC and HVA, but this time the increases were accompanied by a significant elevation in extracellular DA. Finally, when both D-serine and sodium benzoate were administered together, there was a significant increase in levels of DA in the PFC, but no effect on levels of DOPAC or HVA at any individual time point. Together, though complex, these results provided evidence that manipulation of DAO activity and D-serine levels in the VTA had a positive effect on mesocortical DA neuron activity. This provides evidence of a potential role for DAO in the VTA in modulating cortical DA.

These seemingly complex results may be explained by a theory of NMDA receptor internalisation, in which the receptors may rapidly internalise following large elevations in D-serine (see Chapter 4). *In vivo* microdialysis may not have sufficient time resolution to capture the physiological effects that are occurring, according to this hypothesis. The proposed NMDA receptor internalisation theory necessitates the measurement of the firing rates of VTA DA neurons after intra-VTA administration of D-serine and/or sodium benzoate. Additional intra-VTA injections of different doses of D-serine, in combination with a measurement of receptor internalisation, would also be valuable, in order to investigate a potential threshold for maximum D-serine levels in the VTA before receptor internalisation is primed.

Numerous additional experiments may also allow for further characterisation of the link between DAO, the VTA and the mesocortical DA system. Genetically altered animals, such as those with a VTA-specific inactivation of NMDA receptors on DA neurons (Zweifel *et al*., 2011; Wang *et al*., 2011), could be used in order to test the hypothesis that the effects of intra-VTA injections of D-serine or DAO inhibitors are specific to activation of NMDA receptors. In those animals in which NMDA receptors on DA neurons are

inactivated, no effects on levels of DA and its metabolites in the PFC would be expected as a result of administration of D-serine or DAO inhibitors. An inducible VTA-specific DAO knockout mouse would also be invaluable in the investigation of the role of DAO in the VTA. The *in vivo* measurement of extracellular levels of DA in the PFC could again be investigated, but with the ability to specifically manipulate DAO expression in the VTA.

Studies into the role of the cerebellum in regulating levels of DA in the PFC would be interesting, in order to assess this potential further component. Local injections of D-serine or DAO inhibitors directly into the cerebellum while measuring levels of DA and its metabolites in the PFC would probe the potential role of D-serine in the cerebellum in modulating cortical DA. Moreover, a recent study showed that the cerebellar-PFC connection is partially reliant on NMDA receptor function in the VTA (Rogers *et al*., 2011). Intra-VTA injections of D-serine and/or DAO inhibitors could be performed while concurrently stimulating the cerebellum and measuring DA in the PFC, in order to investigate the potential role of DAO and D-serine in modulating the VTA component of the cerebellar-PFC connection. Conversely, measurement of the firing rate of VTA DA neurons could be performed while concurrently measuring DA in the PFC and infusing DAO inhibitors and/or D-serine into the cerebellum. This would permit the reverse assessment, and to consider the importance of DAO and D-serine in the cerebellum as the potential origin of the pathway.

Although *in vivo* microdialysis is an established method for the measurement of extracellular DA in the living brain, it is not without its limitations. As discussed above, the time resolution of the technique is limited, and levels of neurotransmitters in the brain region of interest can only really be measured on the scale of tens of minutes. The employment of techniques with a better time resolution may be useful in taking these studies further; for example *in vivo* electrophysiology, or voltammetry. This may allow for a more detailed investigation of the activity of mesocortical DA neurons, including their

firing rate and release of DA. The current study has also used anaesthetised rats, introducing the possibility that the effects observed would be different if the rat were awake and behaving. An interesting next step would therefore be to perform *in vivo* microdialysis in awake animals, perhaps combining the procedure with some behavioural measures to see if any changes in DA have a functionally relevant effect.

It is important to consider the current study results in the therapeutic context. Administration of D-serine and/or DAO inhibitors in the clinic would be systemic, and so it is important to think about the other potential effects of an increase in D-serine levels in the brain. For example, the VTA is also the source of the mesolimbic DA system, which is believed to be hyperactive in schizophrenia and play a role in the generation of psychotic symptoms. If an elevation in brain D-serine levels also causes an increase in activation of mesolimbic DA neurons in patients with schizophrenia, this could therefore have a potentially detrimental effect. However, evidence from a preclinical study suggests that the opposite may occur. *In vivo* microdialysis in the nucleus accumbens shell (an output region of mesolimbic DA neurons originating in the VTA) revealed that systemic treatment with D-serine attenuated amphetamine-evoked DA release (Smith *et al*., 2009). It is therefore conceivable that systemic treatment with D-serine in the clinical situation would also reduce the activity of the mesolimbic pathway, while concurrently increasing the activity of the mesocortical pathway. An interesting study to investigate this notion would be to use *in vivo* microdialysis with probes located simultaneously in the PFC and nucleus accumbens shell, coupled with systemic injections of D-serine and DAO inhibitors. This would allow for the comparison of the effects of the injection on the two DA systems within each animal, and perhaps give a snapshot of the potential differential effects of systemic D-serine administration and DAO inhibition on the DA systems in the clinic.

It is also important to consider how far the results of the current rodent studies can be extrapolated to humans. A recent paper, published after the completion of the current

laboratory work, proposed a different role for DAO in rodents than in humans based on several differences observed between the rat and human enzymes, and suggested that the rat may not be a good model to use when investigating DAO, D-serine and schizophrenia (Frattini *et al*., 2011). The authors reported, for example, differences in substrate specificity, different kinetic efficiencies and different affinities for DAO inhibitors. However, this *in vitro* study was based on various measures of the biochemical properties of rat DAO that was expressed in *E. Coli* and compared with the results of a different study. Whereas there may indeed be differences in the specific biochemical properties of the enzyme across different species, this does not rule out a role for the rat in providing a valuable insight into potential physiological effects in humans, as it has previously. Although the use of the rat is far from perfect, it still represents a useful tool for the assessment of the potential effects of psychoactive drugs in humans. Until DAO inhibitors are administered to humans, how accurate the rat is as a model will remain unknown.

Together, the results of chapters 3 and 4 provided evidence that manipulation of D-serine levels and DAO activity in the brain, both globally and specifically in the VTA, affected levels of DA and its metabolites in the PFC. This provides support for a link between DAO and the glutamate and DA systems, and provides an interesting explanation for the improvements in negative and cognitive symptoms of schizophrenia seen with clinical D-serine treatment.

6.3 The effects of genetically abolishing DAO activity on markers of DA system function

Chapters 3 and 4 provided pharmacological evidence for a link between DAO activity and the mesocortical DA system. To complement these, pilot investigations were carried out in novel mouse lacking DAO activity in order to assess the effects of a constitutive absence of DAO on the expression of DA genes in the PFC. Alterations were observed in the expression of DA-related genes, with significant increases in the expression of Drd2 and Comt in the PFC of DAO knockout mice when compared with a combined group of wild types and heterozygotes. This final chapter therefore provided further evidence that DAO activity in the brain plays a role in the modulation of DA in the PFC *in vivo*.

However, this study constituted only a small pilot study of the characteristics of the novel DAO knockout mouse. These mice represent a valuable opportunity to further study the physiological relevance of DAO. Numerous additional studies relevant to this thesis could be carried out, which would critically include a detailed characterisation of the pattern of D-serine activity in the knockout brain, including the VTA. qRT-PCR could be employed to probe the expression of other DA genes in the PFC, as well as genes relevant to the NMDA receptor, which may allow for the characterisation of the DAO knockout mouse with relevance to the glutamate and DA systems. Measurements of extracellular levels of DA in the PFC of these mice would be useful for comparing the effects of acute systemic D-serine administration and/or DAO inhibition (as in the current thesis) and a lifetime absence of DAO on cortical DA, as well as the effects of the DAO gene dosage (in heterozygotes and wild-types versus the total knockouts). Measurements of extracellular DA in the nucleus accumbens would also be interesting, in order to assess the effects of the knockout on the mesolimbic DA system, and to compare this with the effects on the mesocortical system. Investigations into the function of VTA DA neurons in the knockout mice, for example by using *in vivo* electrophysiology, could provide an interesting means with which to assess the importance of DAO function in the VTA. The mice would also be

useful for confirming the effects of the DAO inhibitors in use in the current studies, since the administration of a DAO inhibitor to a knockout mouse, be it systemic or local to the VTA, should have no effect. Finally, behavioural tests could be performed with the mice to probe the effects of any molecular and neurochemical changes observed in the proposed experiments above. Ideally, individual mice could be behaviourally assessed before, or during, analysis for neurochemical and molecular changes, to allow for individual correlation of effects.

The current study represents a small pilot investigation into the effects of constitutive DAO knockout in the mouse, but provides results consistent with a role for DAO in the modulation of cortical DA. This novel DAO knockout mouse provides an excellent opportunity for the further study of the physiological relevance of DAO expression.

Summary

In summary, this thesis has advanced our understanding of the expression of DAO and its potential links with the DA system in the context of schizophrenia, although clearly there are many questions left to be answered. These findings provide the basis for numerous further studies into the role of DAO throughout the brain, and how its indirect modulation of NMDA receptors may be physiologically relevant. The data allow for the proposal of a possible mechanism with which to explain the efficacy of D-serine therapy and DAO inhibition in the treatment of negative and cognitive symptoms of schizophrenia, and provide support for its potential future therapeutic use.

References

Abi-Dargham A (2007). Alterations of Serotonin Transmission in Schizophrenia. *Int Rev Neurobiol* **78**:133-164.

Accord Healthcare Ltd. Olanzapine 10 mg Film-coated Tablets Summary of Product Characteristics. Available online at the Electronic Medicines Compendium (eMC; updated 19/07/2012; accessed September 2012).

Adage T, Trillat A C, Quattropani A, Perrin D, Cavarec L, Shaw J, Guerassimenko O, Giachetti C, Greco B, Chumakov I, Halazy S, Roach A and Zaratin P (2008). In Vitro and in Vivo Pharmacological Profile of AS057278, a Selective D-Amino Acid Oxidase Inhibitor With Potential Anti-Psychotic Properties. *Eur Neuropsychopharmacol* **18**:200-214.

Allen NC, Bagade S, McQueen M B, Ioannidis J P, Kavvoura F K, Khoury M J, Tanzi R E and Bertram L (2008). Systematic Meta-Analyses and Field Synopsis of Genetic Association Studies in Schizophrenia: the SzGene Database. *Nat Genet* **40**:827-834.

Almond SL, Fradley R L, Armstrong E J, Heavens R B, Rutter A R, Newman R J, Chiu C S, Konno R, Hutson P H and Brandon N J (2006). Behavioral and Biochemical Characterization of a Mutant Mouse Strain Lacking D-Amino Acid Oxidase Activity and Its Implications for Schizophrenia. *Mol Cell Neurosci* **32**:324-334.

American Psychiatric Association (2000). Diagnostic and statistical manual of mental disorders (4th ed., Text Revision). Washington, DC: Author.

Andreasen NC and Pierson R (2008). The Role of the Cerebellum in Schizophrenia. *Biol Psychiatry* **64**:81-88.

Ascher P and Nowak L (1988). The Role of Divalent Cations in the N-Methyl-D-Aspartate Responses of Mouse Central Neurones in Culture. *J Physiol* **399**:247-266.

Bannai M, Kawai N, Nagao K, Nakano S, Matsuzawa D and Shimizu E (2011). Oral Administration of Glycine Increases Extracellular Serotonin but Not Dopamine in the Prefrontal Cortex of Rats. *Psychiatry Clin Neurosci* **65**:142-149.

Bass NJ, Datta S R, McQuillin A, Puri V, Choudhury K, Thirumalai S, Lawrence J, Quested D, Pimm J, Curtis D and Gurling H M (2009). Evidence for the Association of the DAOA (G72) Gene With Schizophrenia and Bipolar Disorder but Not for the Association of the DAO Gene With Schizophrenia. *Behav Brain Funct* **5**:28.

Bendikov I, Nadri C, Amar S, Panizzutti R, De M J, Wolosker H and Agam G (2007). A CSF and Postmortem Brain Study of D-Serine Metabolic Parameters in Schizophrenia. *Schizophr Res* **90**:41-51.

Bennett S and Gronier B (2007). Effects of Antipsychotic Treatments and D-Serine Supplementation on the Electrophysiological Activation of Midbrain Dopamine Neurons Induced by the Noncompetitive NMDA Antagonist MK 801. *Synapse* **61**:679-688.

Benzel I, Kew J N, Viknaraja R, Kelly F, de B J, Hirsch S, Sanderson T H and Maycox P R (2008). Investigation of G72 (DAOA) Expression in the Human Brain. *BMC Psychiatry* **8**:94.

Bliss TV and Collingridge G L (1993). A Synaptic Model of Memory: Long-Term Potentiation in the Hippocampus. *Nature* **361**:31-39.

Burnet PW, Anderson P N, Chen L, Nikiforova N, Harrison P J and Wood M J (2011). D-Amino Acid Oxidase Knockdown in the Mouse Cerebellum Reduces NR2A MRNA. *Mol Cell Neurosci* **46**:167-175.

Burnet PW, Eastwood S L, Bristow G C, Godlewska B R, Sikka P, Walker M and Harrison P J (2008). D-Amino Acid Oxidase Activity and Expression Are Increased in Schizophrenia. *Mol Psychiatry* **13**:658-660.

Bymaster FP, Katner J S, Nelson D L, Hemrick-Luecke S K, Threlkeld P G, Heiligenstein J H, Morin S M, Gehlert D R and Perry K W (2002). Atomoxetine Increases Extracellular Levels of Norepinephrine and Dopamine in Prefrontal Cortex of Rat: a Potential Mechanism for Efficacy in Attention Deficit/Hyperactivity Disorder. *Neuropsychopharmacology* **27**:699-711.

Caldinelli L, Iametti S, Barbiroli A, Bonomi F, Piubelli L, Ferranti P, Picariello G, Pilone M S and Pollegioni L (2004). Unfolding Intermediate in the Peroxisomal Flavoprotein D-Amino Acid Oxidase. *J Biol Chem* **279**:28426-28434.

Cardno AG and Gottesman I I (2000). Twin Studies of Schizophrenia: From Bow-and-Arrow Concordances to Star Wars Mx and Functional Genomics. *Am J Med Genet* **97**:12-17.

Charléty PJ, Chergui K, Akaoka H, Saunier CF, Buda M, Aston-Jones G, Chouvet G (1993). Serotonin Differentially Modulates Responses Mediated by Specific Excitatory Amino Acid Receptors in the Rat Locus Coeruleus. *Eur J Neurosci* **5**:1024-8.

Charléty PJ, Grenhoff J, Chergui K, De la Chapelle B, Buda M, Svensson TH and Chouvet G (1991). Burst Firing of Mesencephalic Dopamine Neurons is Inhibited by Somatodendritic Application of Kynurenate. *Acta Physiol Scand* **142**: 105-112.

Chergui K, Charlety P J, Akaoka H, Saunier C F, Brunet J L, Buda M, Svensson T H and Chouvet G (1993). Tonic Activation of NMDA Receptors Causes Spontaneous Burst Discharge of Rat Midbrain Dopamine Neurons in Vivo. *Eur J Neurosci* **5**:137-144.

Chumakov I, Blumenfeld M, Guerassimenko O, Cavarec L, Palicio M, Abderrahim H, Bougueleret L, Barry C, Tanaka H, La R P, Puech A, Tahri N, Cohen-Akenine A, Delabrosse S, Lissarrague S, Picard F P, Maurice K, Essioux L, Millasseau P, Grel P, Debailleul V, Simon A M, Caterina D, Dufaure I, Malekzadeh K, Belova M, Luan J J, Bouillot M, Sambucy J L, Primas G, Saumier M, Boubkiri N, Martin-Saumier S, Nasroune M, Peixoto H, Delaye A, Pinchot V, Bastucci M, Guillou S, Chevillon M, Sainz-Fuertes R, Meguenni S, Aurich-Costa J, Cherif D, Gimalac A, Van D C, Gauvreau D, Ouellette G, Fortier I, Raelson J, Sherbatich T, Riazanskaia N, Rogaev E, Raeymaekers P, Aerssens J, Konings F, Luyten W, Macciardi F, Sham P C, Straub R E, Weinberger D R, Cohen N and Cohen D (2002). Genetic and Physiological Data Implicating the New Human Gene G72 and the Gene for D-Amino Acid Oxidase in Schizophrenia. *Proc Natl Acad Sci U S A* **99**:13675-13680.

Collingridge G (1987). Synaptic Plasticity. The Role of NMDA Receptors in Learning and Memory. *Nature* **330**:604-605.

Collins AL, Kim Y, Sklar P, O'Donovan M C and Sullivan P F (2012). Hypothesis-Driven Candidate Genes for Schizophrenia Compared to Genome-Wide Association Results. *Psychol Med* **42**:607-616.

Corvin A, McGhee K A, Murphy K, Donohoe G, Nangle J M, Schwaiger S, Kenny N, Clarke S, Meagher D, Quinn J, Scully P, Baldwin P, Browne D, Walsh C, Waddington J L, Morris D W and Gill M (2007). Evidence for Association and Epistasis at the DAOA/G30 and D-Amino Acid Oxidase Loci in an Irish Schizophrenia Sample. *Am J Med Genet B Neuropsychiatr Genet* **144B**:949-953.

Coyle JT, Tsai G and Goff D (2003). Converging Evidence of NMDA Receptor Hypofunction in the Pathophysiology of Schizophrenia. *Ann N Y Acad Sci* **1003**:318-327.

Coyle JT, Tsai G and Goff D C (2002). Ionotropic Glutamate Receptors As Therapeutic Targets in Schizophrenia. *Curr Drug Targets CNS Neurol Disord* **1**:183-189.

Davis KL, Kahn R S, Ko G and Davidson M (1991). Dopamine in Schizophrenia: a Review and Reconceptualization. *Am J Psychiatry* **148**:1474-1486.

Dobi A, Margolis E B, Wang H L, Harvey B K and Morales M (2010). Glutamatergic and Nonglutamatergic Neurons of the Ventral Tegmental Area Establish Local Synaptic Contacts With Dopaminergic and Nondopaminergic Neurons. *J Neurosci* **30**:218-229.

Dolinska M, Zablocka B, Sonnewald U and Albrecht J (2004). Glutamine Uptake and Expression of MRNA's of Glutamine Transporting Proteins in Mouse Cerebellar and Cerebral Cortical Astrocytes and Neurons. *Neurochem Int* **44**:75-81.

Du W, Aloyo V J and Harvey J A (1997). Harmaline Competitively Inhibits [3H]MK-801 Binding to the NMDA Receptor in Rabbit Brain. *Brain Res* **770**:26-29.

Dunlop DS, Neidle A, McHale D, Dunlop D M and Lajtha A (1986). The Presence of Free D-Aspartic Acid in Rodents and Man. *Biochem Biophys Res Commun* **141**:27-32.

Erhardt S and Engberg G (2002). Increased Phasic Activity of Dopaminergic Neurones in the Rat Ventral Tegmental Area Following Pharmacologically Elevated Levels of Endogenous Kynurenic Acid. *Acta Physiol Scand* **175**:45-53.

Fernandez-Espejo E, Ramiro-Fuentes S, Portavella M and Moreno-Paublete R (2008). Role for D-Serine Within the Ventral Tegmental Area in the Development of Cocaine's Sensitization. *Neuropsychopharmacology* **33**:995-1003.

Fisher GH, D'Aniello A, Vetere A, Padula L, Cusano G P and Man E H (1991). Free D-Aspartate and D-Alanine in Normal and Alzheimer Brain. *Brain Res Bull* **26**:983-985.

Frattini LF, Piubelli L, Sacchi S, Molla G and Pollegioni L (2011). Is Rat an Appropriate Animal Model to Study the Involvement of D-Serine Catabolism in Schizophrenia? Insights From Characterization of D-Amino Acid Oxidase. *FEBS J* **278**:4362-4373.

French ED, Mura A and Wang T (1993). MK-801, Phencyclidine (PCP), and PCP-like Drugs Increase Burst Firing in Rat A10 Dopamine Neurons: Comparison to Competitive NMDA Antagonists. *Synapse* **13**: 108-116.

Fuchs H and Hauber W (2004). Changes in Extracellular Dopamine in the Rat Globus Pallidus Induced by Typical and Atypical Antipsychotic Drugs. *Neurochem Int* **45**:1029-1038.

Fukui K, Watanabe F, Shibata T and Miyake Y (1987). Molecular Cloning and Sequence Analysis of CDNAs Encoding Porcine Kidney D-Amino Acid Oxidase. *Biochemistry* **26**:3612-3618.

Ganote CE, Peterson D R and Carone F A (1974). The Nature of D-Serine--Induced Nephrotoxicity. *Am J Pathol* **77**:269-282.

Garthwaite G, Yamini B, Jr. and Garthwaite J (1987). Selective Loss of Purkinje and Granule Cell Responsiveness to N-Methyl-D-Aspartate in Rat Cerebellum During Development. *Brain Res* **433**:288-292.

Gartside SE, Cole AJ, Williams AP, McQuade R and Judge SJ (2007). AMPA and NMDA Receptor Regulation of Firing Activity in 5-HT Neurons of the Dorsal and Median Raphe Nuclei. *Eur J Neurosci* **25**:3001-8.

Gaunt GL and de Duve C (1976). Subcellular Distribution of D-Amino Acid Oxidase and Catalase in Rat Brain. *J Neurochem* **26**:749-759.

Gejman PV, Sanders A R and Kendler K S (2011). Genetics of Schizophrenia: New Findings and Challenges. *Annu Rev Genomics Hum Genet* **12**:121-144.

Girgenti MJ, Loturco J J and Maher B J (2012). ZNF804a Regulates Expression of the Schizophrenia-Associated Genes PRSS16, COMT, PDE4B, and DRD2. *PLoS One* **7**:e32404.

Gliddon CM, Shao Z, LeMaistre J L and Anderson C M (2009). Cellular Distribution of the Neutral Amino Acid Transporter Subtype ASCT2 in Mouse Brain. *J Neurochem* **108**:372-383.

Goff DC, Herz L, Posever T, Shih V, Tsai G, Henderson D C, Freudenreich O, Evins A E, Yovel I, Zhang H and Schoenfeld D (2005). A Six-Month, Placebo-Controlled Trial of D-Cycloserine Co-Administered With Conventional Antipsychotics in Schizophrenia Patients. *Psychopharmacology (Berl)* **179**:144-150.

Gong N, Gao Z Y, Wang Y C, Li X Y, Huang J L, Hashimoto K and Wang Y X (2011). A Series of D-Amino Acid Oxidase Inhibitors Specifically Prevents and Reverses Formalin-Induced Tonic Pain in Rats. *J Pharmacol Exp Ther* **336**:282-293.

Gould SJ, Keller G A and Subramani S (1988). Identification of Peroxisomal Targeting Signals Located at the Carboxy Terminus of Four Peroxisomal Proteins. *J Cell Biol* **107**:897-905.

Hamase K, Homma H, Takigawa Y, Fukushima T, Santa T and Imai K (1997). Regional Distribution and Postnatal Changes of D-Amino Acids in Rat Brain. *Biochim Biophys Acta* **1334**:214-222.

Hamase K, Inoue T, Morikawa A, Konno R and Zaitsu K (2001). Determination of Free D-Proline and D-Leucine in the Brains of Mutant Mice Lacking D-Amino Acid Oxidase Activity. *Anal Biochem* **298**:253-258.

Hamase K, Konno R, Morikawa A and Zaitsu K (2005). Sensitive Determination of D-Amino Acids in Mammals and the Effect of D-Amino-Acid Oxidase Activity on Their Amounts. *Biol Pharm Bull* **28**:1578-1584.

Harrison PJ and Weinberger D R (2005). Schizophrenia Genes, Gene Expression, and Neuropathology: on the Matter of Their Convergence. *Mol Psychiatry* **10**:40-68.

Hashimoto A, Kumashiro S, Nishikawa T, Oka T, Takahashi K, Mito T, Takashima S, Doi N, Mizutani Y, Yamazaki T, Kaneko T and Ootomo E (1993a). Embryonic Development and Postnatal Changes in Free D-Aspartate and D-Serine in the Human Prefrontal Cortex. *J Neurochem* **61**:348-351.

Hashimoto A, Nishikawa T, Hayashi T, Fujii N, Harada K, Oka T and Takahashi K (1992). The Presence of Free D-Serine in Rat Brain. *FEBS Lett* **296**:33-36.

Hashimoto A, Nishikawa T, Konno R, Niwa A, Yasumura Y, Oka T and Takahashi K (1993b). Free D-Serine, D-Aspartate and D-Alanine in Central Nervous System and Serum in Mutant Mice Lacking D-Amino Acid Oxidase. *Neurosci Lett* **152**:33-36.

Hashimoto A, Nishikawa T, Oka T and Takahashi K (1993c). Endogenous D-Serine in Rat Brain: N-Methyl-D-Aspartate Receptor-Related Distribution and Aging. *J Neurochem* **60**:783-786.

Hashimoto A and Oka T (1997). Free D-Aspartate and D-Serine in the Mammalian Brain and Periphery. *Prog Neurobiol* **52**:325-353.

Hashimoto A, Yoshikawa M, Niwa A and Konno R (2005a). Mice Lacking D-Amino Acid Oxidase Activity Display Marked Attenuation of Stereotypy and Ataxia Induced by MK-801. *Brain Res* **1033**:210-215.

Hashimoto K, Engberg G, Shimizu E, Nordin C, Lindstrom L H and Iyo M (2005b). Reduced D-Serine to Total Serine Ratio in the Cerebrospinal Fluid of Drug Naive Schizophrenic Patients. *Prog Neuropsychopharmacol Biol Psychiatry* **29**:767-769.

Hashimoto K, Fukushima T, Shimizu E, Komatsu N, Watanabe H, Shinoda N, Nakazato M, Kumakiri C, Okada S, Hasegawa H, Imai K and Iyo M (2003). Decreased Serum Levels of D-Serine in Patients With Schizophrenia: Evidence in Support of the N-Methyl-D-Aspartate Receptor Hypofunction Hypothesis of Schizophrenia. *Arch Gen Psychiatry* **60**:572-576.

Hashimoto K, Sawa A and Iyo M (2007). Increased Levels of Glutamate in Brains From Patients With Mood Disorders. *Biol Psychiatry* **62**:1310-1316.

Helboe L, Egebjerg J, Moller M and Thomsen C (2003). Distribution and Pharmacology of Alanine-Serine-Cysteine Transporter 1 (Asc-1) in Rodent Brain. *Eur J Neurosci* **18**:2227-2238.

Heresco-Levy U and Javitt D C (2004). Comparative Effects of Glycine and D-Cycloserine on Persistent Negative Symptoms in Schizophrenia: a Retrospective Analysis. *Schizophr Res* **66**:89-96.

Heresco-Levy U, Javitt D C, Ebstein R, Vass A, Lichtenberg P, Bar G, Catinari S and Ermilov M (2005). D-Serine Efficacy As Add-on Pharmacotherapy to Risperidone and Olanzapine for Treatment-Refractory Schizophrenia. *Biol Psychiatry* **57**:577-585.

Heresco-Levy U, Javitt D C, Ermilov M, Mordel C, Silipo G and Lichtenstein M (1999). Efficacy of High-Dose Glycine in the Treatment of Enduring Negative Symptoms of Schizophrenia. *Arch Gen Psychiatry* **56**:29-36.

Hnasko TS, Chuhma N, Zhang H, Goh G Y, Sulzer D, Palmiter R D, Rayport S and Edwards R H (2010). Vesicular Glutamate Transport Promotes Dopamine Storage and Glutamate Corelease in Vivo. *Neuron* **65**:643-656.

Horiike K, Tojo H, Arai R, Nozaki M and Maeda T (1994). D-Amino-Acid Oxidase Is Confined to the Lower Brain Stem and Cerebellum in Rat Brain: Regional Differentiation of Astrocytes. *Brain Res* **652**:297-303.

Horiike K, Tojo H, Arai R, Yamano T, Nozaki M and Maeda T (1987). Localization of D-Amino Acid Oxidase in Bergmann Glial Cells and Astrocytes of Rat Cerebellum. *Brain Res Bull* **19**:587-596.

Howes OD and Kapur S (2009). The Dopamine Hypothesis of Schizophrenia: Version III--the Final Common Pathway. *Schizophr Bull* **35**:549-562.

Janssen-Cilag Ltd. Risperdal Tablets, Liquid & Quicklet Summary of Product Characteristics. Available online at the Electronic Medicines Compendium (eMC; updated 06/07/2012; accessed September 2012).

Javitt DC (2006). Is the Glycine Site Half Saturated or Half Unsaturated? Effects of Glutamatergic Drugs in Schizophrenia Patients. *Curr Opin Psychiatry* **19**:151-157.

Javitt DC and Zukin S R (1991). Recent Advances in the Phencyclidine Model of Schizophrenia. *Am J Psychiatry* **148**:1301-1308.

Javitt DC, Zylberman I, Zukin S R, Heresco-Levy U and Lindenmayer J P (1994). Amelioration of Negative Symptoms in Schizophrenia by Glycine. *Am J Psychiatry* **151**:1234-1236.

Johnson SW, Seutin V and North R A (1992). Burst Firing in Dopamine Neurons Induced by N-Methyl-D-Aspartate: Role of Electrogenic Sodium Pump. *Science* **258**:665-667.

Kakegawa W, Miyoshi Y, Hamase K, Matsuda S, Matsuda K, Kohda K, Emi K, Motohashi J, Konno R, Zaitsu K and Yuzaki M (2011). D-Serine Regulates Cerebellar LTD and Motor Coordination Through the Delta2 Glutamate Receptor. *Nat Neurosci* **14**:603-611.

Kalivas PW and Duffy P (1998). Repeated Cocaine Administration Alters Extracellular Glutamate in the Ventral Tegmental Area. *J Neurochem* **70**:1497-1502.

Kalivas PW and Stewart J (1991). Dopamine Transmission in the Initiation and Expression of Drug- and Stress-Induced Sensitization of Motor Activity. *Brain Res Brain Res Rev* **16**:223-244.

Kantrowitz JT, Malhotra A K, Cornblatt B, Silipo G, Balla A, Suckow R F, D'Souza C, Saksa J, Woods S W and Javitt D C (2010). High Dose D-Serine in the Treatment of Schizophrenia. *Schizophr Res* **121**:125-130.

Kapoor R, Lim K S, Cheng A, Garrick T and Kapoor V (2006). Preliminary Evidence for a Link Between Schizophrenia and NMDA-Glycine Site Receptor Ligand Metabolic Enzymes, D-Amino Acid Oxidase (DAAO) and Kynurenine Aminotransferase-1 (KAT-1). *Brain Res* **1106**:205-210.

Karler R, Calder LD, Chaudry IA and Turkanis SA (1989). Blockade of "Reverse Tolerance" to Cocaine and Amphetamine by MK801. *Life Sci* **45**:599-606.

Kartvelishvily E, Shleper M, Balan L, Dumin E and Wolosker H (2006). Neuron-Derived D-serine Release Provides a Novel Means to Activate N-Methyl-D-Aspartate Receptors. *J Biol Chem* **281**:14151-14162.

Kashiwabuchi N, Ikeda K, Araki K, Hirano T, Shibuki K, Takayama C, Inoue Y, Kutsuwada T, Yagi T, Kang Y, Aizawa S and Mishina M (1995). Impairment of Motor Coordination, Purkinje Cell Synapse Formation, and Cerebellar Long-Term Depression in GluR Delta 2 Mutant Mice. *Cell* **81**:245-252.

Kim B, Kim H, Joo Y H, Lim J, Kim C Y and Song K (2010). Sex-Different Association of DAO With Schizophrenia in Koreans. *Psychiatry Res* **179**:121-125.

Kim JS, Kornhuber H H, Schmid-Burgk W and Holzmuller B (1980). Low Cerebrospinal Fluid Glutamate in Schizophrenic Patients and a New Hypothesis on Schizophrenia. *Neurosci Lett* **20**:379-382.

Kim Y, Zerwas S, Trace S E and Sullivan P F (2011). Schizophrenia Genetics: Where Next? *Schizophr Bull* **37**:456-463.

Kleckner NW and Dingledine R (1988). Requirement for Glycine in Activation of NMDA-Receptors Expressed in Xenopus Oocytes. *Science* **241**:835-837.

Komuro H and Rakic P (1993). Modulation of Neuronal Migration by NMDA Receptors. *Science* **260**:95-97.

Konno R (1998). Rat D-Amino-Acid Oxidase CDNA: Rat D-Amino-Acid Oxidase As an Intermediate Form Between Mouse and Other Mammalian D-Amino-Acid Oxidases. *Biochim Biophys Acta* **1395**:165-170.

Konno R and Yasumura Y (1983). Mouse Mutant Deficient in D-Amino Acid Oxidase Activity. *Genetics* **103**:277-285.

Krebs HA (1935). Metabolism of Amino-Acids: Deamination of Amino-Acids. *Biochem J* **29**:1620-1644.

Krug AW, Volker K, Dantzler W H and Silbernagl S (2007). Why Is D-Serine Nephrotoxic and Alpha-Aminoisobutyric Acid Protective? *Am J Physiol Renal Physiol* **293**:F382-F390.

Kumashiro S, Hashimoto A and Nishikawa T (1995). Free D-Serine in Post-Mortem Brains and Spinal Cords of Individuals With and Without Neuropsychiatric Diseases. *Brain Res* **681**:117-125.

Kuryatov A, Laube B, Betz H and Kuhse J (1994). Mutational Analysis of the Glycine-Binding Site of the NMDA Receptor: Structural Similarity With Bacterial Amino Acid-Binding Proteins. *Neuron* **12**:1291-1300.

Labrie V, Clapcote S J and Roder J C (2009). Mutant Mice With Reduced NMDA-NR1 Glycine Affinity or Lack of D-Amino Acid Oxidase Function Exhibit Altered Anxiety-Like Behaviors. *Pharmacol Biochem Behav* **91**:610-620.

Laube B, Hirai H, Sturgess M, Betz H and Kuhse J (1997). Molecular Determinants of Agonist Discrimination by NMDA Receptor Subunits: Analysis of the Glutamate Binding Site on the NR2B Subunit. *Neuron* **18**:493-503.

Laube B, Kuhse J and Betz H (1998). Evidence for a Tetrameric Structure of Recombinant NMDA Receptors. *J Neurosci* **18**:2954-2961.

Levinson DF, Duan J, Oh S, Wang K, Sanders A R, Shi J, Zhang N, Mowry B J, Olincy A, Amin F, Cloninger C R, Silverman J M, Buccola N G, Byerley W F, Black D W, Kendler K S, Freedman R, Dudbridge F, Pe'er I, Hakonarson H, Bergen S E, Fanous A H, Holmans P A and Gejman P V (2011). Copy Number Variants in Schizophrenia: Confirmation of Five Previous Findings and New Evidence for 3q29 Microdeletions and VIPR2 Duplications. *Am J Psychiatry* **168**:302-316.

Li Y, Vartanian AJ, White FJ, Xue CJ and Wolf ME (1997). Effects of the AMPA Receptor Antagonist NBQX on the Development and Expression of Behavioural Sensitization to Cocaine and Amphetamine. *Psychopharmacology* **134**:266-276.

Licata SC and Pierce R C (2003). The Roles of Calcium/Calmodulin-Dependent and Ras/Mitogen-Activated Protein Kinases in the Development of Psychostimulant-Induced Behavioral Sensitization. *J Neurochem* **85**:14-22.

Liu X, He G, Wang X, Chen Q, Qian X, Lin W, Li D, Gu N, Feng G and He L (2004). Association of DAAO With Schizophrenia in the Chinese Population. *Neurosci Lett* **369**:228-233.

Liu YL, Fann C S, Liu C M, Chang C C, Wu J Y, Hung S I, Liu S K, Hsieh M H, Hwang T J, Chan H Y, Chen J J, Faraone S V, Tsuang M T, Chen W J and Hwu H G (2006). No Association of G72 and D-Amino Acid Oxidase Genes With Schizophrenia. *Schizophr Res* **87**:15-20.

Madeira C, Freitas M E, Vargas-Lopes C, Wolosker H and Panizzutti R (2008). Increased Brain D-Amino Acid Oxidase (DAAO) Activity in Schizophrenia. *Schizophr Res* **101**:76-83.

Maekawa M, Watanabe M, Yamaguchi S, Konno R and Hori Y (2005). Spatial Learning and Long-Term Potentiation of Mutant Mice Lacking D-Amino-Acid Oxidase. *Neurosci Res* **53**:34-38.

Marek GJ, Behl B, Bespalov A Y, Gross G, Lee Y and Schoemaker H (2010). Glutamatergic (N-Methyl-D-Aspartate Receptor) Hypofrontality in Schizophrenia: Too Little Juice or a Miswired Brain? *Mol Pharmacol* **77**:317-326.

Mathe JM, Nomikos G G, Blakeman K H and Svensson T H (1999). Differential Actions of Dizocilpine (MK-801) on the Mesolimbic and Mesocortical Dopamine Systems: Role of Neuronal Activity. *Neuropharmacology* **38**:121-128.

Mittleman G, Goldowitz D, Heck D H and Blaha C D (2008). Cerebellar Modulation of Frontal Cortex Dopamine Efflux in Mice: Relevance to Autism and Schizophrenia. *Synapse* **62**:544-550.

Miyoshi Y, Hamase K, Tojo Y, Mita M, Konno R and Zaitsu K (2009). Determination of D-serine and D-Alanine in the Tissues and Physiological Fluids of Mice With Various D-Amino-Acid Oxidase Activities Using Two-Dimensional High-Performance Liquid Chromatography With Fluorescence Detection. *J Chromatogr B Analyt Technol Biomed Life Sci* **877**:2506-2512.

Moghaddam B (2003). Bringing Order to the Glutamate Chaos in Schizophrenia. *Neuron* **40**:881-884.

Moghaddam B and Jackson M E (2003). Glutamatergic Animal Models of Schizophrenia. *Ann N Y Acad Sci* **1003**:131-137.

Molla G, Sacchi S, Bernasconi M, Pilone M S, Fukui K and Polegioni L (2006). Characterization of Human D-Amino Acid Oxidase. *FEBS Lett* **580**:2358-2364.

Momoi K, Fukui K, Watanabe F and Miyake Y (1988). Molecular Cloning and Sequence Analysis of CDNA Encoding Human Kidney D-Amino Acid Oxidase. *FEBS Lett* **238**:180-184.

Moreno S, Nardacci R, Cimini A and Ceru M P (1999). Immunocytochemical Localization of D-Amino Acid Oxidase in Rat Brain. *J Neurocytol* **28**:169-185.

Morikawa A, Hamase K, Inoue T, Konno R, Niwa A and Zaitsu K (2001). Determination of Free D-Aspartic Acid, D-Serine and D-Alanine in the Brain of Mutant Mice Lacking D-Amino Acid Oxidase Activity. *J Chromatogr B Biomed Sci Appl* **757**:119-125.

Morikawa A, Hamase K and Zaitsu K (2003). Determination of D-Alanine in the Rat Central Nervous System and Periphery Using Column-Switching High-Performance Liquid Chromatography. *Anal Biochem* **312**:66-72.

Moron JA, Brockington A, Wise R A, Rocha B A and Hope B T (2002). Dopamine Uptake Through the Norepinephrine Transporter in Brain Regions With Low Levels of the Dopamine Transporter: Evidence From Knock-Out Mouse Lines. *J Neurosci* **22**:389-395.

Moses J, Siddiqui A and Silverman P B (1996). Sodium Benzoate Differentially Blocks Circling Induced by D-and L-Dopa in the Hemi-Parkinsonian Rat. *Neurosci Lett* **218**:145-148.

Mothet JP, Parent A T, Wolosker H, Brady R O, Jr., Linden D J, Ferris C D, Rogawski M A and Snyder S H (2000). D-Serine Is an Endogenous Ligand for the Glycine Site of the N-Methyl-D-Aspartate Receptor. *Proc Natl Acad Sci U S A* **97**:4926-4931.

Murphy KC, Jones L A and Owen M J (1999). High Rates of Schizophrenia in Adults With Velo-Cardio-Facial Syndrome. *Arch Gen Psychiatry* **56**:940-945.

Nagata Y, Borghi M, Fisher G H and D'Aniello A (1995). Free D-Serine Concentration in Normal and Alzheimer Human Brain. *Brain Res Bull* **38**:181-183.

Nagata Y, Horiike K and Maeda T (1994a). Distribution of Free D-Serine in Vertebrate Brains. *Brain Res* **634**:291-295.

Nagata Y, Konno R and Niwa A (1994b). Amino Acid Levels in D-Alanine-Administered Mutant Mice Lacking D-Amino Acid Oxidase. *Metabolism* **43**:1153-1157.

Nagata Y, Uehara T, Kitamura Y, Nomura Y and Horiike K (1998). D-Serine Content and D-[3H]Serine Binding in the Brain Regions of the Senescence-Accelerated Mouse. *Mech Ageing Dev* **104**:115-124.

Nagata Y, Yamamoto K, Shimojo T, Konno R, Yasumura Y and Akino T (1992). The Presence of Free D-Alanine, D-Proline and D-Serine in Mice. *Biochim Biophys Acta* **1115**:208-211.

Naur P, Hansen K B, Kristensen A S, Dravid S M, Pickering D S, Olsen L, Vestergaard B, Egebjerg J, Gajhede M, Traynelis S F and Kastrup J S (2007). Ionotropic Glutamate-Like Receptor Delta2 Binds D-Serine and Glycine. *Proc Natl Acad Sci U S A* **104**:14116-14121.

Neims AH, Zieverink W D and Smilack J D (1966). Distribution of D-Amino Acid Oxidase in Bovine and Human Nervous Tissues. *J Neurochem* **13**:163-168.

Nong Y, Huang Y Q, Ju W, Kalia L V, Ahmadian G, Wang Y T and Salter M W (2003). Glycine Binding Primes NMDA Receptor Internalization. *Nature* **422**:302-307.

O'Donovan MC, Craddock N, Norton N, Williams H, Peirce T, Moskvina V, Nikolov I, Hamshere M, Carroll L, Georgieva L, Dwyer S, Holmans P, Marchini J L, Spencer C C, Howie B, Leung H T, Hartmann A M, Moller H J, Morris D W, Shi Y, Feng G, Hoffmann P, Propping P, Vasilescu C, Maier W, Rietschel M, Zammit S, Schumacher J, Quinn E M, Schulze T G, Williams N M, Giegling I, Iwata N, Ikeda M, Darvasi A, Shifman S, He L, Duan J, Sanders A R, Levinson D F, Gejman P V, Cichon S, Nothen M M, Gill M, Corvin A, Rujescu D, Kirov G, Owen M J, Buccola N G, Mowry B J, Freedman R, Amin F, Black D W, Silverman J M, Byerley W F and Cloninger C R (2008). Identification of Loci Associated With Schizophrenia by Genome-Wide Association and Follow-Up. *Nat Genet* **40**:1053-1055.

Oliet SH and Mothet J P (2009). Regulation of N-Methyl-D-Aspartate Receptors by Astrocytic D-Serine. *Neuroscience* **158**:275-283.

Ono K, Shishido Y, Park H K, Kawazoe T, Iwana S, Chung S P, Abou El-Magd R M, Yorita K, Okano M, Watanabe T, Sano N, Bando Y, Arima K, Sakai T and Fukui K (2009). Potential Pathophysiological Role of D-Amino Acid Oxidase in Schizophrenia: Immunohistochemical and in Situ Hybridization Study of the Expression in Human and Rat Brain. *J Neural Transm* **116**:1335-1347.

Overton PG and Clark D (1997). Burst Firing in Midbrain Dopaminergic Neurons. *Brain Res Brain Res Rev* **25**:312-334.

Paxinos C and Watson G (2007). The Rat Brain in Stereotaxic Coordinates (6th ed.). London: Elsevier Inc.

Picchioni MM and Murray R M (2007). Schizophrenia. *BMJ* **335**:91-95.

Pinewood Healthcare. DOLPIN Haloperidol Oral Solution BP 10mg/5ml Summary of Product Characteristics. Available online at the Electronic Medicines Compendium (eMC; updated 31/03/2011; accessed September 2012).

Pollegioni L, Ceciliani F, Curti B, Ronchi S and Pilone M S (1995). Studies on the Structural and Functional Aspects of Rhodotorula Gracilis D-Amino Acid Oxidase by Limited Trypsinolysis. *Biochem J* **310 (Pt 2)**:577-583.

Popiolek M, Ross J F, Charych E, Chanda P, Gundelfinger E D, Moss S J, Brandon N J and Pausch M H (2011). D-Amino Acid Oxidase Activity Is Inhibited by an Interaction With Bassoon Protein at the Presynaptic Active Zone. *J Biol Chem* **286**:28867-28875.

Purcell SM, Wray N R, Stone J L, Visscher P M, O'Donovan M C, Sullivan P F and Sklar P (2009). Common Polygenic Variation Contributes to Risk of Schizophrenia and Bipolar Disorder. *Nature* **460**:748-752.

Rabacchi S, Bailly Y, Delhaye-Bouchaud N and Mariani J (1992). Involvement of the N-Methyl D-Aspartate (NMDA) Receptor in Synapse Elimination During Cerebellar Development. *Science* **256**:1823-1825.

Radley JJ, Williams B and Sawchenko P E (2008). Noradrenergic Innervation of the Dorsal Medial Prefrontal Cortex Modulates Hypothalamo-Pituitary-Adrenal Responses to Acute Emotional Stress. *J Neurosci* **28**:5806-5816.

Rogers TD, Dickson P E, Heck D H, Goldowitz D, Mittleman G and Blaha C D (2011). Connecting the Dots of the Cerebro-Cerebellar Role in Cognitive Function: Neuronal Pathways for Cerebellar Modulation of Dopamine Release in the Prefrontal Cortex. *Synapse* **65**:1204-1212.

Rosemont Pharmaceuticals Ltd. Chlorpromazine Hydrochloride 25mg/5ml Oral Syrup Summary of Product Characteristics. Available online at the Electronic Medicines Compendium (eMC; updated 19/03/2012; accessed September 2012).

Sacchi S, Bernasconi M, Martineau M, Mothet J P, Ruzzene M, Pilone M S, Pollegioni L and Molla G (2008). PLG72 Modulates Intracellular D-Serine Levels Through Its Interaction With D-Amino Acid Oxidase: Effect on Schizophrenia Susceptibility. *J Biol Chem* **283**:22244-22256.

Sakata K, Fukushima T, Minje L, Ogurusu T, Taira H, Mishina M and Shingai R (1999). Modulation by L- and D-Isoforms of Amino Acids of the L-Glutamate Response of N-Methyl-D-Aspartate Receptors. *Biochemistry* **38**:10099-10106.

Sasaki M, Konno R, Nishio M, Niwa A, Yasumura Y and Enami J (1992). A Single-Base-Pair Substitution Abolishes D-Amino-Acid Oxidase Activity in the Mouse. *Biochim Biophys Acta* **1139**:315-318.

Schell MJ, Brady R O, Jr., Molliver M E and Snyder S H (1997). D-Serine As a Neuromodulator: Regional and Developmental Localizations in Rat Brain Glia Resemble NMDA Receptors. *J Neurosci* **17**:1604-1615.

Schell MJ, Molliver M E and Snyder S H (1995). D-Serine, an Endogenous Synaptic Modulator: Localization to Astrocytes and Glutamate-Stimulated Release. *Proc Natl Acad Sci U S A* **92**:3948-3952.

Schumacher J, Jamra R A, Freudenberg J, Becker T, Ohlraun S, Otte A C, Tullius M, Kovalenko S, Bogaert A V, Maier W, Rietschel M, Propping P, Nothen M M and Cichon S (2004). Examination of G72 and D-Amino-Acid Oxidase As Genetic Risk Factors for Schizophrenia and Bipolar Affective Disorder. *Mol Psychiatry* **9**:203-207.

Schwieler L, Erhardt S, Nilsson L, Linderholm K and Engberg G (2006). Effects of COX-1 and COX-2 Inhibitors on the Firing of Rat Midbrain Dopaminergic Neurons--Possible Involvement of Endogenous Kynurenic Acid. *Synapse* **59**: 290-298.

Sebat J, Levy D L and McCarthy S E (2009). Rare Structural Variants in Schizophrenia: One Disorder, Multiple Mutations; One Mutation, Multiple Disorders. *Trends Genet* **25**:528-535.

Sesack SR and Pickel VM (1992). Prefrontal Cortical Efferents in the Rat Synapse on Unlabeled Neuronal Targets of Catecholamine Terminals in the Nucleus Accumbens Septi and on Dopamine Neurons in the Ventral Tegmental Area. *J Comp Neurol* **320**:145-160.

Seutin V, Verbanck P, Massotte L and Dresse A (1990). Evidence for the Presence of *N*-methyl-D-aspartate Receptors in the Ventral Tegmental Area of the Rat: An Electrophysiological *in vitro* study. *Brain Research* **514**: 147-150.

Shi J, Gershon E S and Liu C (2008). Genetic Associations With Schizophrenia: Meta-Analyses of 12 Candidate Genes. *Schizophr Res* **104**:96-107.

Shinkai T, De L, V, Hwang R, Muller D J, Lanktree M, Zai G, Shaikh S, Wong G, Sicard T, Potapova N, Trakalo J, King N, Matsumoto C, Hori H, Wong A H, Ohmori O, Macciardi F, Nakamura J and Kennedy J L (2007). Association Analyses of the DAOA/G30 and D-Amino-Acid Oxidase Genes in Schizophrenia: Further Evidence for a Role in Schizophrenia. *Neuromolecular Med* **9**:169-177.

Shleper M, Kartvelishvily E and Wolosker H (2005). D-Serine Is the Dominant Endogenous Coagonist for NMDA Receptor Neurotoxicity in Organotypic Hippocampal Slices. *J Neurosci* **25**:9413-9417.

Smiley JF and Goldman-Rakic P S (1996). Serotonergic Axons in Monkey Prefrontal Cerebral Cortex Synapse Predominantly on Interneurons As Demonstrated by Serial Section Electron Microscopy. *J Comp Neurol* **367**:431-443.

Smith SM, Uslaner J M, Yao L, Mullins C M, Surles N O, Huszar S L, McNaughton C H, Pascarella D M, Kandebo M, Hinchliffe R M, Sparey T, Brandon N J, Jones B, Venkatraman S, Young M B, Sachs N, Jacobson M A and Hutson P H (2009). The Behavioral and Neurochemical Effects of a Novel D-Amino Acid Oxidase Inhibitor Compound 8 [4H-Thieno [3,2-b]Pyrrole-5-Carboxylic Acid] and D-Serine. *J Pharmacol Exp Ther* **328**:921-930.

Snider RS, Maiti A and Snider SR (1976). Cerebellar Pathways to Ventral Midbrain and Nigra. *Exp Neurol* **53**: 714-728.

Song Y, Feng Y, Lu X, Zhao S, Liu C W and Liu Y M (2008). D-Amino Acids in Rat Brain Measured by Liquid Chromatography/Tandem Mass Spectrometry. *Neurosci Lett* **445**:53-57.

Suaud-Chagny MF, Chergui K, Chouvet G and Gonon F (1992). Relationship Between Dopamine Release in the Rat Nucleus Accumbens and the Discharge Activity of Dopaminergic Neurons During Local in Vivo Application of Amino Acids in the Ventral Tegmental Area. *Neuroscience* **49**:63-72.

Sullivan PF, Kendler K S and Neale M C (2003). Schizophrenia As a Complex Trait: Evidence From a Meta-Analysis of Twin Studies. *Arch Gen Psychiatry* **60**:1187-1192.

Sulzer D, Chen T K, Lau Y Y, Kristensen H, Rayport S and Ewing A (1995). Amphetamine Redistributes Dopamine From Synaptic Vesicles to the Cytosol and Promotes Reverse Transport. *J Neurosci* **15**:4102-4108.

Svensson TH, Mathe J M, Nomikos G G and Schilstrom B (1998). Role of Excitatory Amino Acids in the Ventral Tegmental Area for Central Actions of Non-Competitive NMDA-Receptor Antagonists and Nicotine. *Amino Acids* **14**:51-56.

Tada M, Fukui K, Momoi K and Miyake Y (1990). Cloning and Expression of a CDNA Encoding Mouse Kidney D-Amino Acid Oxidase. *Gene* **90**:293-297.

Tsai G, Lane H Y, Yang P, Chong M Y and Lange N (2004). Glycine Transporter I Inhibitor, N-Methylglycine (Sarcosine), Added to Antipsychotics for the Treatment of Schizophrenia. *Biol Psychiatry* **55**:452-456.

Tsai GE, Yang P, Chang Y C and Chong M Y (2006). D-Alanine Added to Antipsychotics for the Treatment of Schizophrenia. *Biol Psychiatry* **59**:230-234.

Tunbridge EM, Bannerman D M, Sharp T and Harrison P J (2004). Catechol-o-Methyltransferase Inhibition Improves Set-Shifting Performance and Elevates Stimulated Dopamine Release in the Rat Prefrontal Cortex. *J Neurosci* **24**:5331-5335.

Tuominen HJ, Tiihonen J and Wahlbeck K (2005). Glutamatergic Drugs for Schizophrenia: a Systematic Review and Meta-Analysis. *Schizophr Res* **72**:225-234.

Vanoni MA, Cosma A, Mazzeo D, Mattevi A, Todone F and Curti B (1997). Limited Proteolysis and X-Ray Crystallography Reveal the Origin of Substrate Specificity and of the Rate-Limiting Product Release During Oxidation of D-Amino Acids Catalyzed by Mammalian D-Amino Acid Oxidase. *Biochemistry* **36**:5624-5632.

Verrall L, Burnet P W, Betts J F and Harrison P J (2010). The Neurobiology of D-Amino Acid Oxidase and Its Involvement in Schizophrenia. *Mol Psychiatry* **15**:122-137.

Verrall L, Walker M, Rawlings N, Benzel I, Kew J N, Harrison P J and Burnet P W (2007). D-Amino Acid Oxidase and Serine Racemase in Human Brain: Normal Distribution and Altered Expression in Schizophrenia. *Eur J Neurosci* **26**:1657-1669.

Vilella E, Costas J, Sanjuan J, Guitart M, De D Y, Carracedo A, Martorell L, Valero J, Labad A, De F R, Najera C, Molto M D, Toirac I, Guillamat R, Brunet A, Valles V, Perez L, Leon M, de Fonseca F R, Phillips C and Torres M (2008). Association of Schizophrenia With DTNBP1 but Not With DAO, DAOA, NRG1 and RGS4 nor Their Genetic Interaction. *J Psychiatr Res* **42**:278-288.

Wafford KA, Kathoria M, Bain C J, Marshall G, Le B B, Kemp J A and Whiting P J (1995). Identification of Amino Acids in the N-Methyl-D-Aspartate Receptor NR1 Subunit That Contribute to the Glycine Binding Site. *Mol Pharmacol* **47**:374-380.

Wake K, Yamazaki H, Hanzawa S, Konno R, Sakio H, Niwa A and Hori Y (2001). Exaggerated Responses to Chronic Nociceptive Stimuli and Enhancement of N-Methyl-D-Aspartate Receptor-Mediated Synaptic Transmission in Mutant Mice Lacking D-Amino-Acid Oxidase. *Neurosci Lett* **297**:25-28.

Wang LP, Li F, Wang D, Xie K, Wang D, Shen X and Tsien J Z (2011). NMDA Receptors in Dopaminergic Neurons Are Crucial for Habit Learning. *Neuron* **72**:1055-1066.

Wang LZ and Zhu X Z (2003). Spatiotemporal Relationships Among D-Serine, Serine Racemase, and D-Amino Acid Oxidase During Mouse Postnatal Development. *Acta Pharmacol Sin* **24**:965-974.

Weimar WR and Neims A H (1977a). Hog Cerebellar D-Amino Acid Oxidase and Its Histochemical and Immunofluorescent Localization. *J Neurochem* **28**:559-572.

Weimar WR and Neims A H (1977b). The Development of D-Amino Acid Oxidase in Rat Cerebellum. *J Neurochem* **29**:649-656.

Weinberger DR, Egan M F, Bertolino A, Callicott J H, Mattay V S, Lipska B K, Berman K F and Goldberg T E (2001). Prefrontal Neurons and the Genetics of Schizophrenia. *Biol Psychiatry* **50**:825-844.

Williams RE and Lock E A (2005). Sodium Benzoate Attenuates D-Serine Induced Nephrotoxicity in the Rat. *Toxicology* **207**:35-48.

Wolosker H (2006). D-Serine Regulation of NMDA Receptor Activity. *Sci STKE* **2006**:e41.

Wolosker H, Blackshaw S and Snyder S H (1999a). Serine Racemase: a Glial Enzyme Synthesizing D-Serine to Regulate Glutamate-N-Methyl-D-Aspartate Neurotransmission. *Proc Natl Acad Sci U S A* **96**:13409-13414.

Wolosker H, Sheth K N, Takahashi M, Mothet J P, Brady R O, Jr., Ferris C D and Snyder S H (1999b). Purification of Serine Racemase: Biosynthesis of the Neuromodulator D-serine. *Proc Natl Acad Sci U S A* **96**:721-725.

Wood LS, Pickering E H and Dechairo B M (2007). Significant Support for DAO As a Schizophrenia Susceptibility Locus: Examination of Five Genes Putatively Associated With Schizophrenia. *Biol Psychiatry* **61**:1195-1199.

Wood PL, Emmett M R and Wood J A (1994). Involvement of Granule, Basket and Stellate Neurons but Not Purkinje or Golgi Cells in Cerebellar CGMP Increases in Vivo. *Life Sci* **54**:615-620.

World Health Organization (2008). ICD-10: International statistical classification of diseases and related health problems (10th Rev. ed.). New York, NY: Author.

Wu EQ, Birnbaum H G, Shi L, Ball D E, Kessler R C, Moulis M and Aggarwal J (2005). The Economic Burden of Schizophrenia in the United States in 2002. *J Clin Psychiatry* **66**:1122-1129.

Yamada K, Ohnishi T, Hashimoto K, Ohba H, Iwayama-Shigeno Y, Toyoshima M, Okuno A, Takao H, Toyota T, Minabe Y, Nakamura K, Shimizu E, Itokawa M, Mori N, Iyo M and Yoshikawa T (2005). Identification of Multiple Serine Racemase (SRR) MRNA Isoforms and Genetic Analyses of SRR and DAO in Schizophrenia and D-Serine Levels. *Biol Psychiatry* **57**:1493-1503.

Yamaguchi T, Sheen W and Morales M (2007). Glutamatergic Neurons Are Present in the Rat Ventral Tegmental Area. *Eur J Neurosci* **25**:106-118.

Zaar K, Kost H P, Schad A, Volkl A, Baumgart E and Fahimi H D (2002). Cellular and Subcellular Distribution of D-Aspartate Oxidase in Human and Rat Brain. *J Comp Neurol* **450**:272-282.

Zetterström T, Sharp T, Collin A K and Ungerstedt U (1988). In Vivo Measurement of Extracellular Dopamine and DOPAC in Rat Striatum After Various Dopamine-Releasing Drugs; Implications for the Origin of Extracellular DOPAC. *Eur J Pharmacol* **148**:327-334.

Zweifel LS, Fadok J P, Argilli E, Garelick M G, Jones G L, Dickerson T M, Allen J M, Mizumori S J, Bonci A and Palmiter R D (2011). Activation of Dopamine Neurons Is Critical for Aversive Conditioning and Prevention of Generalized Anxiety. *Nat Neurosci* **14**:620-626.