Contributions of COMT and DAT to Regulation of Phasic Dopamine Release and Reward-Guided Behaviour

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A thesis submitted for the degree of Doctor of Philosophy

Michaelmas term 2016

Acknowledgements

One of the things I love most about science is working as part of a team. The team I have had around me during my DPhil have been, quite frankly, awesome – they have made my years in Oxford not only successful but also so very enjoyable.

First and foremost: many, many thanks to Liz and Mark for being superb supervisors, from the first draft of my DPhil proposal to the final days of thesis editing. They have gone above and beyond to support, advise, and teach me at every step along the way. Whether I am working out the details of an experimental protocol, learning a new analysis procedure, wrapping my head around a complex paper, or preparing for a job interview, I always know that I will find good counsel with them. Special thanks to Liz for supporting my explorations of science policy and journalism and for showing me how to do science with an unfailingly positive attitude. And special thanks to Mark for always encouraging me to take the work that little bit farther – one additional analysis, one more round of edits – to make sure it was as good as it could be.

I have had the pleasure of working on this project with a wonderful group of collaborators, including two fantastic master's students. Cristiana Vagnoni assisted me with gathering and analyzing the voltammetry data presented in Chapters 4 and 5. Her enthusiasm, astute questions, and hard work were incredibly helpful in moving this part of the project forward. I am especially grateful to Cristiana for working out the electrode calibration analysis procedure, for showing me how to automate my Matlab scripts (which continues to make my life infinitely easier to this day), and for somehow always understanding what I was going to say before I had even finished saying it.

My second student, Kristian Reveles Jensen, was my partner in every aspect of the 2-step experiment presented in Chapter 7. He helped me to set up the equipment, ran the mice side by side with me, and worked with our collaborators to analyse the data. Kristian was the calm and capable centre point of a complex set of experiments, and his ability to tackle every challenge I threw his way was invaluable during a year when I was juggling many different responsibilities. Many thanks, Kristian, for always looking after both the mice and me.

Thomas Akam was also a key player in the 2-step experiment. Beyond, of course, designing the task, he advised us at every stage of setting up and running the experiment, and he worked with Kristian to analyse all of the data that I present in Chapter 7. Many thanks to Thomas for introducing me to the world of computational modeling, for explaining even the most unfamiliar concepts in terms I could understand, and for useful discussions about chemometrics analysis approaches.

Anna Huber also worked with Thomas, Kristian, and me through all the stages of the 2-step experiment. I am grateful not just for her partnership during the 2-step study but also for laying the groundwork for every experiment I did during my DPhil. Anna, thank you for being a wonderful friend, teacher, and role model throughout my time in Oxford. It has been immensely reassuring to have your companionship at every step along the way – I cannot imagine having done it without you.

I would also like to acknowledge Chris Barkus, Mathieu Baudonnat, and Amy Taylor for assisting me with planning and performing my very first DPhil experiment, the locomotor activity test presented in Chapter 2. Special thanks to Chris for being a great mentor during my first MSc project and throughout my DPhil and for all our conversations about both science and theatre. Thanks also to Greg Daubney, who performed most of the histological processing for the voltammetry experiments presented in Chapters 4 and 5. And an enormous thank you to everyone – Anna, Anushka, Amy W, Emilie, Katie H, Laura, Mathieu, Steve C, Thomas B – who looked after me and my mice during surgeries and made sure that the recording kept going even when I needed a break.

My thanks to the researchers beyond Oxford who have assisted me: to Jaime McCutcheon for helping us optimise the flow cell and advising me on chemometrics; to Richard Keithley for generously sharing his software and taking time to explain all the details of chemometrics analysis to me; and to Bryan Singer and Alex Hoffman for advice on analysing signal kinetics.

A huge thank you to David Bannerman for shouldering the responsibilities of the PPL and for teaching me so much about animal behaviour. Many thanks to Katie Hewitt and Amy Taylor for all the behind the scenes work that keeps this show on the road. Thanks also to all the BMS staff who support our experiments and especially to Kathy Murphy for helping me optimise anaesthesia protocols.

Thank you to Katie Jennings and Paul Harrison, who have accompanied me through all the milestones of my DPhil and provided valuable perspective at each stage of the project. Thank you to Vicky Bajo for her mentorship. Thanks to Phil Burnet for being a great DGS and to Wing Lau for checking in with me every term without fail. And many thanks to Debbie Clarke and Andy King for looking after me from the first day of the MSc onwards, and for all of their assistance and advice along the way.

My labmates in the Walton, Tunbridge, and Bannerman labs have made my DPhil experience an overwhelmingly positive one. Thank you all for your camaraderie and for the many fun times and good memories. Special thanks to Laura for being an awesome conference buddy and for many great conversations, to Anushka for her encouragement and kinship, and to Anna and Steve for welcoming me into their home during the final months of thesis writing.

Thank you to all the friends who have made my time in Oxford so rich – who have sung, danced, and shared good food and good conversation with me. A shout out to the MSc Neuroscience class of 2011-2012, whose curiosity and passion were a great kick-off to my Oxford neuroscience career. Thanks especially to Sana, Friederike, Jovi, Matt, and Luiz, whose friendship has carried me through all the ups and downs of grad school – thank you for your comfort and support during the tough moments and for celebrating all the joyful times with me.

Many thanks to my professors in Claremont, whose teaching nurtured the skills that have allowed me to thrive in grad school, and particularly to Mel Coleman, who continues to be a wonderful mentor. And thank you to the friends and family who have kept in touch no matter which parts of the world we found ourselves in and whose calls, letters, and visits have made me feel that I was never far from home during these years living abroad.

Finally, and most importantly, thank you to my parents and my sister. They are my best teachers and have guided me through every step of my scientific career, from my first visit to a lab through to the submission of my DPhil dissertation. And their commitment and support have allowed me to pursue my studies in Oxford even though it meant living many miles apart. Mommy, Papa, Mirabelle: thank you so very much for all of the Skypes and visits and Snoopy du petit matin, for processing everything with me and for all of your love. This thesis is for you.

Abstract

Fine temporal regulation of dopamine transmission is critical to its effects on behaviour. Dopamine can be cleared from the synapse either by recycling via the dopamine transporter (DAT) or by enzymatic degradation involving catechol-O-methyltransferase (COMT). DAT recycling predominates in striatum and contributes to dopaminergic regulation of reward-guided behaviour, while COMT degradation predominates in cortex and modulates executive functions. However, human functional imaging studies demonstrate interactive effects of DAT and COMT genotype, suggesting that the traditional division between DAT and COMT is not so clear-cut. Given the interdependence of mesolimbic and mesocortical circuitry and the presence of COMT in the striatum, it is possible that DAT and COMT interact to a greater extent than previously thought.

We investigated the contributions of DAT and COMT to regulation of dopamine transmission and reward-guided behaviour by combining *in vivo* electrochemical recording, pharmacology, and behavioural testing in mice. Using fast scan cyclic voltammetry to record evoked dopamine release in anaesthetised animals, we found that systemic DAT blockade increased the size of dopamine transients in the nucleus accumbens (NAc) but not in the medial frontal cortex (MFC), demonstrating that DAT regulates phasic striatal dopamine release and confirming that DAT makes little contribution to regulation of cortical dopamine transmission. Unexpectedly, COMT inhibition did not affect evoked dopamine transients in either the NAc or the MFC. In agreement with these findings, systemic administration of a DAT blocker, but not of a COMT inhibitor, increased

motivation to work for reward in a progressive ratio paradigm. COMT inhibition also had little effect on reinforcement learning (RL) strategies during reward-guided decision making. Intriguingly, however, we found that DAT blockade both decreased the influence of model-free RL and increased the influence of model-based RL on behaviour.

Our study confirms that DAT regulates dopamine transmission in striatum but not in cortex and indicates that sub-second changes in dopamine transmission in both regions are largely insensitive to COMT. However, our behavioural data reveal the importance of striatal dopamine in multiple components of reward-guided behaviour, including both motivational aspects traditionally associated with striatum as well as cognitive aspects heretofore mainly associated with cortical function. Together, these findings emphasise that reward processing occurs across corticostriatal circuits and contribute to our understanding of how striatal dopamine transmission regulates reward-guided behaviours.

Table of contents

Chapter 1: General Introduction……1

1.1 Historical origins of the study of dopamine……1

1.2 Dopaminergic circuitry and transmission……2

1.3 Dopamine clearance mechanisms: recycling versus degradation……8

1.4 The diverse roles of dopamine across brain regions……14

1.5 Potential interaction between DAT and COMT……29

1.6 Aims and hypotheses of this study……36

Chapter 2: Evaluation of drug side effects and optimal dosages……39

2.1 Introduction……39

2.2 Methods……40

2.3 Results……45

2.4 Discussion……49

Chapter 3: Methodological considerations of voltammetry data collection and analysis……51

3.1 Fast scan cyclic voltammetry……51

3.2 Chemometric analysis of voltammetry data……56

3.3 Approaches to chemometric analysis of voltammetry data……57

3.4 Approach used in this study……63

Chapter 4: Effects of DAT blockade and COMT inhibition on dopaminergic transmission in striatum……70

4.1 Introduction……70

4.2 Methods……74

4.3 Results……86

4.4 Discussion……96

Chapter 5: Effects of DAT blockade and COMT inhibition on dopaminergic transmission in cortex……107

5.1 Introduction……107

5.2 Methods……110

5.3 Results……118
5.4 Discussion……131
Chapter 6: Effects of DAT blockade and COMT inhibition on the progressive ratio task……142
6.1 Introduction……142
6.2 Methods……145
6.3 Results……153
6.4 Discussion……160
Chapter 7: Effects of DAT blockade and COMT inhibition on the 2-step decision making task……168
7.1 Introduction……168
7.2 Methods……171
7.3 Results……183
7.4 Discussion……191
Chapter 8: General Discussion……199
8.1 Role of DAT recycling……200
8.2 Role of COMT degradation……205
8.3 Conclusions……209
Abbreviations……210
References……213

Chapter 1:
General Introduction

1.1 Historical origins of the study of dopamine

Although today dopamine is one of the most well-known and widely studied neurochemicals (Marsden, 2006), its importance began to be appreciated only in the mid-20th century. The first half of the century saw experiments characterising the metabolism of dopamine and other amines, the synthesis of dopamine, and demonstrations that dopamine was biologically active (Hornykiewicz, 2002; Marsden, 2006). However, dopamine was for a long time simply considered an intermediate in the synthesis of noradrenaline and adrenaline (Blaschko, 1957; Hornykiewicz, 1958). Hermann Blaschko was one of the first to suggest, in a 1957 review on biogenic amines, that dopamine might in fact have a physiological function of its own (Blaschko, 1957; Hornykiewicz, 2002). A year later, Oleh Hornykiewicz published his findings that administration of both dopamine and L-3,4-dihydroxyphenylalanine (L-DOPA, the precursor from which dopamine is synthesised) depressed arterial blood pressure in guinea pigs (Hornykiewicz, 1958). In his paper, Hornykiewicz made the crucial argument that this effect could not be caused by the oxidation product of dopamine, as had previously been thought (Hornykiewicz, 1958), because administration of an inhibitor of the oxidising enzyme enhanced the effect of dopamine on blood pressure – thus providing evidence of dopamine's physiological importance.

Around the same time, dopamine was localised (albeit as an unnamed compound 'X') in the brains of multiple species, including rats and humans (Montagu, 1957).

Experiments by a team at the University of Lund in Sweden suggested the importance of dopamine to brain function by showing that L-DOPA could reverse the tranquilising effects of reserpine, which was known to deplete catecholamine stores (Carlsson et al., 1957). Carlsson and colleagues went on to develop a fluorimetric method for identifying dopamine in tissue samples and to demonstrate the presence of dopamine in the cat striatum (Carlsson, 1959; Carlsson and Waldeck, 1958). The concentration of dopamine in striatum was confirmed by several other studies, first in rats and other mammals and then in humans (Bertler and Rosengren, 1959a, 1959b; Sano et al., 1959). These studies additionally demonstrated that the localisation of dopamine was different from that of noradrenaline, further supporting the argument that dopamine played its own role in brain function, separate from that of the other catecholamines.

With these early studies, the stage was thus set for the many roles dopamine plays in the brain to be discovered and described. As discussed below, dopamine is now recognized as a critical neurochemical involved in many aspects of brain function.

1.2 Dopaminergic circuitry and transmission

1.2.1 Circuitry

Anatomical studies performed by Nils-Erik Andén and colleagues in the 1960s were the first to localise dopamine neurons to the midbrain (Andén et al., 1964, 1966). We now know that dopaminergic cell bodies are located primarily within the substantia nigra (SN) and ventral tegmental area (VTA) (Tritsch and Sabatini, 2012). For the purposes of this study, I will focus on the mesocorticolimbic circuitry in which VTA dopamine neurons are embedded.

As shown in Figure 1, dopamine neurons project from the VTA to several forebrain regions. The most prominent targets are the nucleus accumbens (NAc) in the striatum and the medial frontal cortex (MFC, consisting of prelimbic and infralimbic cortex – regions that many rodent studies refer to collectively as the medial prefrontal cortex, or PFC), although other regions such as the hippocampus and amygdala also receive dopaminergic input from the VTA (Beier et al., 2015; Russo and Nestler, 2013; Swanson, 1982). The NAc- and MFC-projecting pathways emerging from the VTA are referred to as the mesolimbic and mesocortical dopamine systems, respectively; here, I will refer to them collectively as the mesocorticolimbic system. The VTA receives input in return from the striatum, cortex, and other regions such as the lateral hypothalamus, lateral habenula, dorsal raphe, and tegmental nuclei, among others (Faget et al., 2016; Menegas et al., 2015; Sesack and Grace, 2009; Watabe-Uchida et al., 2012). In addition, the target regions of VTA dopamine neurons are interconnected, particularly via projections from the MFC, hippocampus, and amygdala to the NAc (Russo and Nestler, 2013; Sesack and Grace, 2009).

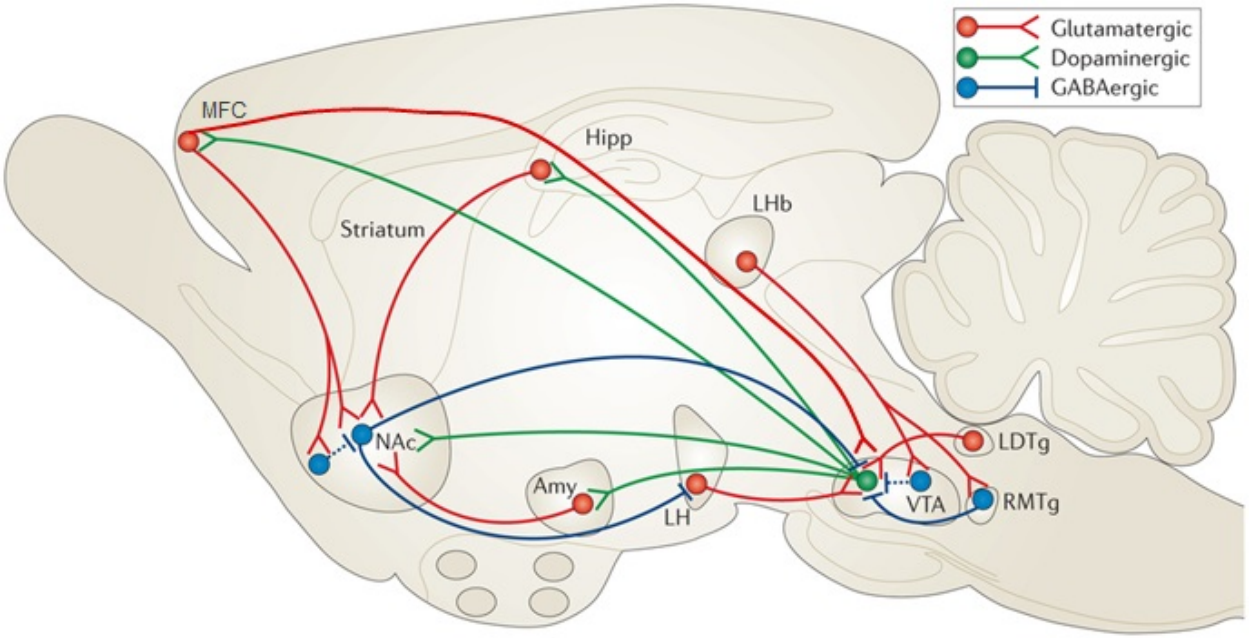


Figure 1: The circuitry of the rodent mesocorticolimbic dopamine system (adapted from (Russo and Nestler, 2013)). Although this illustration does not capture all of the connections involved, it portrays those most relevant to this study, particularly the dopaminergic projections from the VTA to both the NAc and MFC, the projections from each of these regions back to the VTA, and the projection from the MFC to the NAc. Note that incoming afferents may synapse on either the projection neurons or the interneurons in a particular area, as is illustrated in the VTA and NAc. Amy = amygdala; Hipp = hippocampus; LDTg = lateral dorsal tegmentum; LH = lateral hypothalamus; LHb = lateral habenula; MFC = medial frontal cortex; NAc = nucleus accumbens; RMTg = rostromedial tegmentum; VTA = ventral tegmental area.

The past few decades have seen great advances in our understanding of the complexities of the mesocorticolimbic system, spurred on both by careful tracing and electron microscopy studies as well as by the recent advent of tools such as transgenic rodents and optogenetic constructs. These tools have helped researchers uncover the neuronal diversity within each hub of the system and begin to untangle local microcircuitry. The VTA is now known to contain not just dopaminergic but also glutamatergic and GABAergic neurons, the latter of which both act as interneurons and project to other regions (Swanson, 1982). To further complicate matters, researchers have discovered that some dopamine neurons can co-release other transmitters, including both

glutamate and GABA (Stuber et al., 2010; Tecuapetla et al., 2010; Tritsch et al., 2016). When considering how incoming afferents might affect dopamine neuron activity and transmission, therefore, it is important to take into account both where these afferents come from and which VTA cell types they synapse onto. Indeed, the different inputs to the VTA have been shown to target not only different cell types but also subpopulations within each cell type that project to different target regions (Carr and Sesack, 2000; Faget et al., 2016; Lammel et al., 2012; Menegas et al., 2015; Sesack et al., 2003; Watabe-Uchida et al., 2012). The subpopulations of VTA dopamine neurons projecting to distinct target regions appear to be largely separate (i.e. few neurons send collaterals to multiple target regions) (Swanson, 1982). They are located in somewhat distinct but overlapping regions of the VTA and exhibit different physiological properties based on their projection targets (Lammel et al., 2008, 2011, Margolis et al., 2006, 2008).

For this study's purposes, the relationships between the VTA, NAc, and MFC are the most relevant. Briefly, dopamine neurons projecting to the NAc synapse on GABAergic medium spiny neurons (MSNs – the primary output neurons of the striatum), which also receive glutamatergic input from the MFC (as well as other areas), allowing for a convergence of glutamatergic and dopaminergic modulation of NAc output (Kelley, 2004; Sesack and Grace, 2009). (Note that regulation of dopamine release within the NAc itself is complex and mediated by numerous factors. Both cholinergic and GABAergic striatal interneurons are known to influence release at NAc dopamine terminals, and glutamatergic input to the NAc can indirectly affect dopamine release via MSNs. In addition, opioids and various neuropeptides appear to contribute to the regulation of dopamine release within the NAc, and dopamine itself can influence its own release via autoreceptors. See (Sulzer et

al., 2016) for a detailed review of this topic.) The input to NAc-projecting dopamine neurons includes glutamatergic, GABAergic, and cholinergic afferents from many subcortical structures (Beier et al., 2015; Menegas et al., 2015). However, NAc-projecting dopamine neurons do not appear to receive much cortical input (Sesack and Grace, 2009; Sesack et al., 2003) – although recent work indicates that some NAc-projecting dopamine cells, particularly those that project to the lateral shell, do receive direct input from cortex (Beier et al., 2015).

MFC-projecting dopamine neurons receive cortical as well as subcortical input, providing a means for the MFC to regulate the dopaminergic input it receives (Beier et al., 2015; Carr and Sesack, 2000; Menegas et al., 2015). The connections dopaminergic terminals make in the cortex are less well understood than those they make in the NAc: although dopaminergic transmission appears to act in concert with glutamate, as well as with inputs from GABAergic interneurons, to modulate cortical output, it is unclear which glutamatergic afferents in the cortex interface with dopamine (Sesack et al., 2003).

1.2.2. Transmission

Dopamine is typically considered a neuromodulator (Tritsch and Sabatini, 2012). As noted in the discussion of dopaminergic circuitry above, dopamine is positioned to mediate the influence of glutamatergic input onto the neurons it targets. Indeed, accumulating evidence indicates that dopamine can influence synaptic plasticity, particularly in the striatum (Kelley, 2004; Reynolds et al., 2001; Shen et al., 2008; Shiflett and Balleine, 2011; Surmeier et al., 2007). Dopamine exerts much of its influence via volume transmission: rather than acting only within the synapse in which it is released,

dopamine can diffuse through the extracellular fluid to act over a larger area (Cragg et al., 2001; Rice and Cragg, 2008; Sesack et al., 2003; Zoli et al., 1998).

One implication of this mode of transmission is that a steady baseline level of dopamine can be maintained and could carry important information within the circuitry (Descarries et al., 1996). This is now known to constitute one of the two main modes of transmission by which dopamine operates. This so-called tonic mode of transmission is composed of small, regular release events that maintain basal dopamine levels and act via high-affinity receptors to regulate network responsivity (Moore et al., 1999). Phasic transmission, by contrast, consists of large but short-lived bursts of release that act via lower affinity receptors (because high affinity receptor are already occupied by tonically released dopamine) and that are often linked to behaviourally relevant events (Moore et al., 1999). These two types of release appear to be driven by, respectively, the tonic and phasic firing patterns that have been recorded from dopaminergic neurons in the VTA (Goto et al., 2007; Sulzer et al., 2016) – although some evidence indicates that the firing of midbrain dopamine neurons does not fall into such a neat dichotomy (Brown et al., 2009). However, the extent to which dopamine release in target regions depends on neuronal activity at the level of the cell body is an area of continued investigation: evidence indicates that firing and release can be dissociated, as dopamine release can be regulated at the level of the terminals (Marinelli and McCutcheon, 2014; Moore et al., 1999; Sulzer et al., 2016).

The tonic-phasic dichotomy has been best characterised in the striatum, but whether it applies to cortical dopamine as well remains unknown. One key regional difference is that dopamine levels in cortex are substantially lower than they are in striatum (Moghaddam et al., 1990). In addition, MFC-projecting dopamine neurons lack

somatodendritic D2 autoreceptors, which regulate phasic firing and release in the striatal circuitry (Gainetdinov and Caron, 2003; Grace, 1991; Lammel et al., 2008). This lack of autoinhibition may contribute to the higher firing rates seen in MFC-projecting dopamine cells compared to dopamine neurons that project to other targets (Marinelli and McCutcheon, 2014). Furthermore, dopamine transmission in cortex is more dependent on dopamine synthesis capability than it is in striatum, as the pool of releasable vesicles in dopamine terminals is smaller in the cortex than in the striatum (Jones et al., 1998a). Finally, dopamine receptor expression in cortical output neurons is not as clearly patterned as it is in striatal output neurons. Whereas striatal MSNs can be divided fairly neatly into two populations expressing D1 and D2 dopamine receptions, respectively, that project to different targets, cortical pyramidal cells do not exhibit such a clear division of D1 and D2 receptor expression (Tritsch and Sabatini, 2012). These two types of dopamine receptors appear to have opposing effects on cortical function (Durstewitz and Seamans, 2008), but the mechanisms underlying these effects require further clarification.

In summary, it is becoming ever clearer that the circuitry in which dopamine is embedded is multifaceted and interlinked; that dopamine neuron activity, dopaminergic release, and the downstream effects of this release vary widely depending on the context in which they occur; and that these complexities exhibit regional differences, particularly between the striatum and cortex.

1.3 Dopamine clearance mechanisms: recycling versus degradation

One of the key factors that contributes to and regulates the complexities of dopaminergic transmission is dopamine clearance. To ensure precise signaling, dopamine,

like all neurotransmitters, must be cleared away after its release. Dopamine can either be recycled, most prominently via the dopamine transporter (DAT), or degraded by enzymes, particularly by catechol-*O*-methyltransferase (COMT). (Other enzymes such as monoamine oxidase also play an important role in dopamine degradation (Tunbridge et al., 2006); however, they are beyond the scope of this study and so will not be discussed in detail.) These mechanisms are illustrated in Figure 2.

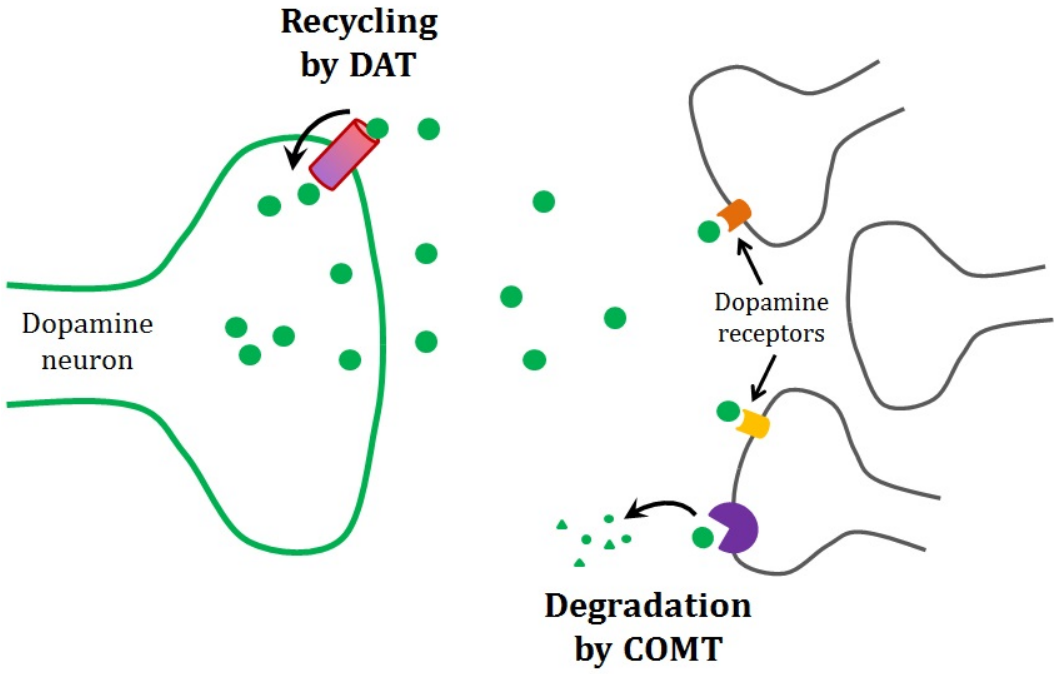


Figure 2: The two main mechanisms by which dopamine can be cleared following release: recycling into the presynaptic neuron via the dopamine transporter (DAT) or degradation by the enzyme catechol-*O*-methyltransferase (COMT). Note that there is uncertainty about whether the COMT protein is located pre- or postsynaptically and about whether it is situated in the cellular membrane or in an intracellular membrane (see section 1.3.2 for further discussion). I have depicted it in one of the postsynaptic neurons for illustrative purposes.

1.3.1 DAT recycling

The DAT, along with other monoamine transporters, is part of a family of transporters that use the sodium gradient across the plasma membrane to drive transport

of their respective substrates into the cell (Amara and Sonders, 1998). DAT expression is highly specific to dopamine neurons (Sulzer et al., 2016) and appears to be localised to the perisynaptic region (just outside the synapse) in striatum, indicating that dopamine diffuses out of the synaptic cleft before being transported back into the presynaptic terminal (Torres et al., 2003). As well as mediating reuptake at the axon terminals in dopamine neurons' target regions, DAT is also expressed in dopaminergic cell bodies and participates in the reuptake of somatodendritic dopamine release within the VTA (Rice and Patel, 2015). It should be noted that the DAT is able to transport noradrenaline as well as dopamine (Torres et al., 2003), although the extent to which this occurs *in vivo* is unclear (Fentress et al., 2013).

Research interest in DAT is underpinned not only by its importance in the regulation of dopamine transmission under normal conditions but also by its role in the use and abuse of addictive drugs, many of which exert their primary effects by blocking the DAT (Amara and Sonders, 1998). Cocaine, amphetamines, and other psychostimulants are among the compounds that can disrupt dopamine transmission by binding to DAT (Torres et al., 2003).

1.3.2 COMT degradation

Since its discovery in 1957 (Axelrod, 1957), the COMT enzyme has been implicated in several steps along dopamine's metabolic pathway, as illustrated in Figure 3. Despite this prominent role in dopamine's degradation, however, COMT's subcellular localization is controversial. COMT has both membrane-bound (MB-COMT) and soluble (S-COMT) isoforms. The MB-COMT protein predominates in the brain, and COMT mRNA has been localised primarily to neurons rather than glia (Matsumoto et al., 2003; Rivett et al., 1983).

However, whether COMT is pre- or postsynaptic, and whether it sits within the cellular member or in an intracellular membrane, remains controversial (Tunbridge et al., 2006).

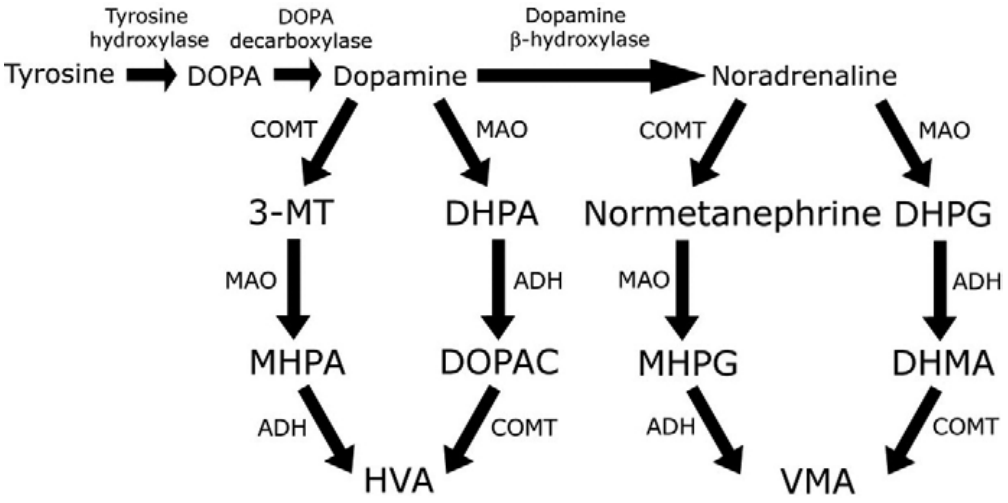


Figure 3: Metabolic pathways in which COMT is involved (figure from (Tunbridge et al., 2006)). COMT catalyses several steps in the breakdown of both dopamine and noradrenaline. ADH = aldehyde dehydrogenase; COMT = catechol-*O*-methyltransferase; DHMA = 3,4-dihydroxymandelic acid; DHPA = 3,4-dihydroxyphenylacetaldehyde; DHPG = dihydroxyphenylglycol; DOPA = dihydroxyphenylalanine; DOPAC = 3,4-dihydroxyphenylacetic acid; HVA = homovanillic acid; MAO = monoamine oxidase; MHPA = 3-methoxy-4-hydroxyphenylacetaldehyde; 3-MT = 3-methoxytyramine; VMA = vanillylmandelic acid.

The widespread research interest in COMT's function arises from a polymorphism in the human population, which possesses two allelic variants of the COMT gene. The polymorphism arises from a single G to A nucleotide substitution that results in either a valine (Val) or a methionine (Met), respectively, at position 158 in MB-COMT (Lachman et al., 1996). The identity of this single amino acid results in a substantial difference in the enzyme's activity: Met-COMT is less active than Val-COMT due to its lower thermostability (Chen et al., 2004; Gothelf et al., 2014; Lotta et al., 1995). Differential COMT activity has been linked to differences in cortical dopamine levels as well as to behavioural differences, as will be discussed in more detail in sections 1.3.3 and 1.4.2 below.

1.3.3 Regional segregation of dopamine clearance mechanisms and effects on transmission

The prominence of dopamine recycling by DAT and enzymatic degradation by COMT varies across brain regions. In the NAc and other striatal regions, DAT expression is high (Nirenberg et al., 1997), and reuptake provides the primary mechanism for clearing dopamine from the synaptic cleft (Sulzer et al., 2016). This has been dramatically demonstrated using DAT knockout mice, which have greatly slowed dopamine clearance, as well as altered dopamine tissue content and extracellular dopamine levels, in the striatum (Giros et al., 1996; Torres et al., 2003). Numerous pharmacological experiments using DAT blockers have confirmed DAT's importance in regulating striatal dopamine levels (Budygin et al., 1999; Huotari et al., 1999; Nakachi et al., 1995; Nomikos et al., 1990; Owesson-White et al., 2012; Raevskii et al., 2002). Recycling of dopamine in the striatum is rapid – the half-life of evoked dopamine transients in this region is on the order of 50 milliseconds (Garris and Wightman, 1994; Sulzer et al., 2016; Wightman and Zimmerman, 1990) – and DAT thus provides an efficient means of clearing the high levels of dopamine released during striatal transmission. Indeed, DAT is key to maintaining stable tonic dopamine levels in the striatum as well as to clearing the surges in dopamine produced by phasic transmission (Moore et al., 1999; Sulzer et al., 2016).

Enzymatic degradation is traditionally thought to play at most a small role in regulating striatal dopamine transmission. The enzyme monoamine oxidase can contribute to clearance of dopamine in the striatum, but its influence is limited and only becomes apparent when DAT's action is impaired (Sulzer et al., 2016). COMT, meanwhile, is also expressed at low levels in striatum (Hong et al., 1998; Matsumoto et al., 2003) and appears to play a minimal role in regulating striatal dopamine locally (Cass et al., 1993; Tunbridge

et al., 2006) – although, as will be discussed further below, COMT may affect dopaminergic transmission in the striatum by other means. In Chapter 4, I will report our findings on the relative contributions of DAT and COMT to regulation of phasic dopamine transmission in the NAc.

The situation is reversed in the cortex. COMT is far more prevalent, with expression levels in MFC substantially higher than those seen in striatum (Matsumoto et al., 2003). As one would expect from these higher expression levels, COMT degradation plays a major role in regulating cortical dopamine transmission. Manipulations of COMT activity levels in rodents, whether achieved through genetic knockout or pharmacological inhibition of the enzyme, alter dopamine levels in MFC (but not in striatum) (Käenmäki et al., 2010; Lapish et al., 2009; Tammimaki et al., 2016; Tunbridge et al., 2004, 2006), and the $Val^{158}Met$ polymorphism has also been linked to differential dopamine transmission in cortex in humans (Slifstein et al., 2008). The slower rates of clearance seen in cortex compared to striatum – studies have estimated the half-life of cortical dopamine transients to be on the order of several seconds (Garris and Wightman, 1994; Mundorf et al., 2001; Sulzer et al., 2016) – support a prominent role for enzymatic degradation in this region.

DAT is sparsely expressed in the rodent MFC (Sesack et al., 1998), indicating that it plays a relatively minor role in clearance of cortical dopamine. Studies have indeed found that pharmacological blockers of DAT are far less effective at altering dopamine levels in MFC than they are in striatum (Cass and Gerhardt, 1995; Izenwasser et al., 1990; Mundorf et al., 2001; Sulzer et al., 2016). An important implication of the low DAT expression levels in cortex, particularly within synapses, is that cortical dopamine released at a particular site can likely diffuse over larger distances – and thus act at longer timescales over larger

areas of tissue – than it would be able to do in striatum (Jones et al., 1998a; Mundorf et al., 2001; Sesack et al., 1998). However, an additional factor must be taken into account when considering cortical dopamine clearance: the noradrenaline transporter (NET) is widely expressed in cortex and can transport dopamine as well as noradrenaline, and some studies indicate that NET is more important for cortical dopamine uptake than DAT (Morón et al., 2002; Mundorf et al., 2001; Raiteri et al., 1977; Williams and Steketee, 2004). I will return to this topic in Chapter 5, which describes our findings on the relative influences of COMT and DAT on phasic dopamine transients in the MFC.

Overall, evidence indicates that DAT recycling is the predominant mechanism for clearing dopamine in the striatum, while enzymatic degradation by COMT plays a prominent role in clearing cortical dopamine. As will be discussed below, however, there is evidence that the actions of these two clearance mechanisms are not as segregated as has traditionally been thought.

1.4 The diverse roles of dopamine across brain regions

Dopamine is notable for both the large number and wide range of functions in which it is implicated. As Charles Marsden noted in a 2006 review, dopamine cells make up less than 1% of the brain's neurons yet make a great impact on brain function and behaviour (Marsden, 2006). The functions in which dopamine is involved include movement, reward, cognition, motivation, and emotion (Berridge, 2006; Hornykiewicz, 2002; Robbins, 2003; Ungless et al., 2010). Here, I will focus on the roles that dopamine plays in reward and cognition and on how DAT recycling and COMT degradation influence these functions. Although reward is most prominently associated with striatal dopamine and cognition with

cortical dopamine, it should be noted that both the striatum and cortex contribute to reward-guided behaviour and to cognitive function.

1.4.1 Reward

The mesolimbic dopamine pathway, comprised of neurons running from the VTA to the NAc, plays a leading role in mediating how organisms learn about and pursue rewards. One of the first clues to dopamine's role in reward was James Olds and Peter Milner's demonstration that rats would work for intracranial self-stimulation (ICSS) of particular brain regions – so called 'pleasure centres' (Olds and Milner, 1954; Wise, 2008). Researchers soon determined that dopamine was a key neurochemical underlying ICSS reward (Wise, 2008), and this finding, combined with studies of neuroleptic drugs, led to the classic anhedonia hypothesis of dopamine (Wise, 1982; Wise et al., 1978). Interestingly, the original articulation of the hypothesis by Wise linked dopamine to motivation, reinforcement, and goal-directed behaviour – all concepts that would later emerge as primary players in theories about dopamine and reward (Wise, 2008). However, it was the piece of the hypothesis describing the link between dopaminergic drugs and subjective pleasure that initially gained traction, and dopamine was thus for a long time thought to underlie the pleasure of obtaining a reward. Although the media and lay public still often associate dopamine with pleasure, the research community now generally agrees that dopamine's primary reward-related function is not to mediate the hedonic experience produced by rewards (Berridge and Robinson, 1998; Salamone et al., 2007).

What role dopamine plays in reward processing and reward-guided behaviour, however, remains a lively topic of discussion. For the past 20 years, the debate has been

dominated by two distinct (although not necessarily mutually exclusive) ideas: 1) that dopamine drives behavioural activation, motivation, and the exertion of effort to obtain reward, and 2) that dopamine mediates learning about reward-predictive cues and about actions that will lead to reward (Berridge, 2006; Salamone et al., 2007). (Of course, there are many more than two theories about dopamine's role in reward. I summarize some of the most prominent here, but more fine-grained assessments can be found in the numerous reviews on this topic (Berridge, 2006; Salamone et al., 2007; Wise, 2008).)

The first view of dopamine's role in reward is espoused by several prominent groups that have argued for a key role for dopamine in activation and energisation of reward-guided behaviours (Berridge, 2006; Robbins and Everitt, 2006; Salamone et al., 2007). This argument is based on evidence that dopamine motivates animals to pursue rewards and allows them to overcome effort costs to achieve a goal. For instance, John Salamone and colleagues have demonstrated that the degree to which blockade of dopamine transmission interferes with instrumental responding in rodents increases with the effort required to obtain reward in a task. Specifically, dopamine antagonists affect performance when significant effort is required to obtain reward (such as numerous lever presses per reward) but not when rewards are freely available or only require a small exertion of effort (such as a single lever press) (Salamone et al., 2007). Dopamine antagonists do not cause such effects by changing primary motivation or motor function; rather, they appear to alter cost/benefit calculations about whether and how to allocate effort in the pursuit of reward (Phillips et al., 2007). For example, dopamine antagonists cause animals to choose a freely available but less palatable food over a highly palatable food that must be obtained through work (Salamone et al., 2007). Similarly, dopamine

antagonists make animals more likely to choose a smaller reward that is immediately available over a larger reward that is only accessible after a delay (Denk et al., 2005).

This sort of cost/benefit decision making is thought to occur across corticostriatal circuits, involving both the NAc and regions of the frontal cortex with which it is connected (Salamone et al., 2007). Indeed, cortical lesions in rats can alter willingness to exert effort in the pursuit of a reward (Walton et al., 2002). The anterior cingulate cortex (ACC) appears to be particularly important in this regard (Walton et al., 2003). Interestingly, however, ACC lesions only affect animals' willingness to pursue rewards under certain circumstances. Lesions reduce selection of a high-effort/high-reward option only when there is an alternative low-effort/low-reward option to choose (Schweimer et al., 2005), and ACC lesions do not affect animals' ability to tolerate a delay before the delivery of reward (in contrast to the effect of dopamine antagonists described above) (Walton et al., 2007). Although there is some evidence that cortical dopamine contributes to the ACC's mediation of cost/benefit decision making in the pursuit of reward, this has not been definitively established (Schweimer et al., 2005; Walton et al., 2005).

The concept of incentive salience, developed by Kent Berridge and Terry Robinson, is an important component of the view that dopamine's key role in reward is in activating and energising behaviour. Incentive salience is the attribution of motivational value to a stimulus or action that is associated with reward, which can then drive behaviour to pursue and obtain the reward (Berridge, 2006). This involves a psychological phenomenon of 'wanting' a reward that is distinct from the hedonic 'liking' of it and from learned associations about what cues or actions lead to it. Although not all in the field accept the details of the incentive salience concept, the notion that dopamine is involved in the

motivational drive induced by reward is widespread. Trevor Robbins and Barry Everitt, for example, use a similar concept in much of their work (Robbins and Everitt, 2006). The body of evidence supporting these theories is largely comprised of studies showing that dopamine can modulate the influence that reward-associated cues have over animals' behaviour (Berridge, 2006; Robbins and Everitt, 2006).

There is a great deal of data implicating DAT in the motivation to pursue and work for rewards. DAT knockdown mice, which have elevated striatal dopamine levels, are willing to perform more work for a given reward and will choose a high-effort/high-palatability option over a low-effort/low-palatability option more often in comparison to wildtype mice (Cagniard et al., 2006a, 2006b; Zhuang et al., 2001). Pharmacological blockade of DAT produces similar results (Young and Geyer, 2010). In addition, Berridge and colleagues have linked DAT function to incentive salience using DAT knockdown mice, which they found were more motivated in their pursuit of a reward in a runway task – the knockdown mice proceeded down the runway towards the reward more directly and efficiently and were less susceptible to distraction than wildtype animals – but failed to display different orofacial reactions while consuming the reward (Peciña et al., 2003). This finding is interpreted as enhanced 'wanting' but unchanged 'liking' in the DAT knockdown animals. Together, these data indicate that DAT-mediated regulation of striatal dopamine transmission affects animals' motivational drive and willingness to exert effort in the pursuit of reward.

Previous rodent studies have not investigated whether COMT can also influence motivational drive and cost/benefit decisions about whether to expend effort in reward-

guided tasks. I will return to this topic in Chapter 6, where I will report our findings on the roles of both DAT and COMT in mediating willingness to work for reward.

The second leading view of dopamine's role in reward – that dopamine is involved in reward learning – revolves around the seminal discovery by Peter Dayan and Read Montague, based on Wolfram Schultz's data, that dopamine neuron firing correlates with a reward prediction error (RPE) term in reinforcement learning models (Montague et al., 1996; Schultz et al., 1997). Schultz and colleagues observed that firing of putative dopamine neurons shifted from receipt of a reward to the presentation of a reward-predicting cue as animals learned the cue-reward association and found that firing rate decreased when a reward was unexpectedly omitted (Schultz et al., 1997). The authors interpreted this finding in the context of temporal difference reinforcement learning models and posited that dopamine might mediate learning about reward availability and contingencies (Schultz et al., 1997). RPE-like signals have now been observed not only in neuronal firing but also in measurements of release at dopamine terminals in the NAc core (Clark et al., 2010; Day et al., 2007; Hart et al., 2014) and even in fMRI BOLD signals in the striatum (Delgado et al., 2000; Gottfried et al., 2002; Knutson et al., 2001; O'Doherty et al., 2004, 2003; Schönberg et al., 2007).

Although striatal dopamine is most clearly implicated in reward learning, cortical dopamine appears to play a role as well. The frontal cortex is certainly implicated in reward processing: recordings in monkeys demonstrate that neurons in the prefrontal, cingulate, and orbitofrontal cortices can code RPEs and other reward-related signals (Asaad and Eskandar, 2011; Kennerley et al., 2011; Tremblay and Schultz, 2000). In addition, microdialysis measurements in rats have shown that MFC dopamine levels are

elevated while animals consume appetitive foods (Ahn and Phillips, 1999; Bassareo et al., 2002). Some studies indicate that cortical dopamine does not just reflect receipt of a reward but also influences reward learning. For example, infusion of dopamine antagonists into the prefrontal cortex impairs learning of cue-reward associations in monkeys (Puig and Miller, 2012; Puig et al., 2014).

Indications that DAT can influence reward learning come from human imaging studies of the effects of a polymorphism in the DAT gene, which involves a variable number of tandem repeats (VNTR) that most often results in either 9 or 10 repeats (Vandenbergh et al., 1992). Although the DAT VNTR is located in an untranslated portion of the gene, it is thought to influence transcription, and several studies indicate that it can affect DAT expression levels both *in vitro* and *in vivo* (Fuke et al., 2001; Heinz et al., 2000; Jacobsen et al., 2000; Michelhaugh et al., 2001; VanNess et al., 2005) – although findings are mixed (Costa et al., 2011; Martinez et al., 2001; Mill et al., 2005). Several studies have associated DAT VNTR genotype with individual variability in reward-related activity in the ventral striatum, as assessed by the fMRI BOLD signal, including during reward anticipation and reward delivery (Dreher et al., 2009; Forbes et al., 2009; Hahn et al., 2011). However, DAT function has not yet been clearly linked to reward-guided learning. Meanwhile, experiments examining reward-guided task performance in DAT knockdown mice – which display clear differences in willingness to work for reward – have produced mixed results, with some studies finding enhanced learning but others reporting no change (Cagniard et al., 2006a, 2006b; Peciña et al., 2003; Yin et al., 2006a). The extent to which DAT mediates dopamine signaling underlying reward learning therefore remains to be determined.

In sum, dopamine transmission – most prominently in the striatum, but also in the cortex – is central to reward processing and associated behaviours. Reuptake by DAT influences many of the reward-related functions in which striatal dopamine is implicated, particularly motivational drive and willingness to work for reward.

1.4.2 Cognition

The mesocortical dopamine pathway is comprised of neurons projecting from the VTA to the cortex, particularly to the MFC. Cortical dopamine has been linked to various aspects of cognitive function such as working memory, attention, and decision making (as well as to reward processing, as discussed in the previous section). Consistent with a role for cortical dopamine in mediating cognition, variability in COMT activity appears to influence these functions.

The study of cortical dopamine was pioneered by Patricia Goldman-Rakic and colleagues (Robbins, 2005), who found that dopamine depletion in the association cortex impaired performance on a working memory task in monkeys but could be rescued by administration of dopaminergic drugs such as L-DOPA (Brozoski et al., 1979). Goldman-Rakic et al. built on this early finding by locally injecting dopamine antagonists into the cortex, which they found both altered memory-associated neural activity and specifically impaired memory-dependent elements of behavioural tasks (Sawaguchi and Goldman-Rakic, 1991; Wang et al., 2004; Williams and Goldman-Rakic, 1995). Rodent studies have confirmed these findings: MFC dopamine levels, measured by microdialysis, correlate with memory performance and, as in monkeys, lesions of cortical dopamine terminals in rodents disrupt acquisition and performance of tasks testing working memory (Bubser and Schmidt, 1990; Phillips et al., 2004). (Note that the term 'working memory' refers to

different processes in rodents compared to primates: while in rodents it denotes holding of spatial or directional information in short-term memory and may actually reflect short-term habituation, in primates it refers to retention and manipulation of abstract information in attention (Floresco and Jentsch, 2011; Sanderson and Bannerman, 2011).)

COMT appears to play an important role in modulating cortical dopamine levels for optimal working memory function (although the extent of COMT's involvement in cognitive function is controversial (Barnett et al., 2007, 2008)). Individuals homozygous for the Met allele of the Val^{158}Met polymorphism – who have less active COMT and thus, presumably, higher baseline levels of cortical dopamine – exhibit better working memory than individuals homozygous for the high activity Val-COMT allele (Diaz-Asper et al., 2008; Farrell et al., 2012; Goldberg et al., 2003). Pharmacological inhibition of COMT can also improve working memory task performance in humans (Apud et al., 2006). COMT activity has been linked specifically to cortical function during working memory in a study showing that, while subjects performed a working memory task with similar levels of accuracy, Met homozygotes had a smaller BOLD response in the prefrontal and cirgulate cortices than Val homozygotes, indicating that individuals with less active COMT had more efficient cortical function (Egan et al., 2001).

Cortical dopamine is additionally implicated in mediating attentional aspects of cognitive function such as the ability to optimally maintain focus on a task but to then shift attention to another task when required, which is key to being able to adapt to a changing environment. A classic measure of this function is the attentional set shifting test, in which subjects must learn which stimuli are predictive of reward and then adapt their behaviour as the identity and salient feature of the reward-predicting stimulus is changed (Birrell and

Brown, 2000). Lesions of MFC dopamine terminals alter performance on this task in monkeys (Crofts et al., 2001) and rodents (Birrell and Brown, 2000). COMT modulates set shifting as well: pharmacological inhibition of COMT in rats improves performance on certain elements of the set shifting task (Tunbridge et al., 2004), and individuals homozygous for the low-activity Met-COMT allele perform better on a human analogue of the task (Egan et al., 2001). Both cortical dopamine and COMT have also been shown to influence performance on other attentional tasks such as the 5-choice serial reaction time task (Barkus et al., 2016; Granon et al., 2000).

A useful concept for tying together these various findings on dopamine's role in cognitive function is that of a balance between cognitive stability and cognitive flexibility that is influenced by cortical dopamine levels. This has been explored using computational models based on biophysical studies of the effects of dopamine on cortical circuits as well as using behavioural tasks. Higher cortical dopamine levels are generally thought to enhance cognitive stability by stabilizing representations within the cortical circuitry, while lower cortical dopamine levels shift the balance towards increased cognitive flexibility and make attention more susceptible to change, likely via differential effects at different types of dopamine receptors (Durstewitz and Seamans, 2008; Durstewitz et al., 2000; Frank and Fossella, 2011; Robbins, 2005). The COMT Val^{158}Met polymorphism has been linked to this concept, as Val-COMT homozygotes – who should have lower levels of cortical dopamine – were found to be more cognitively flexible than Met-COMT homozygotes (Colzato et al., 2010).

Another important theme that has emerged from studies of dopamine's role in cognition is that the relationship between cortical dopamine levels and performance of

cognitive tasks is not linear; instead, it appears to follow an inverted U shape function (Arnsten, 1998; Goldman-Rakic et al., 2000; Robbins, 2005). The inverted U concept was first articulated in 1908 by Robert Yerkes and John Dodson, who proposed that optimal cognitive performance was supported by intermediate levels of mental arousal or stress and that performance suffered when arousal levels were either too low or too high (Yerkes and Dodson, 1908). Accumulating evidence indicates that there is a similar inverted U relationship between cortical dopamine and cognitive performance (although some argue that an inverted U function is insufficient to capture the complexities of cortical dopamine's effects on cognitive performance (Floresco, 2013)) – perhaps not surprising given that stress can increase cortical dopamine levels (Thierry et al., 1976). For example, although the studies mentioned above demonstrate that depleting cortical dopamine impairs working memory, other studies have found that excessive dopamine transmission in cortex can also have deleterious effects on memory task performance (Murphy et al., 1996; Zahrt et al., 1997).

The concept of the inverted U can be used to explain why the effects of dopaminergic manipulations on cognitive function often depend on individual differences in baseline performance (Haddon and Killcross, 2011; Onur et al., 2011), as these performance differences are theorised to arise from baseline differences in cortical dopamine transmission. Studies of COMT support this idea: numerous investigations have found opposite effects of dopaminergic drugs – including amphetamine and the COMT inhibitor tolcapone – on performance depending on COMT genotype (Apud et al., 2006; Barkus et al., 2016; Farrell et al., 2012; Mattay et al., 2003). Individuals with low COMT activity (such as Met-COMT homozygotes) are proposed to have optimal baseline levels of

cortical dopamine and to, on average, sit at the top of the inverted U, while individuals with higher COMT activity (such as Val-COMT homozygotes) have suboptimal baseline dopamine and sit on the left of the curve, as show in Figure 4. Conditions that increase cortical dopamine, such as stress or the administration of amphetamine or tolcapone, should move all individuals to the right along the curve, which can improve performance in those with low baseline dopamine but impair performance when dopamine levels were already optimal at baseline (Tunbridge et al., 2006).

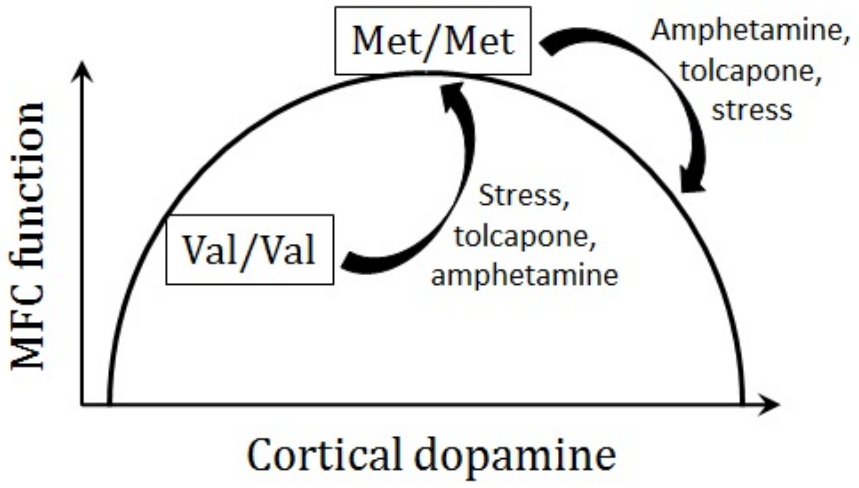


Figure 4: Illustration of the proposed inverted U function relating cortical dopamine signaling to MFC function. Individuals with different COMT alleles are thought to sit at different points along the curve at baseline, with Met-COMT homozygotes near the peak and Val-COMT homozygotes on the left side. Conditions that increase cortical dopamine such as stress, amphetamine, or the COMT inhibitor tolcapone move everyone rightward along the curve. Figure adapted from (Tunbridge et al., 2006).

Although most of the research on dopamine's role in cognition focuses on cortical dopamine, there are indications that striatal dopamine can also influence cognitive processes. A particularly interesting line of evidence comes from a schizophrenia mouse model in which D2 dopamine receptors are overexpressed selectively in striatal MSNs (Simpson and Kellendonk, 2016). D2 receptor overexpressor (D2R-OE) animals exhibit

impairments in several cognitive functions, including spatial working memory, conditional associative learning, behavioural flexibility, and problem solving (Bach et al., 2008; Ben Abdallah et al., 2011; Kellendonk et al., 2006). These changes likely arise from the alterations in cortical dopamine transmission that are observed in D2R-OE mice (Simpson and Kellendonk, 2016), which I will describe in more detail in section 1.5.2 below. In addition, the changes in striatal dopamine transmission caused by D2 receptor overexpression may affect cognitive function via their effects on reward processing and motivation. D2R-OE mice are less motivated to work for reward (similarly to animals treated with dopamine antagonists, as discussed in section 1.4.1 above), likely due to deficits in cost/benefit decision making that arise from impairments in their ability to represent the value of future rewards (Simpson et al., 2011; Ward et al., 2012). Some researchers have proposed that motivation is a key mediator of cognitive function, particularly in the context of schizophrenia (Simpson and Kellendonk, 2016). In support of this view, experiments with the D2R-OE mice indicate that their motivational impairments can negatively impact cognitive function (Ward et al., 2015). Conversely, manipulating motivation in the D2R-OE mice – either by increasing reward magnitude or by administering a drug that increases motivation – improves cognitive function in these animals (Avlar et al., 2015).

These findings emphasize that reward processing and cognitive function are not neatly separable but may interact to influence behaviour. Similarly, as the experiments discussed in this section demonstrate, striatal dopamine not only mediates reward-guided behaviour but can impact cognition as well, and cortical dopamine's roles extend beyond cognitive functions to encompass aspects of reward processing. I will further explore this

intertwining of striatal and cortical dopamine transmission, and of the functions they mediate, in the next section.

1.4.3 Reinforcement learning and decision making

A particularly interesting intersection of reward processing and cognitive function is in reinforcement learning (RL). RL is the process by which subjects learn about the reward contingencies associated with different actions and then incorporate this knowledge into decision making in order to maximise positive outcomes (Daw et al., 2005). Different types of RL are thought to underlie the habitual and goal-directed behavioural modes into which decision making is often classified. Briefly, model-free RL, which is thought to underlie habitual behaviour, learns associations between actions and outcomes by using RPEs to retrospectively update cached estimates of the values of actions, leading to reinforcement of actions that result in positive outcomes (Daw et al., 2005). By contrast, model-based RL, which is thought to underlie goal-directed decision making, relies on state prediction errors (the difference between predicted and experienced states, rather than particular outcomes) to build an internal model of the world that can then be used to prospectively simulate the various potential outcomes of an action (Daw et al., 2005). Model-free and model-based RL are useful under different circumstances – model-free RL when rapid responses are required and when computational capacity is limited, model-based RL when time and cognitive resources are sufficient to allow more accurate responding (O'Doherty et al., 2017). Indeed, decision making may shift from one strategy to the other over time, as the flexibility of model-based RL is particularly useful early in learning, and the more efficient model-free RL may take over once the subject has sufficient experience of a given situation.

Model-free and model-based RL are each mediated by both striatum and cortex, although model-free RL is particularly influenced by striatum, while model-based RL relies on a more diverse network of cortical and subcortical regions (Daw et al., 2011; Gläscher et al., 2010; Knutson et al., 2001; Lee et al., 2014; O'Doherty et al., 2017; Yin et al., 2004, 2005, 2006b). Model-free RL has long been linked with striatal dopamine and the RPE signals it carries (Daw et al., 2011; Deserno et al., 2015). Model-based RL is also subject to mediation by dopamine (Sharp et al., 2016; Wunderlich et al., 2012) but is typically associated with cortical dopamine because model-based RL is influenced by working memory function (Eppinger et al., 2013; Otto et al., 2013a, 2013b; Smittenaar et al., 2013) and, as covered in section 1.4.2 above, working memory relies on cortical dopamine. In support of this view, the COMT Val^{158}Met polymorphism has been associated with differential model-based capacity (Doll et al., 2016). However, a recent PET imaging study has shown that higher presynaptic dopamine levels in the ventral striatum are correlated with enhanced model-based behavioural control (Deserno et al., 2015), suggesting that striatal dopamine may also influence model-based RL.

These findings indicate that studying model-based decision making, as well as the balance between model-free and model-based RL, provides an opportunity to examine how striatal and cortical dopamine transmission interact to mediate behaviour. As such, RL-guided decision making is also an excellent paradigm in which to assess the relative contributions of DAT and COMT to behaviour. I will return to this topic in Chapter 7, which covers our findings on how interference with DAT recycling and COMT degradation affects RL in mice.

1.5 Potential interaction between DAT and COMT

Most of the evidence that I have discussed so far supports the view that DAT and COMT regulate dopamine transmission in striatum and cortex, respectively, thereby mediating the distinct functions in which these regions are involved. However, human imaging studies, as well as some rodent experiments, suggest that this division may not be so clear-cut. Furthermore, the complex and interlinking circuitry of the mesocorticolimbic system provides routes by which DAT and COMT could interactively influence dopamine transmission and the behaviours it mediates.

1.5.1 Evidence of DAT-COMT interaction

Human studies have investigated the relative contributions of DAT and COMT to brain function and behaviour by taking advantage of the functional DAT VNTR and COMT Val^{158}Met polymorphisms (see sections 1.3.2 and 1.4.1 above for details) in order to look for additive or interactive effects of these two polymorphisms. Such effects were observed on fMRI BOLD signal in the cortex – including in the MFC – of healthy volunteers performing working memory and other cognitive tasks (Bertolino et al., 2006, 2008; Caldú et al., 2007; Prata et al., 2009). In addition, several studies have found interactive effects of the DAT and COMT polymorphisms on the BOLD signal in regions such as the midbrain, ventral striatum, and frontal cortex during performance of tasks involving reward processing (Dreher et al., 2009; Yacubian et al., 2007). However, other studies have failed to find evidence of interactive influences on the BOLD signal (Forbes et al., 2009), and genotype effects on brain activity do not always extend to behavioural differences (Blanchard et al., 2011; Schott, 2006).

Given the variability in the imaging data as well as the uncertainty about how exactly these polymorphisms alter dopamine transmission in humans (particularly in the case of the DAT VNTR polymorphism), it is advantageous to consider animal studies in which information about protein expression and neurotransmitter levels is more readily available. As neither the VNTR nor the Val^{158}Met polymorphisms are present in rodents (Lotta et al., 1995), it is necessary to alter DAT and COMT function either pharmacologically or using transgenic lines in order to study potential interactive effects on transmission and behaviour in rats and mice. Several groups have reported effects of the COMT inhibitor tolcapone – either on its own or in combination with a DAT blocker – on dopamine transmission in the striatum, as assessed by changes in levels of dopamine itself or of its metabolites using microdialysis (Acquas et al., 1992; Budygin et al., 1999; Huotari et al., 1999, 2001; Li et al., 1998; Raevskii et al., 2002). The ability of a COMT inhibitor to potentiate the effects of a DAT blocker has been observed during behavioural testing as well as in changes in transmission (Budygin et al., 1999; Raevskii et al., 2002). To our knowledge, potential interactive effects of COMT and DAT on dopamine transmission in cortex have not previously been investigated. However, DAT blockers, although typically less effective in cortex, have nevertheless been shown to increase cortical dopamine levels in a number of microdialysis studies (Carboni et al., 2006; Tanda et al., 1997; Valentini et al., 2004). Furthermore, DAT knockout mice exhibit impaired behaviour on tasks testing spatial cognition, which involve the frontal cortex as well as the hippocampus (Dzirasa et al., 2009; Gainetdinov et al., 1999). Together, these data indicate that DAT and COMT could act interactively in the striatum and/or cortex.

1.5.2 Possible substrates underlying DAT-COMT interactions

There are several mechanims by which DAT and COMT could produce interactive effects. The most straightforward is by local interactive effects on dopamine transmission – i.e. by hitherto unappreciated effects of COMT in the striatum or by DAT in the cortex. As noted above, COMT is expressed, and is functional, in striatum (Laatikainen et al., 2013; Matsumoto et al., 2003). Local infusions of a COMT inhibitor into the striatum have been shown to increase dopamine levels (Huotari et al., 2001), indicating that COMT could influence striatal transmission locally. However, the extent to which this occurs under native conditions remains unclear (Tunbridge et al., 2006). Meanwhile, although the evidence to date indicates that the little DAT that is expressed in cortex has minimal influence on dopamine transmission in this region (Sulzer et al., 2016), it is possible that local DAT could make some contribution to cortical transmission dynamics.

A potentially more important – and more intriguing – substrate for putative DAT-COMT interactions is the extensive network of feedback loops within the mesocorticolimbic circuitry. Let us first consider how projections from MFC to the NAc and VTA provide a route by which COMT's effects on cortical dopamine transmission could feed forward to affect striatal transmission. As discussed in section 1.2, MFC pyramidal cells project to the dopaminergic cell bodies in the VTA. These MFC afferents appear to synapse mainly on MFC-projecting rather than NAc-projecting dopamine neurons (Carr and Sesack, 2000) (although see (Beier et al., 2015)), indicating that MFC may not provide much direct modulation of the mesolimbic dopamine cell population. However, MFC afferents do target NAc-projecting GABAergic cells in the VTA and may also synapse on GABAergic VTA neurons that make local connections to dopamine cells within the VTA (Carr and Sesack,

2000), providing two routes by which MFC could indirectly influence NAc-projecting dopamine cells, either at the level of the cell body or at the terminals. Furthermore, MFC projections to the NAc itself could also influence release at dopamine terminals (Sulzer et al., 2016). Finally, the MFC may influence striatal dopamine through its projections to NAc output neurons, some of which in turn project back to the VTA (Moore et al., 1999).

Although it is not as frequently considered, the relationship between the mesolimbic and mesocortical dopamine systems could operate in the other direction as well – striatal dopamine, regulated by DAT, could induce downstream circuit effects on cortical dopamine transmission. Experiments with the D2R-OE mouse model (introduced in section 1.4.2 above) support this idea. Alteration of striatal dopamine transmission by overexpression of D2 dopamine receptors exerts downstream effects throughout the mesocorticolimbic system in this mouse line (Simpson and Kellendonk, 2016). Both tonic firing rates and phasic burst firing in VTA dopamine neurons are reduced in the D2R-OE mice (Krabbe et al., 2015). In addition, dopamine turnover is diminished, and D1 dopamine receptor sensitivity enhanced, in the MFC in these animals (Kellendonk et al., 2006). Furthermore, as discussed previously, the D2R-OE mice exhibit behavioural changes consistent with these alterations in dopamine transmission, including deficits in both cognitive function and motivation to work for reward (Bach et al., 2008; Ben Abdallah et al., 2011; Kellendonk et al., 2006; Simpson et al., 2011; Ward et al., 2012). Such effects are likely mediated by projections from the NAc back to the VTA, which could influence MFC-projecting dopamine neurons either directly or indirectly via local connections within the VTA. Evidence of which VTA cell types are targeted by afferents arriving from the NAc is mixed, but it appears that both dopamine and GABA neurons receive NAc input (Bocklisch et al., 2013;

Faget et al., 2016; Xia et al., 2011). Several recent studies have demonstrated that the NAc provides direct input to MFC-projecting dopamine neurons in the VTA (Beier et al., 2015; Menegas et al., 2015).

1.5.3 An inverse relationship between cortical and striatal dopamine?

The potential influence of MFC dopamine on NAc transmission has attracted particular interest due to a prominent theory postulating an inverse relationship between MFC dopamine levels and phasic transmission in the NAc. Early evidence for this idea came from experiments in which dopamine terminals in the frontal cortex were selectively lesioned, which resulted in striking alterations to dopamine transmission in the striatum – including higher levels of dopamine, increased dopamine turnover, and enhanced dopamine uptake capacity – as well as changes in behavioural responses to dopaminergic drugs (Carter and Pycock, 1980; Pycock et al., 1980a, 1980b). The inverse relationship theory was elaborated by Anthony Grace as a means of explaining the cortical hypodopaminergia but striatal hyperdopaminergia that is believed to occur in schizophrenia. Low levels of dopamine in MFC are proposed to reduce cortical output to the NAc, which diminishes tonic dopamine release evoked by cortical afferents, and this reduced tonic transmission then acts via autoreceptors to enhance the responsivity of the system, ultimately resulting in increased phasic transmission (Grace, 1991). Grace and colleagues have also linked their theory to the COMT Val^{158}Met polymorphism, arguing that the low activity Met-COMT allele, acting both in the MFC and in NAc, should increase striatal tonic transmission and thus decrease phasic transmission, while the high activity Val-COMT allele should decrease tonic and increase phasic transmission (Bilder et al., 2004). Although this theory offers an attractive account of the relationship between

cortical and striatal dopamine (and has been extremely influential in the neuroimaging literature), evidence that this is what occurs *in vivo* is limited. For example, although a key tenet of the original proposal is that tonic transmission in the NAc is primarily regulated by cortical afferents, it is not currently known exactly how regulation at the terminals of NAc dopamine neurons balances with regulation at the cell bodies to mediate tonic transmission.

In contrast, there is considerable evidence that cortical activity, including cortical dopamine transmission, does influence dopamine in NAc, although these effects cannot always be parsed into distinct changes to tonic versus phasic transmission. As noted above, lesions of cortical dopamine terminals, including lesions targeted specifically to the MFC, increase NAc dopamine levels (Carter and Pycock, 1980; Pycock et al., 1980a, 1980b; Thompson and Moss, 1995). In addition, infusion of a D1 dopamine receptor antagonist into the MFC increases NAc dopamine levels, whereas infusion of a D2 receptor agonist into MFC decreases NAc dopamine (Del Arco and Mora, 2005; Doherty and Gratton, 1996). Several studies indicate that the MFC's influence on NAc dopamine is mediated via the VTA (Carr and Sesack, 2000; Karreman and Moghaddam, 1996; Murase et al., 1993; Taber et al., 1995), although there is also evidence that MFC projections to the NAc can influence release at dopamine terminals both directly via glutamatergic receptors on the terminals and indirectly via MSNs and interneurons (Sulzer et al., 2016).

The detailed dynamics within these circuits are not yet fully explained. Nevertheless, there is an accumulation of evidence that cortical and striatal dopamine transmission can be mutually influential, particularly in the case of MFC influences on NAc, and this provides a means by which the regulatory actions of COMT and DAT could

plausibly interact, in addition to any combined effects they may have locally within these regions.

1.5.4 Implications of potential DAT-COMT interaction

The potential interaction between DAT and COMT is particularly relevant to advancing understanding of how COMT might influence reward-guided behaviours (Tunbridge et al., 2012). Although COMT's role in cognition is fairly well established, evidence that it is involved in reward processing is sparser, as this topic has not been as extensively investigated. COMT's potential role in reward merits further exploration, as it could shed light on the extent to which cortical dopamine – regulated by COMT – participates in top-down control over reward-guided behaviours. For example, it is of interest to know to what extent COMT and cortical dopamine interact with DAT to influence motivation and willingness to exert effort in the pursuit of reward. Conversely, DAT-mediated regulation of striatal dopamine – and the motivational drive and reward processing in which it is involved – may impact cognitive function, as suggested by the experiments with D2R-OE mice discussed above. Evidence of interactive effects of DAT and COMT could provide further support for the idea that motivation can mediate cognition. Furthermore, DAT and COMT may interface in regulating the balance between model-free and model-based RL strategies during reward-guided decision making, a process that recruits both striatal and cortical circuitry.

Insights into these sorts of issues are of considerable interest due to their potential relevance to disease states associated with altered dopamine function. Addiction, for example, is thought to involve aberrant striatal dopamine function and reward processing, a loss of cortical control over behaviour, and a shift from goal-directed to habitual action

(Everitt and Robbins, 2005; Lüscher and Malenka, 2011). A better understanding of the extent of COMT's influence on reward-guided behaviours might therefore shed light on differential susceptibility to addiction – arising, for example, from different COMT genotypes – or even point towards possible treatment options (Tunbridge et al., 2012). In addition, knowledge of how DAT and COMT mediate the balance between habitual (model-free) and goal-directed (model-based) decision making could provide insights into how to redress the imbalance that develops in addiction.

By helping to clarify the relationship between the mesocortical and mesolimbic dopamine pathways, a better understanding of DAT-COMT interactions could also aid efforts to understand how this relationship is altered in schizophrenia. As discussed above, this disorder is believed to involve changes in both striatal and cortical dopamine transmission, but where these changes originate remains to be determined. Grace's theory of schizophrenia proposes that cortical hypodopaminergia induces striatal hyperdopaminergia (Grace, 1991), and study of how DAT and COMT affect both striatal and cortical dopamine (particularly phasic transmission) could provide evidence of whether or not this is likely to occur – or whether, conversely, changes in striatal dopamine might precede and influence alterations in cognition, as suggested by the D2R-OE experiments.

1.6 Aims and hypotheses of this study

The evidence reviewed above suggests that the influences of DAT recycling and COMT degradation might not be neatly limited to the striatum and cortex, respectively, but may in fact interact to regulate dopaminergic transmission in these regions as well as related behaviours. We investigated this potential interaction using a combination of *in*

vivo electrochemical recording and behavioural testing in mice. We conducted our experiments in wildtype animals and used pharmacological agents to modulate the activity of DAT and/or COMT in order to study the effects of acute disruption of these molecules on dopamine transmission and behaviour.

In the preliminary experiments presented in Chapter 2, we assessed dosages and potential side effects of the DAT blocker and COMT inhibitor that were used throughout the study. We then examined the effects of DAT blockade and COMT inhibition on dopaminergic transmission in a series of experiments in which we used fast scan cyclic voltammetry (FCV) to record evoked dopamine release in anaesthetised animals, first in the NAc and then in the MFC. FCV methodology is discussed in Chapter 3, while the data from the NAc and MFC recordings are presented in Chapters 4 and 5, respectively. We next turned to the question of how DAT blockade and COMT inhibition affect motivation to work for reward using the progressive ratio (PR) task, as described in Chapter 6. Finally, we investigated how DAT and COMT contribute to the balance between model-free and model-based decision making strategies using a 2-step decision making task; these data are presented in Chapter 7.

Based on previous findings, we expected that our FCV study would confirm that DAT blockade enhances dopamine transmission in NAc and that COMT inhibition does the same in MFC. We further hypothesised, however, that we might observe effects of the COMT inhibitor on NAc transmission and of the DAT blocker on MFC transmission as well as interactive effects – particularly potentiation of the DAT blocker's effects by COMT inhibition. In addition, we anticipated that DAT blockade would increase motivation to work for reward in the PR task and that the COMT inhibitor might potentiate this effect.

Finally, we predicted that both the DAT blocker and COMT inhibitor would influence behaviour and decision making strategy in the two-step task.

Chapter 2:
Evaluation of drug side effects and optimal dosages

2.1 Introduction

This study aimed to assess the contributions of enzymatic breakdown by catechol-*O*-methyltransferase (COMT) and of recycling by the dopamine transporter (DAT) to dopamine transmission and reward-guided behaviour. In both anaesthetised voltammetry recordings (Chapters 4 and 5) and behavioural experiments (Chapters 6 and 7), we manipulated COMT and DAT function by administering pharmacological inhibitors to wildtype mice. Specifically, we used the COMT inhibitor tolcapone and the DAT blocker GBR-12909. In this chapter I will explain the rationale behind our choice of drugs and then present data from preliminary experiments used to evaluate potential side effects and to select optimal dosages for use in the study's main experiments.

Tolcapone is a selective, brain-penetrant inhibitor that is routinely used in experimental studies of COMT function as well as therapeutically in the treatment of Parkinson's disease (Kaakkola et al., 1994; Männistö and Kaakkola, 1999; Truong, 2009). It has been shown to modulate levels of dopamine metabolites – and, in some cases, of dopamine itself – and to affect behaviours in which COMT is implicated (Farrell et al., 2012; Huotari et al., 1999, 2001, Laatikainen et al., 2012, 2013; Lapish et al., 2009; Tunbridge et al., 2004). GBR-12909 is a selective and high potency DAT blocker that produces moderately sized and relatively long lasting increases in extracellular dopamine levels without inducing the intense euphoric effects associated with DAT blockers like cocaine (Andersen, 1989; Baumann et al., 1994; Izenwasser et al., 1990; Nomikos et al., 1990;

Rothman et al., 1989; Singh, 2000), making GBR-12909 an ideal tool for interfering with DAT function without addictive complications.

Stimulant DAT blockers like GBR-12909 cause hyperactivity and may induce stereotypy (repetitive behaviour) at high doses (Heikkila and Manzino, 1984; Huotari et al., 2002a; Nakachi et al., 1995; Salahpour et al., 2008), and tolcapone may potentiate these effects (Budygin et al., 1999; Raevskii et al., 2002). Excessive hyperactivity or stereotypy could interfere with performance on behavioural tasks. We therefore tested several doses of GBR-12909, with and without tolcapone, for motor effects using a locomotor activity (LMA) and stereotypy test. In addition, the effects of tolcapone and GBR-12909 on preference for sucrose solution over water were investigated to ensure that there were no drug effects on sucrose preference *per se* that might interfere with behavioural tasks in which sucrose was used as a reinforcer.

For tolcapone injections, we chose a dose of 30mg/kg, which has been widely used in both behavioural and physiological studies of COMT function in rodents (Barkus et al., 2016; Benoit-Marand et al., 2000; Budygin et al., 1999; Huotari et al., 1999; Lapish et al., 2009; Tunbridge et al., 2004). We selected two different doses of GBR-12909 for assessment in the LMA-stereotypy and sucrose preference tests based on previous literature (Budygin et al., 1999; Raevskii et al., 2002; Salahpour et al., 2008).

2.2 Methods

2.2.1 Subjects

Male C57BL/6 mice were obtained from Envigo (formerly Harlan). Mice were aged 9-16 weeks at the time of testing. Animals were housed on a 12/12-hr light/dark cycle

(lights on at 0700hr) in groups of 4 mice per cage and were given food and water *ad libitum* expect where otherwise stated. Tests were conducted during the light phase. Care and testing of all animals was conducted under the auspices of the UK Home Office laws and guidelines for the treatment of animals under scientific procedures and of the local ethical review board at the University of Oxford.

A total of 40 mice were used for the preliminary experiments; all animals underwent both the LMA-stereotypy and sucrose preference tests. Of these, 1 mouse was excluded from the analyses of both tests due to an ineffective injection.

2.2.2 Drugs

Drugs were dissolved in 20% hydroxypropyl-beta-cyclodextrin (Acros Organics) in 0.9% saline (AquPharm). This served as a vehicle control in all experiments. The COMT inhibitor tolcapone (Ro 40-7592; 4'-methyl-3,4-dihydroxy-5-nitro-benzophenone) (TRC Inc) was used at a dose of 30mg/kg. The DAT blocker GBR-12909 (1-[2-(bis[4-fluorophenyl]methoxy)ethyl]-4-[3-phenylpropyl]piperazine) dihydrochloride (Tocris) was used at doses of 6mg/kg and 8mg/kg. The dihydrochloride was not taken into account when calculating the dosage, so GBR-12909 injections contained either 6mg/kg or 8mg/kg of GBR-12909 dihydrochloride. Drugs and vehicle were delivered by intraperitoneal injection, with an injection volume of 5mL/kg.

Drugs were administered using a between-subjects design as described in Table 1. The first injection (vehicle or tolcapone) was given 1 hour prior to the second injection (vehicle or GBR-12909) to account for differences in the drugs' time courses of action (Acquas et al., 1992; Männistö and Kaakkola, 1999; Nakachi et al., 1995; Raevskii et al.,

2002). In the LMA-stereotypy test, two doses of GBR-12909 (6mg/kg and 8mg/kg) were assessed. In the sucrose preference test, only the lower dose (6mg/kg) was used.

Group	**Drug 1** **at Time 1**	**Drug 2** **at Time 2 (Time 1 + 1hr)**
Veh-Veh	Vehicle	Vehicle
Tolc-Veh	Tolcapone	Vehicle
Veh-GBR	Vehicle	GBR-12909
Tolc-GBR	Tolcapone	GBR-12909

Table 1: Drug treatment groups.

2.2.3 Locomotor activity test

Assessment of LMA was carried out in 46 x 25 cm plastic boxes with sawdust covering the floor. Boxes were placed within a beam break system (SD Instruments Inc). Beam break data were collected in 15sec intervals.

Mice were food deprived to approximately 90% of free feeding weight for LMA testing. All mice were habituated to handling – including the restraint position used during injections – before the LMA experiment began.

Testing was carried out between 1000hr and 1500hr over 5 days, with 8 mice tested each day. Mice received an initial injection of either tolcapone or vehicle and were then placed in individual boxes for recording of beam breaks. The testing boxes were a completely novel environment to the mice at the start of the session. After 1hr, mice were removed from the boxes and received a second injection of either GBR-12909 (6mg/kg or 8mg/kg) or vehicle and were then placed back in their respective boxes, at which point recording recommenced for an additional 4hrs.

2.2.4 Stereotypy test

Stereotypy was assessed during LMA testing (see above). Stereotypic behaviours were scored manually at 10min intervals over the 4hrs following the second drug injection. The scorer was blind to the second injection. Scoring criteria were adapted from Creese and Iversen, 1974 for use in mice (Table 2).

Stereotypy score	**Criteria use in this study**	**Criteria used by Creese and Iversen 1974**
0	Asleep, stationary, or (non-stereotyped) grooming	Asleep or stationary
1	Active or very active in random fashion	Active
2	Active with bouts of repeated rearing or sniffing or digging	Predominantly active with bursts of stereotyped sniffing or rearing
3	Continuous rearing or sniffing or digging along fixed path or in a particular part of the cage, with hunched posture; may see hopping	Stereotyped activity e.g. sniffing along a fixed path
4	Continuous rearing or sniffing or digging in one location, with hunched posture	Stereotyped sniffing or rearing in one location
5	N.A. – not seen with drug doses used in this study	Stereotyped behaviour in one location with bursts of gnawing or licking
6	N.A. – not seen with drug doses used in this study	Continued gnawing or licking of cage bars

Table 2: Comparison of stereotypy scoring criteria used in this study with those used by (Creese and Iversen, 1974).

2.2.5 Sucrose preference test

Sucrose preference was assessed in open-top cages equipped with two water bottles, one in the position normally occupied by the drinking bottle and one in the food hopper. Bottles contained approximately 100mL of liquid – sufficient to allow consumption throughout the test.

Mice underwent the sucrose preference test either immediately after or 5 weeks after the LMA-stereotypy experiment. The mice had thus been habituated to handling and scruffing and had each received 2 drug or vehicle injections prior to sucrose preference testing. Groups were counterbalanced for previous drug treatment. All animals were sucrose-naïve at the start of the sucrose preference experiment. Mice were water deprived for 3hrs prior to each testing session.

Mice were tested each day between 1100hr and 1800hr for a total of 7 days. The preference test was performed by placing mice individually into testing cages for 5hrs, during which time they had free access to the two bottles. Bottles were weighed immediately before and after testing to determine consumption. On days 1-3, both bottles contained water. On days 4-7, the contents of one bottle were replaced with 10% weight/volume sucrose solution (Sigma Aldrich). On day 7, mice received two injections – the first (tolcapone or vehicle) 1hr before testing and the second (GBR-12909 or vehicle) immediately before testing.

2.2.6 Data Analysis

Data were analysed using Microsoft Excel 2010 and Matlab R2012a and plotted using GraphPad Prism version 5.04. Statistical comparisons were performed using SPSS versions 20 and 24 with significance set at $\alpha = 0.05$. For repeated-measures ANOVAs, Greenhouse-Geisser corrections were applied where data failed Mauchley's test of sphericity; degrees of freedom are reported to two decimal places in such cases. Simple main effects analyses were conducted as necessary when significant interactions were found. Least square difference pairwise comparison tests were used to assess which groups were driving significant main and interactive effects.

The total number of beam breaks made in the LMA test were analysed in 30min bins. Drug effects on LMA levels were assessed using a repeated-measures ANOVA with the first and second drug injections included as separate between-subjects factors: Drug 1 (either vehicle or tolcapone) and Drug 2 (either vehicle, 6mg/kg GBR-12909, or 8mg/kg GBR-12909); time bin was included as a within-subjects factor. Stereotypy scores were analysed in 1hr bins, and drug effects on stereotypy were assessed using the same approach taken with the LMA data. Sucrose preference was assessed as a ratio of sucrose consumption to total consumption (sucrose consumption plus water consumption). Drug effects on sucrose preference were assessed using a univariate ANOVA with two between-subjects factors: Drug 1 (either vehicle or tolcapone) and Drug 2 (either vehicle or 6mg/kg GBR-12909).

2.3 Results

2.3.1 Locomotor activity

GBR-12909 dose-dependently increased LMA (Figure 1). A repeated-measures ANOVA found a significant main effect of drug 2 (vehicle or GBR-12909) on the total number of beam breaks (data in 30min bins; $F_{2,32} = 12.3$, $p < 0.001$). Posthoc tests revealed that activity in groups that received 6mg/kg GBR-12909 was significantly higher than in those that received 8mg/kg GBR-12909 ($p = 0.047$) and that both displayed significantly higher activity than groups that did not receive GBR-12909 (p's < 0.007). The lower activity levels observed in the 8mg/kg groups compared to the 6mg/kg groups can be attributed to stereotypy in the 8mg/kg group (see below) – movement around the testing box became limited as mice engaged in repetitive behaviours in one area of the box.

There was a significant interaction between the effect of drug 2 and time ($F_{4.46,71.31}$ = 7.9, $p < 0.001$), and posthoc testing showed that activity levels in the GBR-12909 treated groups became significantly higher than the activity of groups that did not receive GBR-12909 beginning approximately 45 minutes after administration of the drug (p's < 0.05). During the final 2 hours of the session, groups treated with 6mg/kg GBR-12909 were significantly more active than those treated with the 8mg/kg dose (p's < 0.006).

There were no significant main or interactive effects of drug 1 (vehicle or tolcapone) (F's < 0.9, p's > 0.48), indicating that tolcapone had no effect on locomotor activity levels, either alone or in combination with GBR-12909. There were no other significant main or interactive effects of time or animal weight (F's < 1.7, p's > 0.20).

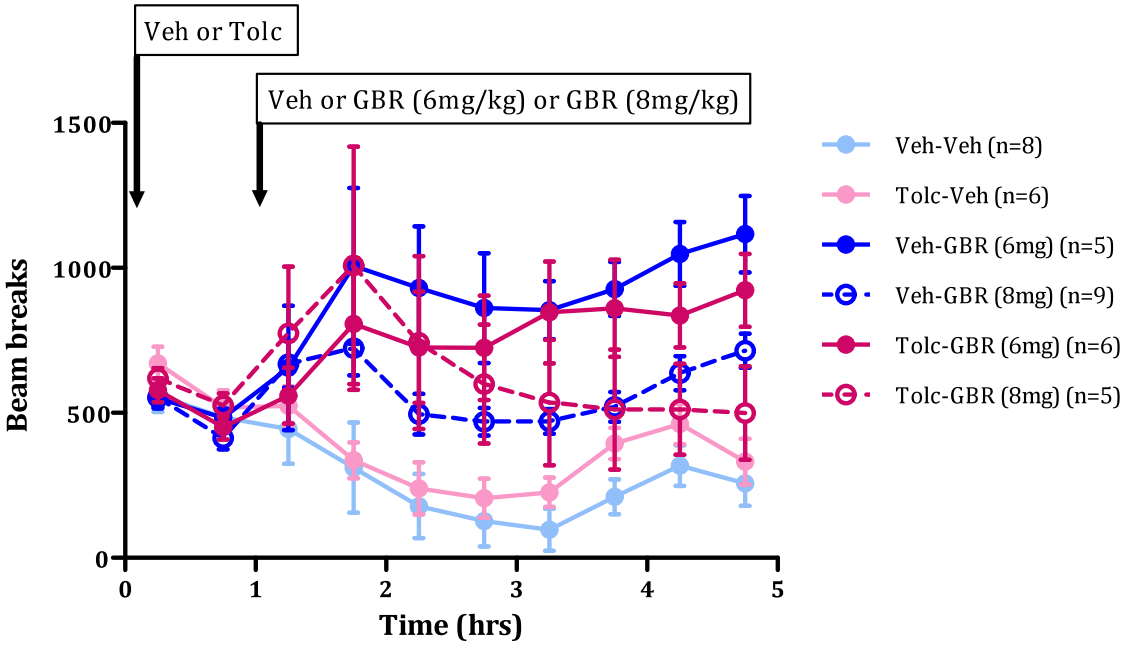


Figure 1: Locomotor activity measured in beam breaks (mean ± SEM). Drug 1 (vehicle or tolcapone) was administered immediately prior to the start of the test. Drug 2 (vehicle, 6mg/kg GBR-12909, or 8mg/kg GBR-12909) was administered 1 hour later. Beam break data are presented in 30min bins.

2.3.2 Stereotypy

GBR-12909 induced stereotypy at 8mg/kg, whereas animals administered the 6mg/kg dose were hyperactive but did not engage in repetitive behaviours (Figure 2). There was a significant main effect of drug 2 on stereotypy score (data in 1hr bins; repeated-measures ANOVA, $F_{2,32} = 74.1$, $p < 0.001$). Posthoc tests showed that stereotypy scores were significantly higher in animals administered 8mg/kg GBR-12909 than in animals treated with the 6mg/kg dose ($p < 0.001$), and all GBR-12909-treated groups scored significantly higher than those that did not receive GBR-12909 (p's < 0.001). There was a significant interaction of drug 2 with time ($F_{6,96} = 7.1$, $p < 0.001$), and posthoc testing revealed significant differences in stereotypy scores between vehicle-, 6mg/kg GBR-12909-, and 8mg/kg GBR-12909-treated animals at all time points (p's < 0.03).

Tolcapone had no main effect on stereotypy score ($F_{1,32} = 0.3$, $p = 0.599$) and no interactive effects with GBR-12909 or time (F's < 2.2, p's > 0.13). There were no other significant main or interactive effects of time or animal weight (F's < 0.9, p's > 0.48).

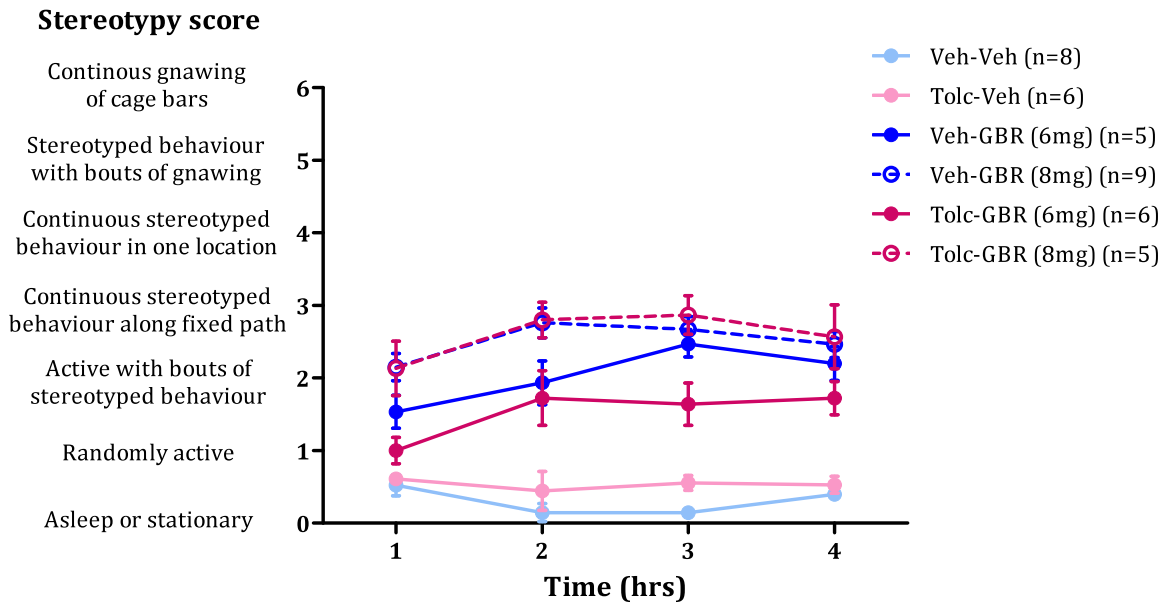


Figure 2: Stereotypy scores (mean ± SEM) over the final 4 hours of the locomotor activity test. Scoring began from the time of administration of drug 2 (vehicle, 6mg/kg GBR-12909, or 8mg/kg GBR-12909), 1 hour after the start of the test. Stereotypy score data are presented in 1hr bins.

2.3.3 Sucrose preference

As can be seen in Figure 3a, mice in all drug groups consumed more sucrose than water with equal preference, by a factor of approximately 4:1. The consumption ratio (sucrose consumption divided by total consumption) was significantly greater than 0.5 for all groups (one-sample t-test, $p < 0.001$), indicating a preference for sucrose. Neither GBR-12909 (6mg/kg) nor tolcapone treatment affected this preference (univariate ANOVA, F's < 1.7, p's > 0.21). Furthermore, neither GBR-12909 nor tolcapone affected the absolute amount of sucrose consumed (univariate ANOVA, F's < 0.6, p's > 0.44) (Figure 3b) or the absolute amount of water consumed (univariate ANOVA, F's < 1.4, p's > 0.26) (Figure 3c).

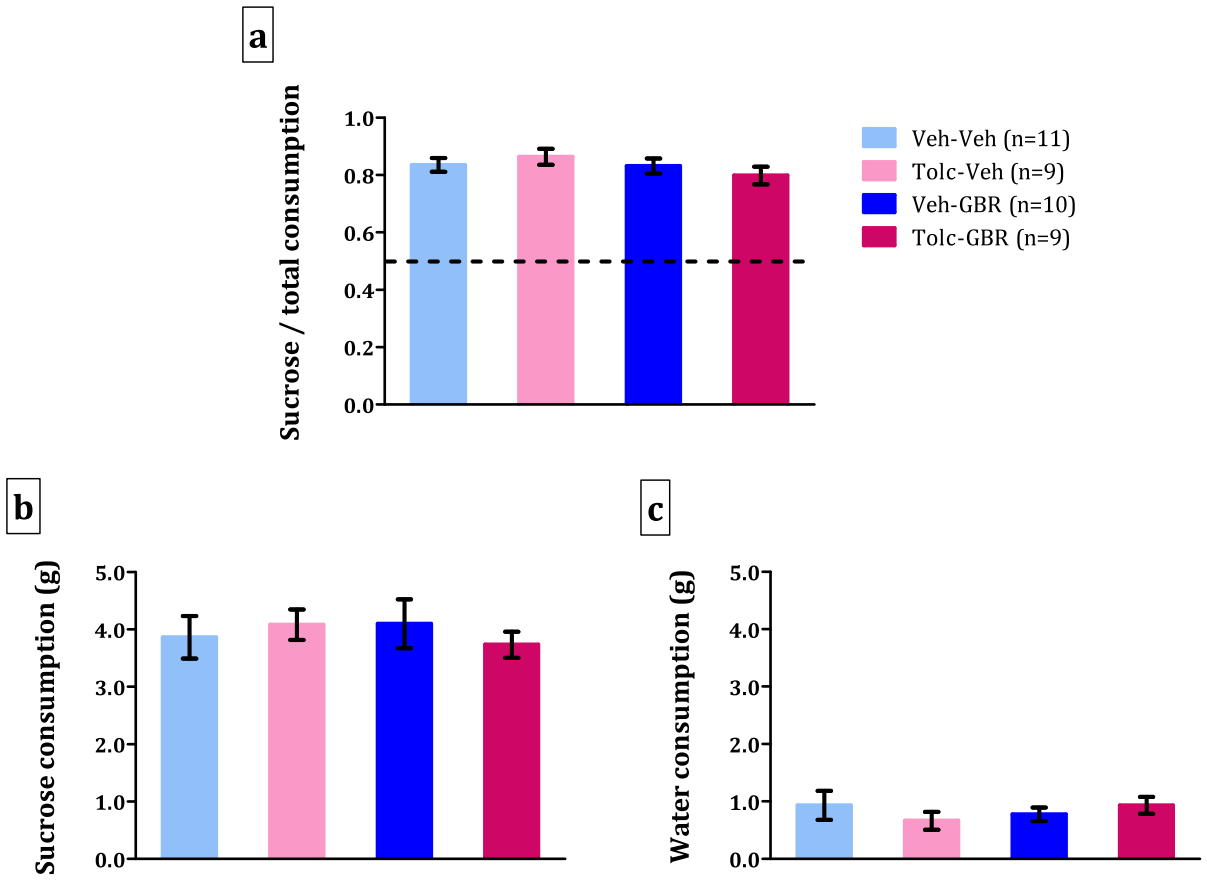


Figure 3: Sucrose preference, assessed by a) the ratio of sucrose consumption to total consumption (sucrose consumption + water consumption), b) absolute sucrose consumption, and c) absolute water consumption. All data presented as mean ± SEM by drug group. The dashed line at 0.5 in (a) indicates equal preference for sucrose and water.

2.4 Discussion

We found that the DAT blocker GBR-12909 significantly increased LMA levels (Figure 1). Activity levels were higher in animals that received the 6mg/kg dose compared to those that received the 8mg/kg dose of GBR-12909, and stereotypy scores revealed that this was due to stereotyped behaviours induced by the 8mg/kg dose (Figure 2). The COMT inhibitor tolcapone did not influence GBR-12909's effects on either LMA levels or stereotypy. In addition, neither GBR-12909 nor tolcapone affected sucrose preference.

Based on the LMA data, we selected the 6mg/kg dose of GBR-12909 for subsequent experiments. It should be noted that this dose is lower than those used in previous studies, which ranged from 10 to 50 mg/kg (Budygin et al., 1999; Heikkila and Manzino, 1984; Huotari et al., 2002a; Nakachi et al., 1995; Raevskii et al., 2002; Salahpour et al., 2008). We found, however, that the 6mg/kg dose produced a discernable behavioural effect (increased LMA) without inducing the stereotypies seen with the 8mg/kg dose (Figures 1 and 2). This indicated that 6mg/kg GBR-12909 would have behavioural effects in the main behavioural experiments of the study without producing confounding motor side effects.

Unexpectedly, and in contrast to previous findings in rats (Budygin et al., 1999; Huotari et al., 1999; Raevskii et al., 2002), 30mg/kg tolcapone had no potentiating effects on GBR-12909-induced hyperactivity or stereotypy. This discrepancy may be attributable to species differences or, potentially, to the higher doses of GBR-12909 (10 and 20 mg/kg) used in these earlier studies.

The sucrose preference test showed that all mice preferentially drank sucrose over water, as anticipated based on previous findings (Sclafani, 2006; Smith and Sclafani, 2002). We did not expect DAT blockade or COMT inhibition to affect this preference, and our findings confirmed this prediction, as neither GBR-12909 nor tolcapone influenced sucrose preference (Figure 3). This result rules out confounding drug effects on the hedonic properties of the reinforcer (sucrose) from subsequent behavioural experiments.

Chapter 3:
Methodological considerations of voltammetry data collection and analysis

In this study, we used fast scan cyclic voltammetry (FCV) to assess the effects of catechol-*O*-methyltransferase (COMT) inhibition and dopamine transporter (DAT) blockade on dopamine transmission. We evoked dopamine release by stimulating dopamine neurons in anaesthetized mice and used FCV to record this release in either the nucleus accumbens (NAc) or medial frontal cortex (MFC); these data are reported in Chapters 4 and 5, respectively. In this chapter, I will provide an in-depth explanation of FCV as a technique and will then discuss chemometrics, a procedure that is a key step in the analysis of FCV data. As there is currently some controversy in the field about how the chemometric analysis of FCV data should be done, I will consider both sides of the debate and then present the approach that I have taken in analyzing our datasets.

3.1 Fast scan cyclic voltammetry

FCV is an electrochemical technique that originated in the 1970s from the work of analytical chemists with an interest in neuroscience (Adams, 1976; Conti et al., 1978; Keller et al., 1976; Kissinger et al., 1973; Lane et al., 1976; LeRoy Blank and Pike, 1976; Oke et al., 1978; Weintraub et al., 1975). FCV was then adapted for use *in vivo* (Kuhr and Wightman, 1986; Kuhr et al., 1986; Millar et al., 1985; Stamford et al., 1984) and has since undergone numerous modifications and refinements (Atcherley et al., 2015; Clark et al., 2010; Phillips

et al., 2003; Stamford and Justice, 1996; Willuhn et al., 2010). It is now widely used to study dopamine transmission across species in both *ex vivo* and *in vivo* preparations.

FCV is performed by passing a small voltage ramp across a carbon fibre microelectrode, which transiently oxidizes electroactive compounds in the tissue surrounding the electrode (Figure 1a) (Robinson et al., 2003). In this study, for instance, we oxidized dopamine to its *o*-quinone by ramping the electrode voltage from –0.4V to +1.3V (versus an Ag/AgCl reference electrode) and then reduced the *o*-quinone back to dopamine by stepping the voltage back to –0.4V (Phillips et al., 2003). The faradaic current generated by this redox reaction can be recorded with the same electrode, and the current at dopamine's peak oxidation potential (between +0.6V and +0.7V versus Ag/AgCl) is linearly proportional to the dopamine surrounding the electrode (Phillips et al., 2003). Because the voltage step is performed continuously at a fast scan rate (in our study, with a speed of 400 V/sec and a frequency of 10Hz), the recorded current provides an approximate real-time readout of this information (Figure 1b) (Robinson et al., 2003).

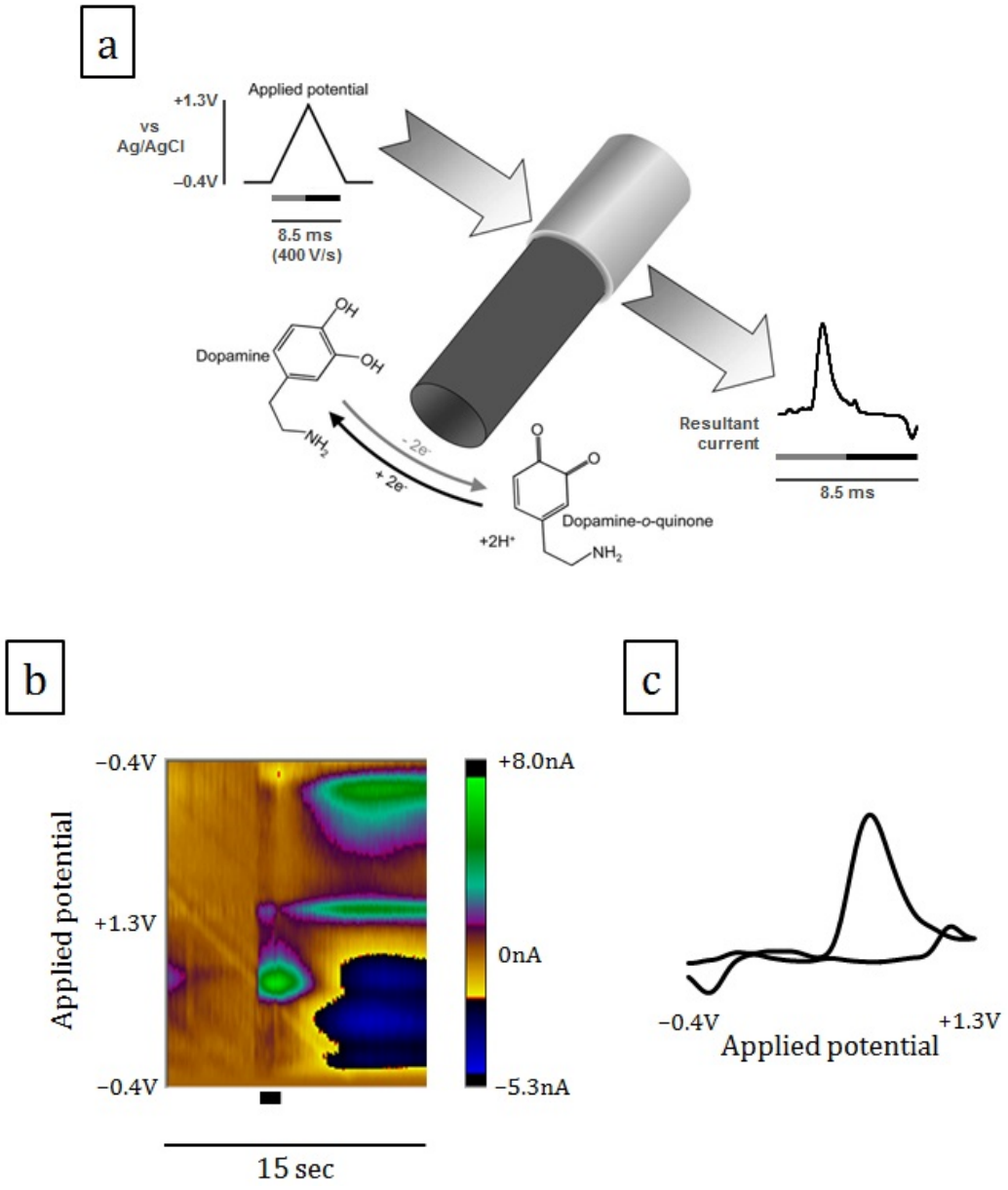


Figure 1: Schematic representation of how FCV is used to measure dopamine. a) A triangular waveform is applied to the carbon fibre electrode. As the voltage is stepped from –0.4V to +1.3V, dopamine molecules surrounding the electrode are oxidized to dopamine-*o*-quinone and then reduced back to dopamine as the voltage is stepped down to –0.4V. The current produced by this redox reaction is recorded as a measure of dopamine levels. b) FCV data can be usefully presented in a pseudocolour plot: the recorded current is plotted as a function of the applied potential of the triangular waveform (y axis) over time (x axis). In this example, electrical stimulation of the midbrain (timing and duration of stimulation indicated by thick black bar below colour plot) evoked dopamine release in the NAc. The stimulation also produced a pH change (blue in the colour plot) that closely follows the dopamine transient; as will be discussed below, the dopamine and pH components of the recording are separated during analysis. c) The chemicals detected at the electrode are identified by their cyclic voltammograms; this voltammogram was extracted at the peak of the dopamine signal, 1.1sec after the time of stimulation. Part (a) of this figure is adapted from Phillips and Wightman, 2003.

FCV can be used to both identify and quantify the neurochemical of interest. The chemical is identified by its cyclic voltammogram – a plot of the recorded current as a function of the voltage step (Figure 1c). FCV can detect numerous neurologically relevant chemicals: dopamine and its fellow monoamines adrenalin, noradrenaline, and serotonin; several of their metabolites; and other chemicals such as encephalins and ascorbic acid (Heien et al., 2003; Stamford, 1990). Most of these compounds have distinguishable voltammograms because they oxidize and reduce at different points along the voltage step; the notable exceptions are dopamine and noradrenaline, whose voltammograms are nearly identical (Robinson et al., 2003). It should be noted that, in our study, although this similarity does not impact the identification of dopamine release recorded in the NAc – which contains little noradrenaline, particularly in the core region to which we targeted our recordings (Berridge et al., 1997; Delfs et al., 1998; Hnasko et al., 2006; McKittrick and Abercrombie, 2007) – it does complicate the interpretation of recordings made in the MFC – which is innervated by both dopaminergic and noradrenergic fibres (Lindvall et al., 1978; Slopsema et al., 1982). See Chapter 5 for further discussion of this topic.

Quantification of FCV signals is performed by calibrating the electrode *in vitro* in a known quantity of the chemical of interest and then using the resulting calibration factor to convert the signal recorded *in vivo* into units of concentration (Robinson et al., 2003). For dopamine, the relationship between current and concentration is linear, making this conversion straightforward (Phillips et al., 2003; Sinkala et al., 2012). Nevertheless, note that the quantification procedure always produces an estimate of dopamine concentration because the sensitivity of FCV electrodes is altered when they are implanted in brain tissue,

likely due to adherence of proteins and other substances to the electrode surface as well as to temperature changes (Phillips et al., 2003).

FCV is of great value to neuroscience because of its spatial and temporal precision. This technique offers excellent spatial resolution due to the small size of the electrodes (Wassum and Phillips, 2015). The carbon fibre tip of modern FCV electrodes is less than 10μm in diameter and, unlike a microdialysis probe, produces no discernable tissue damage even when implanted for extended periods of time (Clark et al., 2010; Robinson et al., 2003). Because of their small size, FCV electrodes can be used to investigate differences in transmission between or even within specific brain regions (Stamford, 1990).

Although other techniques can more accurately identify and quantify neurochemicals, FCV exhibits far greater temporal resolution: it can detect sub-second changes in dopamine release, whereas techniques like microdialysis operate on the order of minutes (Robinson et al., 2003). FCV is currently best suited to measuring *changes* in transmitter levels. For example, FCV can be used to monitor phasic dopamine transmission – large, fast, and short-lived dopamine transients that accompany behaviourally relevant events such as an unexpected stimulus or a cue signaling reward (Robinson et al., 2003; Willuhn et al., 2010). Microdialysis, by contrast, is a better option for measuring *absolute* levels of a neurochemical and is therefore used to assess tonic dopamine transmission – the smaller, slower, and more continuous dopamine release that establishes the baseline levels of dopamine on top of which phasic transients occur (Robinson et al., 2003; Willuhn et al., 2010). (This distinction between FCV and microdialysis may someday be overcome, however: recently, voltammetrists have begun adapting FCV so that it can be used to detect both phasic and tonic release at the same electrode (Atcherley et al., 2015)).

It should be noted that, although its temporal resolution is better than that of microdialysis, FCV does not provide the temporal precision of electrophysiology. However, because FCV can directly detect neurotransmitter release, it offers critical insights into neural output that electrophysiology cannot provide. The relationship between neural activity and neurotransmitter release is complex, as release can be influenced by many factors at the axon terminal (Melchior et al., 2015) – for example, glutamatergic corticostriatal afferents can cause dopamine release at striatal terminals even in the absence of dopamine neuron spiking (Grace, 1991) – and it is therefore necessary to study release itself as well as neural activity (Salamone, 1996). Furthermore, because it has only recently become feasible – and remains technically challenging – to trace the projection of a neuron from which one is recording (Dautan et al., 2016), electrophysiology often cannot provide information on how neural activity affects transmission in a particular target region, whereas FCV does offer such specificity.

FCV thus provides neuroscientists with a tool for studying dopamine transmission in well-defined spatial regions and at behaviourally relevant timescales.

3.2 Chemometric analysis of voltammetry data

As discussed above, FCV is capable of detecting numerous chemicals in addition to dopamine. When recording in a heterogeneous chemical environment like the brain, it is therefore necessary to extract the dopaminergic component of the recorded signal from components attributable to other chemical fluctuations and to changes in the microenvironment of the electrode that are coincident with phasic dopamine release. Notable among these is the pH change that is frequently observed in FCV recordings just

after dopamine transients (Jones et al., 1994; Takmakov et al., 2010; Venton et al., 2003). The neural activity associated with these transients alters blood flow, leading to changes in local carbon dioxide concentrations and, as a result, pH shifts (Robinson et al., 2003). If the pH change overlaps the dopamine signal, it distorts and diminishes this signal, which can significantly impact calculations of the size and kinetics of the dopamine response (Michael et al., 1998). It is therefore important to separate pH shifts, as well as noise and other chemical changes, from the recorded signal before assessing dopaminergic transmission.

The discipline of chemometrics is used to perform this separation. Chemometrics is a set of mathematical and statistical methods developed by chemists to identify and quantify different analytes in a dataset (Keithley et al., 2009). As the use of FCV has expanded beyond the electrochemical laboratory to neuroscience research, so too has the use of chemometrics. However, the use of these techniques beyond their discipline of origin has generated challenges and, occasionally, controversies. I will therefore discuss the alternative approaches taken to analyzing FCV data before explaining the approach we applied to our dataset.

3.3 Approaches to chemometric analysis of voltammetry data

Principal component regression (PCR) is the key instrument in the chemometrics toolbox. It is a multivariate calibration technique involving two steps: principal component analysis (PCA) and least-squares regression (Heien et al., 2004; Keithley et al., 2009). PCA is the process of calculating orthogonal vectors, called principal components (PCs), that together describe the variance of a dataset. The initial PCs describe variance that can be ascribed to meaningful chemical changes, such as dopamine release or a pH shift, whereas

the variance contained in PCs that fall below a certain threshold is ascribed to noise (Keithley et al., 2010a). A regression is then performed to determine the relationship between distances along PCs and the concentration of the analytes of interest.

Traditional chemometrics involves running PCA on a training set comprised of sample cyclic voltammograms of the chemical analytes that are expected to be present in the experimental dataset – usually dopamine and pH, in the case of FCV studies of striatal dopamine release (Figure 2) (Keithley et al., 2009; Willuhn et al., 2010). These model voltammograms essentially instruct the analysis on which chemical signatures should be identified and separated from one another. Once the PCA and least-squares regression are complete, the experimental data is projected back onto the PCs that fall above the noise threshold, allowing identification of which portions of the recorded signal can be attributed to dopamine and which to pH, and which portions can be discarded as noise (Keithley et al., 2010a).

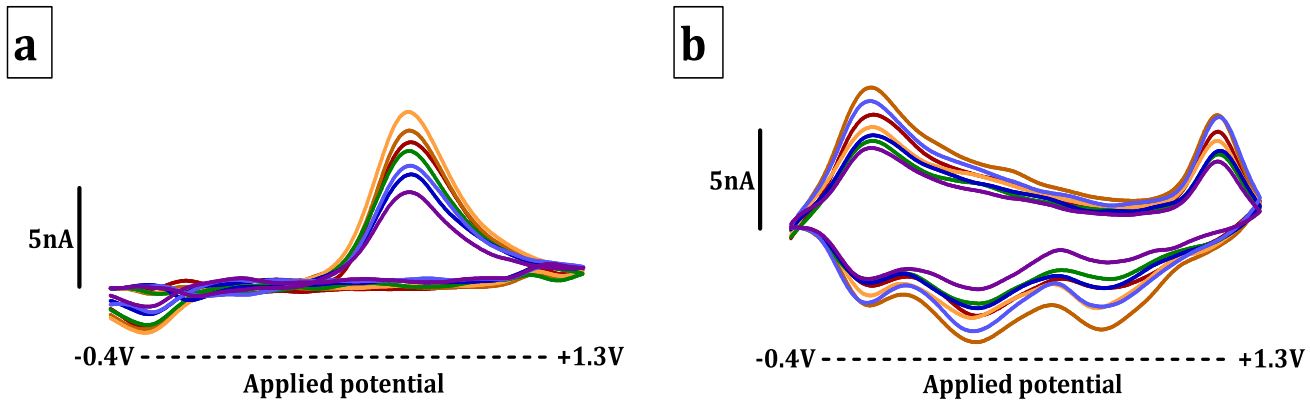


Figure 2: Example of cyclic voltammograms included in a training set for one of the NAc data sets. a) Dopamine voltammograms. b) pH voltammograms. The current at the electrode (y axis) is shown as a function of the applied potential of the triangular waveform (the step from –0.4V to +1.3V and back; x axis). Training sets voltammograms – both dopamine and pH – should ideally cover a range of signal sizes comparable to the range found in the data set.

The alternative approaches that have developed for the analysis of FCV data – and the controversies that have grown around them – arise at their root from a contradiction inherent in the procedure described above. In order for chemometrics to work optimally, the sample cyclic voltammograms contained in the training set must be as close a match to pure dopamine (and pure pH) voltammograms as possible – the more accurate the representation of the chemical signature of dopamine, the more faithfully PCR will extract the portion of the recorded signal that is actually attributable to dopamine. One might imagine that an ideal FCV training set would therefore be composed of dopamine voltammograms (alongside pH voltammograms) obtained by recording pure dopamine in a flow cell or other *in vitro* apparatus. However, dopamine voltammograms in such recordings may differ considerably from the voltammograms in recordings made *in vivo* due to differences in temperature, chemical composition, and so forth between the *in vitro* apparatus and living brain tissue, and *in vitro* training sets therefore often fail to accurately extract dopamine from *in vivo* data sets (Heien et al., 2005). The ideal training set must thus paradoxically contain cyclic voltammograms that are both pure enough to accurately identify the analyte of interest and representative enough to account for the particular *in vivo* microenvironment in which data set recordings were made.

The solution devised by Mark Wightman and colleagues was to aim to record a training set in each animal on each day of an experiment by stimulating dopamine release, usually using an electrode targeted to the midbrain dopaminergic nuclei (Heien et al., 2005; Johnson et al., 2016; Rodeberg et al., 2015; Wightman et al., 2007). This 'individual' training set approach ensured that the many factors that might affect the voltammograms – noise levels on the day of the recording, the properties of the electrode used, the brain

region in which recordings were made – would be constant between the voltammograms included in the training set and those found in the experimental data it would be used to analyze. The use of electrode- and recording session-specific training sets fulfilled the requirement for representative voltammograms, while the experienced eye of the researcher, trained to recognize the characteristic shapes of dopamine and pH voltammograms from *in vitro* data, in principle provided a subjective purity check.

This approach, although admirable for its rigor and specificity, has several critical disadvantages that became apparent when FCV was adapted for use in longitudinal behavioural recordings. Traditionally, researchers have used glass electrodes that, due to their size and fragility, must be inserted and then removed for each recording session using a microdrive (Clark et al., 2010; Phillips et al., 2003). This acute preparation cannot be used to monitor dopamine transmission in the same brain region over days or weeks, as one might want to do in studies of learning or of disease progression (Wassum and Phillips, 2015). In addition, the manual process of inserting and optimizing the position of the electrode can cause stress to the animal that may disrupt behaviour (Wassum and Phillips, 2015). It should also be noted that, because training set voltammograms must cover a range of signal amplitudes in order to capture the range found in the experimental data, training sets are typically generated by electrically stimulating dopamine release and varying the stimulation parameters to generate a stimulus-response curve of differently sized signals (Heien et al., 2005; Willuhn et al., 2010). Many neuroscientists attempting to record behaviourally-evoked dopamine release in freely moving animals were therefore reluctant to implant relatively large (~0.15mm diameter) stimulating electrodes for the sole purpose of making training sets when the tissue damage such electrodes do could

interfere with both the dopamine transmission and the behaviours that they were interested in studying (Mark Walton, personal communication).

In response to these drawbacks, Paul Phillips and colleagues developed small silica-based electrodes that could be chronically implanted and used to study dopamine transmission in a given region for as long as several months (Clark et al., 2010). Users of these chronic FCV electrodes generally do not implant an additional stimulating electrode (Flagel et al., 2011; Howe et al., 2013; Parker et al., 2010). Because of these changes, an alternative approach to chemometrics had to be devised. Rather than using a different training set to analyze every animal's data, Phillips and colleagues generated a 'standard' training set by electrically evoking dopamine release in a separate animal and used this to analyze data sets from animals implanted with chronic FCV electrodes for behavioural experiments (Clark et al., 2010, 2013; Hart et al., 2014; Willuhn et al., 2012). Several additional criteria are used alongside standard training sets to accurately identify dopamine transients in recordings made using chronically implanted electrodes: there must be a high correlation between behaviourally-evoked dopamine voltammograms in the data set and a standard dopamine voltammogram evoked by electrical stimulation, and the peak of the data set voltammograms must fall within the portion of the voltage step where dopamine is typically oxidized (between +0.6V and +0.7V versus Ag/AgCl) (Clark et al., 2010; Gan et al., 2010).

The adjustments made to record and analyze dopamine release in longitudinal behavioural studies using chronically implanted electrodes have thus required several departures from the initial principles of chemometrics. The standard training set, recorded on a different electrode in a different animal, is likely not as representative of the

voltammograms in a given data set as a training set recorded with the same electrode in the same animal. While some researchers have generally considered this an acceptable concession, others maintain that standard training sets fail to accurately extract the dopaminergic component of a signal and warn that use of such training sets may lead to erroneous conclusions (Johnson et al., 2016; Rodeberg et al., 2015).

Unfortunately, it is impossible to directly measure how much of an FCV recording is truly attributable to dopamine, and it is therefore challenging to compare the individual training set approach with the standard training set method. Because recordings must be background-subtracted in order to resolve small changes in dopamine levels from the large capacitive currents generated during the recording process, FCV can only provide measurements of changes in dopamine transmission, not of absolute levels (Wightman et al., 2007). One can therefore determine that different approaches to chemometrics yield different results (although these differences are often very small (Keithley and Wightman, 2011)) but not which is more accurate, as there is no ground truth against which to check them. In addition, because the stimulation used to evoke dopamine release causes concurrent changes in other neurochemicals – including, of course, pH – the voltammograms used to construct training sets will in practice never be perfectly pure, and chemometrics will thus always be based on an approximated model of dopamine and pH, regardless of whether an individual or standard training set it used. Therefore, while it is likely that an individual training set more accurately extracts dopamine than a standard one in most cases, given that its voltammograms are more representative of those found in that animal's data set, it is difficult to assess how large this difference is and how much it may affect the overall analysis.

3.4 Approach used in this study

In this study, it was possible to make a training set for each animal used, as experiments were conducted in anaesthetized animals, and a stimulating electrode was implanted regardless. Since individual training sets are considered to be more reliable, I chose to take this approach. I consulted with Dr Jaime McCutcheon (University of Leicester, UK) and also extensively with Dr Richard Keithley (Roanoke College, Virginia, USA), a former postdoc in the Wightman research group whose statistical expertise allowed the group to refine and improve its initial procedure for doing PCR, and developed a slightly modified version of the Keithley-Wightman approach – one as faithful to their procedure as possible but adjusted to suit the constraints of this study's data set – which is described here as well as in the methods sections of Chapters 4 and 5.

This study's anaesthetized FCV recording experiments involved a long stabilization period, lasting 1.0-2.5 hours, before collection of experimental data could begin. A rundown in the size of the evoked release was observed over the course of the day, particularly in NAc recordings. NAc signals were especially unstable during the first few hours of recording, and baseline measurements and drug injections were therefore delayed until the signal had stabilized (see Figure 2 in Chapter 4); MFC recordings were also allowed to stabilize for an hour before baseline measurements began (see Figure 1 in Chapter 5). These stabilization periods provided a set of recordings from which to extract dopamine and pH voltammograms for each animal's training set and were theoretically advantageous because they allowed us to use training set voltammograms from recordings separate from those included in the experimental data set. However, in practice there were numerous cases (74% of NAc data sets and 64% of MFC data sets) in which

voltammograms extracted from the stabilization recordings were either of insufficient quality or did not cover the necessary range of signal sizes, and in these instances additional voltammograms from the data set recordings were added to the training set. In addition, two of the NAc training sets were constructed using only dopamine voltammograms, as it was not possible to extract pH voltammograms from these datasets due to the large dopamine release and comparatively negligible pH change evoked by stimulation.

Training sets were evaluated and refined using several tools developed by Keithley and colleagues. Malkowski's F test was used to determine the cut-off point between PCs whose variance is statistically larger than error (i.e. that contain chemically relevant information), which are retained by the analysis, and PCs whose variance is not significantly larger than error (i.e. that contain only noise), which are rejected and extracted from the data (Keithley et al., 2010b, 2010a). Voltammograms that deviated too significantly from other voltammograms in the training set, thus exerting excessive influence on the direction of the PCs, were identified by Cook's distance (Figure 3a) (Keithley and Wightman, 2011). K matrices – a representation of the dopamine (or pH) voltammogram that is extrapolated from the sample voltammograms in the training set and that guides the extraction of the dopamine (or pH) signal from the data set (Hermans et al., 2008; Keithley and Wightman, 2011) – were inspected, and training sets were modified as necessary to achieve K matrices that matched pure dopamine and pH voltammograms as closely as possible (Figure 3b). Finally, the residuals (those elements of the signal not accounted for by the retained PCs) were assessed to ensure that the training

set was effectively separating dopamine and pH without erroneously rejecting either of these signals along with noise (Figure 3c) (Keithley et al., 2009).

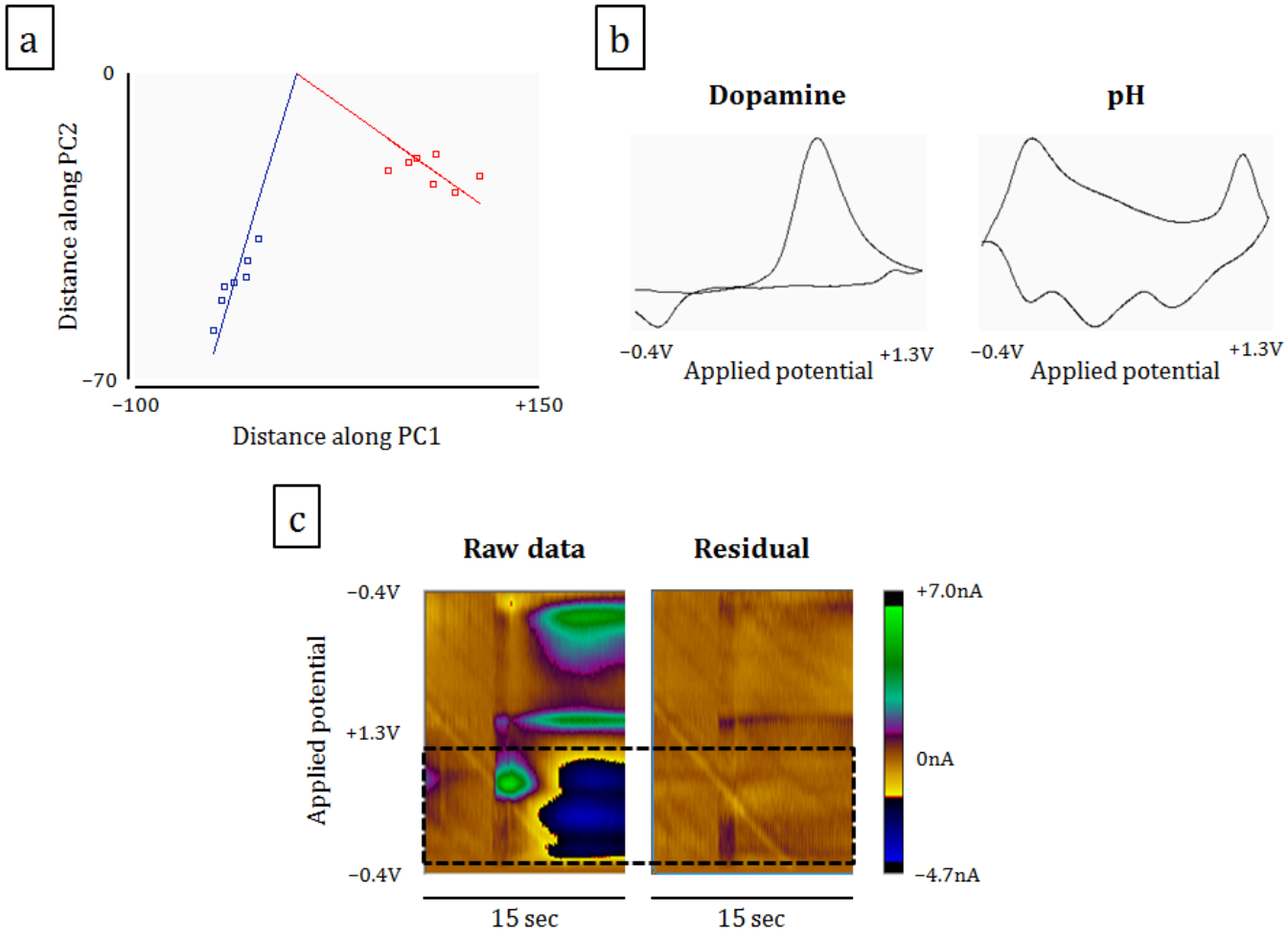


Figure 3: Tools provided by Richard Keithley's customized version of the CV Analysis software that can be used to assess the quality of a training set, illustrated using one of the NAc training sets. a) The dopamine (blue) and pH (red) voltammograms included in the training set plotted as a function of the first two PCs generated by PCA. This plot can be used to visualize how the individual voltammograms for each analyte relate to each other. If an individual voltammogram deviates excessively from the other voltammograms in the set, this should be visible in the plot and will be flagged up by the software based on the Cook's distance calculation. b) K matrices. In this example, the K matrices closely resemble pure voltammograms for dopamine and pH, respectively, indicating that the training set is working well. c) A comparison of the raw data with the residual signal remaining after chemometric analysis. Each plot shows current (in colour) as a function of the applied potential of the triangular waveform (y axis) over time (x axis). In the raw data, both the dopamine transient (green) and pH shift (blue) are visible within the boxed portion of the plot. In the residual plot, by contrast, there are no visible dopaminergic or pH changes, indicating that the chemometric analysis has successfully identified and separated the dopamine and pH components of the recording.

When chemometrics is performed, a Q value is generated for each data point that is calculated by summing the squares of the residuals; this value must fall below a statistical threshold, Qα, for that data point to be viable for further analysis. Q values below Qα indicate that the retained PCs satisfactorily account for all of the relevant information in the data set (dopamine release, pH changes, etc.), while those that exceed Qα indicate that meaningful information is being discarded in the excluded PCs along with noise (Keithley et al., 2009). Qα values are typically in the hundreds (Keithley et al., 2010a) (Richard Keithley, personal correspondence). In this study, however, Qα sometimes exceeded this range: for 56% of NAc training sets and 36% of MFC training sets, Qα values were greater than 1000. The highest Qα obtained in this study was equal to 11,959. However, this training set was an outlier, as the raw signals were extremely large, and in other training sets Qα values that were greater than 1000 did not exceed 6000. These high Qαs may arise from a number of factors, including variability across voltammograms in the training sets, large signal sizes (an issue particularly prevalent in NAc recordings), and, potentially, the presence of analytes other than dopamine and pH in the recorded signals (a factor particularly likely in MFC recordings).

In practice, the process of refining a training set by optimizing the various measures described above often requires compromise. This can be illustrated using the example training set, made for one of the NAc data sets, that is depicted in Figure 4. The initial version of this training set contained 7 dopamine voltammograms and 7 pH voltammograms, retained 5 PCs (as calculated by Malikowski's F test), and had a Qα value of 271. However, inspection of the K matrices for this initial version showed that they diverged from pure dopamine and pH voltammograms (Figure 4a, top). The training set

was therefore refined by removal of 2 dopamine and 2 pH voltammograms that were skewing the K matrices. This resulted in 2 PCs being retained by Malikowski's F test. The Qα value was higher (1202), which is less desirable, but the K matrices were much cleaner and closely resembled pure dopamine and pH voltammograms (Figure 4a, bottom). The refined version of the training set was therefore used for chemometric analysis of this data set. Figure 4b shows the dopamine signal extracted from one of the data set's recordings by the initial and final versions of the training set. The extracted signal is similar across the two training sets, although the final version extracts a slightly larger signal late in the recording.

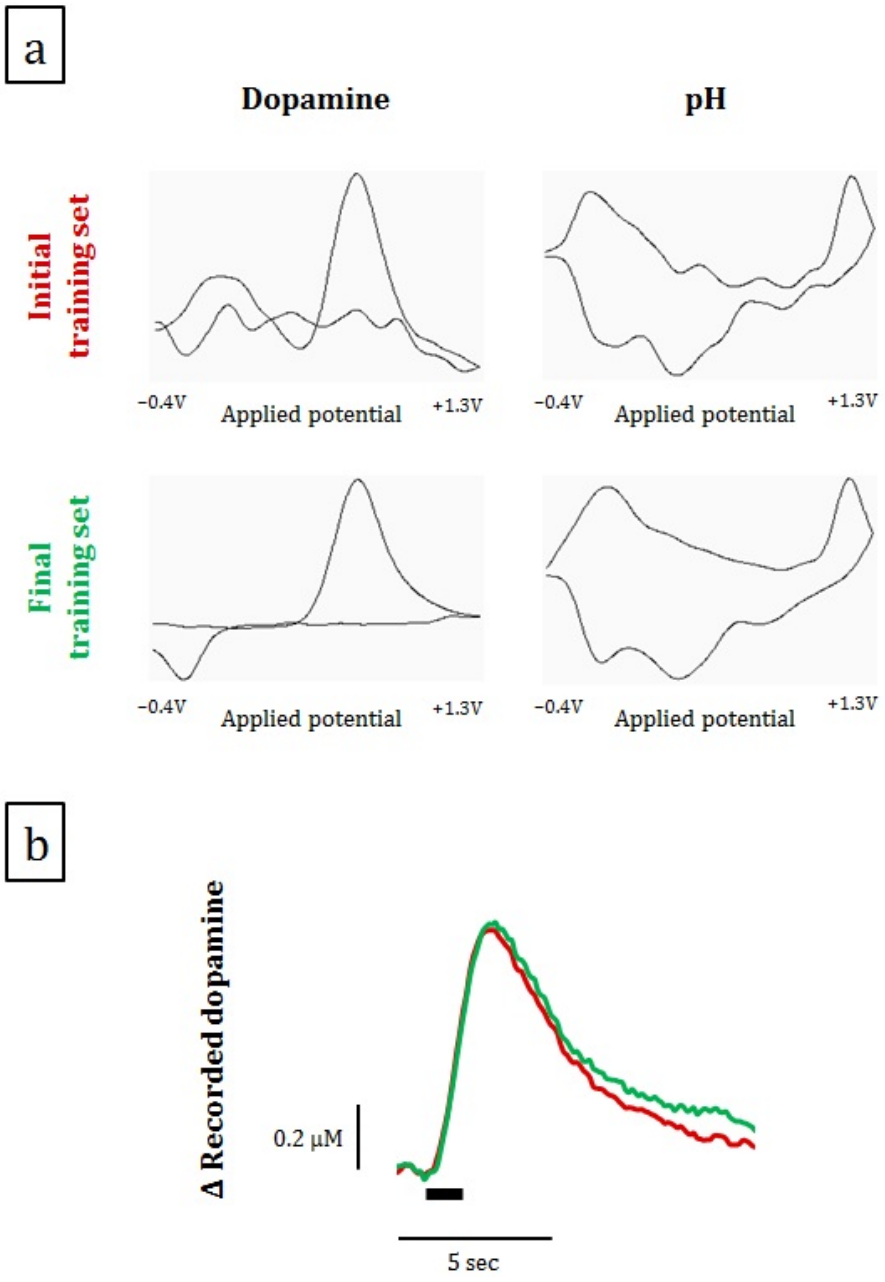


Figure 4: A training set before and after being refined using the tools described in the text and illustrated in Figure 3. a) Dopamine and pH K matrices for the initial (top) and final (bottom) versions of the training set. b) The dopamine signal extracted using the initial (red) and final (green) versions of the training set, displayed as a change in concentration over time. Timing and duration of stimulation indicated by thick black bar.

During chemometric analysis, FCV signals were converted from nA into nM using calibration factors obtained by pre-calibrating electrodes in a flow cell (Sinkala et al.,

2012); see the methods section of Chapter 4 for further details. Electrodes were calibrated for dopamine but not pH, as it was not relevant to the study to quantify the pH component of recordings, and pH was thus represented in arbitrary units during chemometric analysis.

Chapter 4:
Effects of DAT blockade and COMT inhibition on dopaminergic transmission in striatum

4.1 Introduction

Striatal dopamine, particularly in the nucleus accumbens (NAc), is involved in many aspects of reward processing, such as motivation to pursue and work for rewards as well as learning about reward-related cues (Berridge, 2006; Salamone et al., 2007). Two main modes of striatal dopamine transmission have been identified: tonic transmission, which involves small but regular release events that maintain baseline levels of dopamine, and phasic transmission, which consists of short-lived but high intensity bursts of dopamine release that occur in conjunction with behaviourally relevant events (Moore et al., 1999; Robinson et al., 2003). Although dopamine clearance in the striatum is achieved primarily through recycling via the dopamine transporter (DAT) (Sulzer et al., 2016), there is evidence that degradation by the enzyme catechol-*O*-methyltransferase (COMT) may also contribute to the regulation of striatal dopamine (Acquas et al., 1992; Budygin et al., 1999; Huotari et al., 1999, 2001; Li et al., 1998; Raevskii et al., 2002). However, findings are mixed: COMT effects have been observed on tonic striatal dopamine but not on phasic transmission, and these effects often only emerge when manipulations of COMT degradation are combined with additional dopaminergic manipulations. We therefore assessed the effects of pharmacological inhibition of DAT and/or COMT on evoked dopamine release in the striatum in order to clarify to what extent COMT contributes to the regulation of dopamine transmission in this region.

4.1.1 Evidence that COMT may impact striatal dopamine

Extensive evidence indicates that DAT recycling is the primary means of dopamine clearance in the striatum: interference with DAT's function by either pharmacological blockade or genetic knockdown produces marked increases in striatal dopamine levels (Budygin et al., 1999; Giros et al., 1996; Huotari et al., 1999; Nakachi et al., 1995; Nomikos et al., 1990; Owesson-White et al., 2012; Raevskii et al., 2002; Torres et al., 2003). However, some evidence indicates that enzymatic degradation by COMT might also affect transmission in this region. Several human imaging studies have found interactive effects of DAT and COMT polymorphisms on fMRI BOLD signal in the striatum (Dreher et al., 2009; Yacubian et al., 2007). Recording studies in rodents support the idea that interactive effects of DAT and COMT on striatal dopamine might underlie these imaging data. Direct injection of a COMT inhibitor into striatum can increase tonic striatal dopamine levels (Huotari et al., 2001). When COMT is inhibited systemically, however, tonic dopamine levels in the striatum are only increased when an additional dopamine-boosting drug, such as a DAT blocker, is administered alongside the COMT inhibitor (although systemic COMT inhibition does, on its own, consistently alter striatal levels of the dopamine metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC)) (Acquas et al., 1992; Budygin et al., 1999; Huotari et al., 1999; Li et al., 1998; Raevskii et al., 2002). All of these studies used microdialysis, which takes measurements at the timescale of tonic transmission (at ~10 minute intervals). The few studies that used fast-scan cyclic voltammetry (FCV), which operates at the sub-second timescale of phasic transmission (Robinson et al., 2003), have failed to find an effect of COMT inhibition on striatal

dopamine, either on its own or when combined with DAT blockade (Budygin et al., 1999; Garris and Wightman, 1995).

Indications that COMT may contribute to the regulation of striatal dopamine are intriguing not just because they suggest that the low levels of COMT expressed in the striatum (compared to the cortex) (Matsumoto et al., 2003) may participate in dopamine clearance in this region but also because they could provide insights into a leading theory about the relationship between cortical and striatal dopamine. As I described in Chapter 1, this theory posits that dopamine levels in the medial frontal cortex (MFC) and phasic transmission in the striatum are inversely related: increased cortical dopamine is thought to increase tonic transmission in striatum via corticostriatal afferents, and this increased tonic tone in turn acts via autoreceptors to decrease phasic release (Grace, 1991). Because COMT is an important regulator of cortical dopamine levels (Tunbridge et al., 2006), it is further proposed that variability in COMT activity, whether genetic or pharmacologically induced, could impact striatal dopamine transmission through this pathway (Bilder et al., 2004). Although this theory is an attractive model, there is little direct evidence to support it. Information is particularly limited on what effect, if any, COMT-mediated degradation has on phasic dopamine transmission in the striatum.

4.1.2 Aim and approach of this experiment

In this study, we evoked dopamine release in anaesthetised mice by stimulating dopamine neurons in the ventral tegmental area (VTA) and used FCV to record the resulting phasic dopamine release in the NAc core. We then administered the DAT blocker GBR-12909, the COMT inhibitor tolcapone, or both drugs in combination in order to assess

the relative contributions of DAT recycling and COMT degradation to regulation of phasic dopamine transmission in the striatum.

In contrast to the two previous studies that have examined the effects of tolcapone inhibition on striatal dopamine using FCV, which targeted their recordings to the dorsal striatum (Budygin et al., 1999; Garris and Wightman, 1995), we targeted our recording electrodes to the NAc. This region – sometimes referred to as the ventral striatum, particularly in primates – is involved in numerous aspects of reward processing. The NAc is critical for the exertion of effort in the pursuit of reward (Salamone et al., 2007); mediates incentive salience, the process by which reward-related cues motivate behaviour to obtain reward (Berridge, 2006); exhibits reward prediction error signals, which are thought to underlie reward learning, in both dopamine release and fMRI BOLD signal (Day et al., 2007; Knutson and Cooper, 2005); and is implicated as a key site in the aberrant reward processing seen in addiction (Lüscher and Malenka, 2011). Furthermore, because NAc is closely linked to MFC within the mesocorticolimbic reward circuitry (Sesack and Grace, 2009; Sulzer et al., 2016), it is a likely site for potential downstream effects of COMT-mediated cortical dopamine transmission on subcortical regions. A better understanding of how DAT affects phasic dopamine signals in the NAc and to what extent COMT contributes to the regulation of these signals could therefore yield insights not only into the dynamics of dopamine transmission underlying reward processing but also into what roles DAT and COMT might play in psychiatric disorders like addiction and schizophrenia (Tunbridge et al., 2012).

Based on the previous work discussed above, we expected GBR-12909 to increase the size and slow the clearance of the dopamine transients we recorded. We anticipated

that, although tolcapone might not affect NAc dopamine signals on its own, it would potentiate GBR-12909's effects.

4.2 Methods

4.2.1 Subjects

Male C57BL/6 mice were obtained from Envigo (formerly Harlan). Mice were aged 10-16 weeks at the time of testing. Mice were housed on a 12/12-hr light/dark cycle (lights on at 0700hr) in groups of 2-6 mice per cage and were given food and water *ad libitum*. Care and testing of all animals was conducted under the auspices of the UK Home Office laws and guidelines for the treatment of animals under scientific procedures and of the local ethical review board at the University of Oxford.

A total of 43 mice were used for FCV recordings in the NAc. Data from 25 of these animals were included in the final analysis. We excluded 9 animals from the analysis due either to death prior to the completion of the experiment (n = 3) or to ineffective drug injection (n = 6). In addition, 9 animals were excluded because the quality of their recorded signals failed to meet the inclusion criteria described in Table 1.

Criterion type:	**Absolute peak timing**	**Consistency of peak timing**	**Noise level**
Included if:	Peak ≤ 3sec after stimulation	Peak timing varies by ≤ 2sec across bins	Ratio of standard deviation of pre-stimulation noise to peak height ≤ 0.5

Table 1: Criteria used to assess signal quality. Following chemometric analysis (see below), data were averaged across recordings into 30min bins and graphed, and the peak timing was then assessed in each animal individually. Noise levels were assessed in unbinned, raw data prior to chemometric analysis.

4.2.2 Drugs

Drugs were dissolved in 20% hydroxypropyl-beta-cyclodextrin (Acros Organics) in 0.9% saline (AquPharm). This served as a vehicle control in all experiments. Tolcapone (Ro 40-7592; 4'-methyl-3,4-dihydroxy-5-nitro-benzophenone) (TRC Inc), which inhibits the enzyme COMT, was used at a dose of 30mg/kg. GBR-12909 (1-[2-(bis[4-fluorophenyl]methoxy)ethyl]-4-[3-phenylpropyl]piperazine) dihydrochloride (Tocris), which blocks the transporter DAT, was used at a dose of 6mg/kg. The dihydrochloride was not taken into account when calculating the dosage, so GBR-12909 injections contained 6mg/kg of GBR-12909 dihydrochloride. See Chapter 2 for further information on tolcapone and GBR-12909 and on how these dosages were selected. Drugs and vehicle were delivered by intraperitoneal injection with an injection volume of 5mL/kg.

Drugs were administered as described in Table 2. The first injection (vehicle or tolcapone) was given 1.5 hours prior to the second injection (vehicle or GBR-12909) to account for differences in the drugs' time courses of action (Acquas et al., 1992; Männistö and Kaakkola, 1999; Nakachi et al., 1995; Raevskii et al., 2002).

Group	**Drug 1** **at Time 1**	**Drug 2** **at Time 2 (Time 1 + 1.5hrs)**
Veh-Veh	Vehicle	Vehicle
Tolc-Veh	Tolcapone	Vehicle
Veh-GBR	Vehicle	GBR-12909
Tolc-GBR	Tolcapone	GBR-12909

Table 2: Drug treatment groups.

4.2.3 Electrodes

Recording and reference electrodes were made in-house. Voltammetry recording electrodes consisted of a 7µm carbon fibre (Goodfellow) encased in a polyamide-coated

fused silica capillary (outer diameter = 90µm diameter, inner diameter = 20µm; Composite Metal Services Ltd) cut to a length of 1cm. One end of the capillary was sealed with epoxy glue (Devcon), and the exposed carbon fibre protruding from that end was trimmed to a length of 150-200µm. The other end was glued to a silver pin (Farnell) with silver epoxy (MG Chemicals) and insulated with clear epoxy. Ag/AgCl reference electrodes consisted of a silver wire (Sigma Aldrich) treated with sodium hypochlorite (Fisher Scientific), affixed to a gold pin (Farnell) with silver epoxy, and insulated with clear epoxy. The stimulating electrodes were untwisted, bipolar, stainless steel electrodes measuring 0.150mm in diameter (PlasticsOne).

4.2.4 Calibration

Recording electrodes were pre-calibrated in a flow cell (Sinkala et al., 2012) to allow conversion of recorded signals from current (nA) into concentration (nM). Electrodes were lowered into artificial cerebrospinal fluid (ACSF) (154.7mM Na^+, 0.82mM Mg^{2+}, 2.9mM K^+, 132.49mM Cl^-, and 1.1mM Ca^{2+} in deionized water, brought to pH ~7.4 with hydrochloric acid; Sigma Aldrich), which was allowed to flow continuously through the cell. Data was acquired as described under 'Data acquisition' below. Electrodes were cycled at 60Hz in the ACSF for 5-10 minutes before calibration testing. For the calibration, a recording was made during which the flow of pure ACSF was turned off for 30sec while a 1000nM solution of dopamine hydrochloride (Sigma Aldrich) in ACSF was allowed to flow through the cell.

Calibration factors were determined using the current at the 'inflection point' in the dopamine-induced signal, illustrated in the example recording below (Figure 1). Signals failed to plateau at a stable maximum during the 30sec recording period, so the inflection

point was used *in lieu* of a maximum. Up to this point, the signal likely reflects the concentration of dopamine flowing past the electrode, whereas after the inflection point the continued increase in signal size is likely due to adherence of dopamine to the electrode (Phillips et al., 2003). The inflection point was defined as the point at which the rate of increase of the signal reached a plateau and quantified using the first and second derivatives of the signal curve. When calibrations factors were unavailable (in 9 out of the 79 electrodes used in this study across both NAc and MFC recordings), an average calibration factor – calculated from those electrodes that were successfully calibrated – was used.

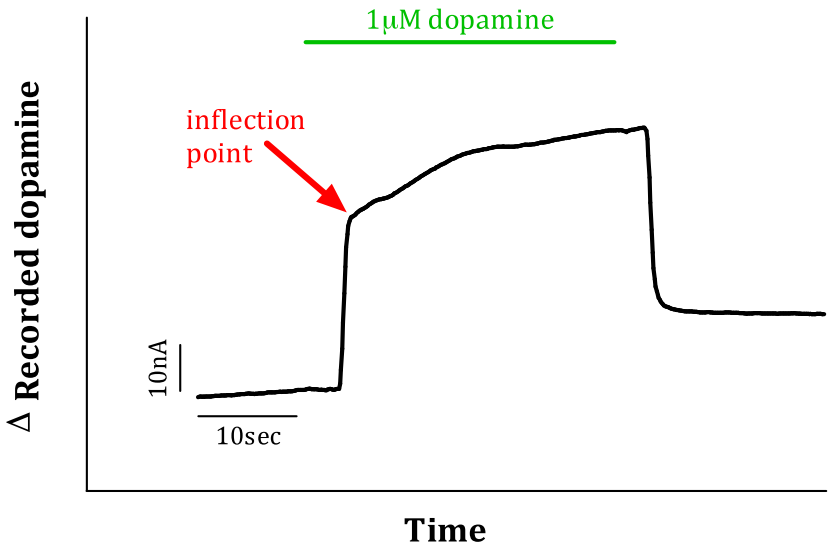


Figure 1: Example of a signal recorded during electrode calibration. Dopamine, at a concentration of 1000nM in ACSF, was allowed to flow past the electrode for a 30sec period, as indicated by the green bar. The red arrow shows the 'inflection point' at which the calibration factor for this electrode was measured.

4.2.5 Surgery

Mice were lightly anaesthetised with isoflurane (3% in oxygen; Abbott Laboratories Ltd) and then received an intraperitoneal injection of urethane (25% weight/volume

solution; Sigma Aldrich) at a dose of 1.5-1.8g/kg. Anaesthesia was maintained by a combination of the initial urethane dose and isoflurane (0.5-1.5% in oxygen, flowing at a rate of 0.5-2.5L/min, delivered via a facemask) during the initial portion of the surgery. The isoflurane was turned off before FCV recordings began, and urethane top-ups of 10-20% of the initial dose were given as necessary to maintain anaesthesia during the remainder of the experiment. Glycopyrronium bromide (MercuryPharm Ltd) was administered by intraperitoneal injection at a dose of 0.01-0.02mg/kg at the same time as the initial urethane injection to minimize the adverse effects of bronchial secretions. Glucosesaline (0.5% in 0.9% saline; Aquapharm) was administered at doses of approximately 0.7mL every 3 hours to maintain hydration, and the animals' temperature was maintained at 35.0-37.0°C with a rectal probe and heating blanket.

Once anaesthetised, mice were placed in a stereotaxic frame (Kopf Instruments). The local anaesthetic bupivacaine (AstraZeneca) (2mg/kg) was administered under the scalp and craniotomy was performed, with holes drilled for recording, stimulating, and reference electrodes as well as for an anchoring screw (Precision Technology Supplies). The screw and reference electrode were fixed in place using dental cement (Associated Dental Products Ltd). Recording and stimulating electrodes – aimed at the NAc core and VTA, respectively – were always placed in the left hemisphere, while the reference electrode and screw were placed in the right hemisphere. Stereotaxic coordinates used for the placement of the electrodes and screw are listed in Table 3.

	Anterior-posterior (mm)	Medial-lateral (mm)	Dorsal-ventral (mm)
NAc core	+1.40	-0.75	-3.50 to -4.75 from skull
VTA	-3.50	-0.35	-4.00 to -4.80 from brain
Reference	+4.80	+1.00	N.A.
Screw	+2.80	+2.00	N.A.

Table 3: Coordinates of regions targeted for placement of recording (NAc core), stimulating (VTA), and reference electrodes and of the stabilizing screw. All measurements were made relative to bregma.

We targeted stimulating electrodes to the posterior-medial portion of the VTA, where the cell bodies of dopamine neurons projecting to both NAc and MFC are located (Lammel et al., 2008, 2011, 2014). NAc- and MFC-projecting populations are largely separate, as few dopamine neurons send collaterals to multiple target areas (Swanson, 1982). However, the spatial overlap of these two populations within the VTA allowed us to use the same stimulating electrode coordinates to evoke dopamine release in both the NAc experiments presented here and in the MFC experiments presented in Chapter 5.

After FCV recordings were completed, the animal was overdosed with pentobarbital (Merial Animal Health Ltd), and the recording electrode was either removed for post-calibration or left in the brain so that its location could be marked by electrolytic lesion. A lesion-making device (designed and built by Scott Ng-Evans, University of Washington, Seattle, U.S.A.) was connected to the recording electrode and to a metal rectal probe to complete the circuit, and a 300V stimulus was passed across the electrode for 30sec. The animal was then transcardially perfused with either 10% formalin (VWR Chemicals BDH Prolab) in saline or 4% paraformaldehyde (TAAB Laboratories Equipment Ltd) in 0.1M phosphate buffer, and the brain was removed for histological analysis.

4.2.6 Histology

Approximately 24hrs before sectioning, fixed brains were transferred to a 30% weight/volume solution of sucrose (Fisher Scientific) in either 10% formalin-saline or paraformaldehyde. Brains were sectioned on a freezing microtome (Leitz Wetzlar) or a cryostat (Leica) into 40μm sections, which were then mounted onto 1.5% gelatin-coated slides (Raymond A Lamb Ltd). Most sections were stained with cresyl violet (Sigma Aldrich) and mounted in DPX mounting medium (VWR International) enclosed with coverslips.

A subset of sections containing the midbrain underwent immunostaining for tyrosine hydroxylase (TH) to confirm the proximity of the stimulating electrode to the dopaminergic cell bodies in the VTA. Sections were washed for 3 x 10min in phosphate buffered saline (PBS), immersed in blocking serum (1% foetal calf serum, 5% normal rabbit serum, and 0.3% Triton in PBS) for 1.5hrs, and incubated in a 1:2000 dilution of the primary antibody (chicken anti-TH polyclonal antibody; Abcam) in blocking serum for 1hr at room temperature and then overnight at 4°C. The sections were then washed (3 x 10min in PBS), quenched for 30min in 0.3% hydrogen peroxide in 20% methanol-PBS, washed again (3 x 10min in PBS), and then incubated in a 1:700 dilution of the secondary antibody (rabbit anti-chicken hydrogen peroxidase-coupled antibody; Sigma Aldrich) in blocking serum for 1.5hrs at room temperature. Finally, sections were washed (3 x 10min in PBS), immersed in a DAB (diaminobenzidine; Sigma Aldrich) solution (0.5mg/mL DAB, 2mg/mL ammonium nickel sulphate, and 0.01% hydrogen peroxide in imidazole) for 4-6min, and washed in PBS to stop the oxidation reaction before being mounted onto slides, dried, dehydrated in alcohol, and prepared with coverslips.

4.2.7 Data acquisition

An in-depth discussion of FCV as a technique and of the approach we have taken in the collection and analysis of FCV data can be found in Chapter 3. Here, I report the specific methods used for FCV data collection and analysis in this study.

Dopamine release was induced by passing current through the stimulating electrode via a DS3 Stimulator (Digitimer) (stimulation parameters: pulse number = 60 pulses, frequency = 50Hz, amplitude = 300μA, pulse width = 2ms, pulse phase = biphasic). Stimulation parameters were selected based on previous literature (Yavich et al., 2007; Yorgason et al., 2011a) and on pilot experiments in which different levels of stimulation were tested until a set of parameters was found that reliably evoked dopamine release in both the NAc (for the experiments presented in this chapter) and the MFC (for the experiments presented in Chapter 5). Dopamine release was recorded by applying a triangular waveform (voltage step from –0.4V to +1.3V and back to –0.4V [vs. Ag/AgCl], sweep rate = 400 V/sec, scan rate frequency = 10Hz) to the recording electrode. This causes transient oxidation of electroactive molecules surrounding the tip of the electrode, producing a current that serves as a measure of the levels of these chemicals in the tissue. The component of this signal that is attributable to dopamine can be identified by its distinctive oxidation-reduction profile, as different chemicals oxidise and reduce at different points along the triangular voltage step. Cyclic voltammograms, which plot the current as a function of the applied potential of the voltage step, were used to assess oxidation-reduction profiles and to identify dopamine. Stimuli were generated and recordings collected using Tarheel CV (National Instruments).

Recording electrodes were initially lowered to 0.1mm above the target region and were cycled by applying the waveform at 60Hz for 20min before any recordings were made. The carbon surface of FCV electrodes undergoes changes when it is implanted in tissue, and cycling at a high frequency helps speed the equilibration of the carbon surface so that recordings can then be made at a well-conditioned electrode (Owesson-White et al., 2012). After cycling, stimulation of the VTA was triggered, and the resulting dopamine release in NAc was recorded. The stimulating and recording electrodes were lowered by increments of 0.05-0.1mm (stimulation applied every 2-3min) to locate a site of reliable dopamine release.

4.2.8 Experiment structure

We observed a rundown of the signal size over the course of the recording session that we hypothesise is due to a combination of continued changes at the electrode surface and adaptation of the neural circuit to repeated stimulation. In NAc recordings, signal size was particularly variable during the first few hours of the session and often decreased by >50% during this time (Figure 2). We therefore allowed the signal to stabilise for approximately 2.5hrs – stimulating every 5-10min during this period – before acquiring pre-drug baseline recordings and then administering experimental drugs.

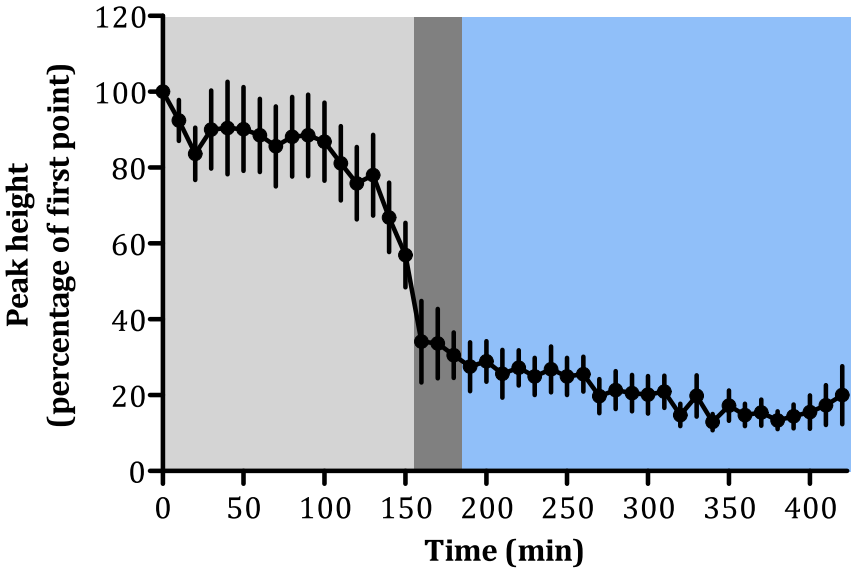


Figure 2: Rundown of signal size across the session in NAc recordings. Peak height at each time point is expressed as a percentage of the peak height of the recording made at time 0 and then averaged across animals. The light grey box frames the stabilisation period (n=13), the dark grey box frames the pre-drug baseline recordings (n=5), and the blue box frames recordings made following drug administration in animals that received only vehicle injections (n=5). Refer to Figure 3 for further details on the structure of the experiment. Note that here peak height is calculated from raw data that has not undergone chemometric analysis.

Following stabilisation, recordings were made every 5min. We recorded pre-drug baseline signals for 30min before injecting drug 1 (tolcapone or vehicle), then recorded for a further 90min before injecting drug 2 (GBR-12909 or vehicle) and continued to record for several hours following the second injection (Figure 3). Data were averaged into 15min bins, with each bin covering 3 stimulations. The analysis focused on two time points. The first, labelled 'Effect of Drug 1' in Figure 3, is an average of 3 recordings made approximately 85min after administration of drug 1 (i.e. the last bin before the second injection), by which point any effects of tolcapone should emerge (Acquas et al., 1992; Huotari et al., 1999; Li et al., 1998). The second time point, labelled 'Effect of Drug 1 + Drug 2' in Figure 3, is an average of 3 recordings made approximately 25min after

administration of drug 2, when GBR-12909's effects should begin to become apparent (Budygin et al., 1999; Huotari et al., 1999, 2002a; Nakachi et al., 1995; Raevskii et al., 2002).

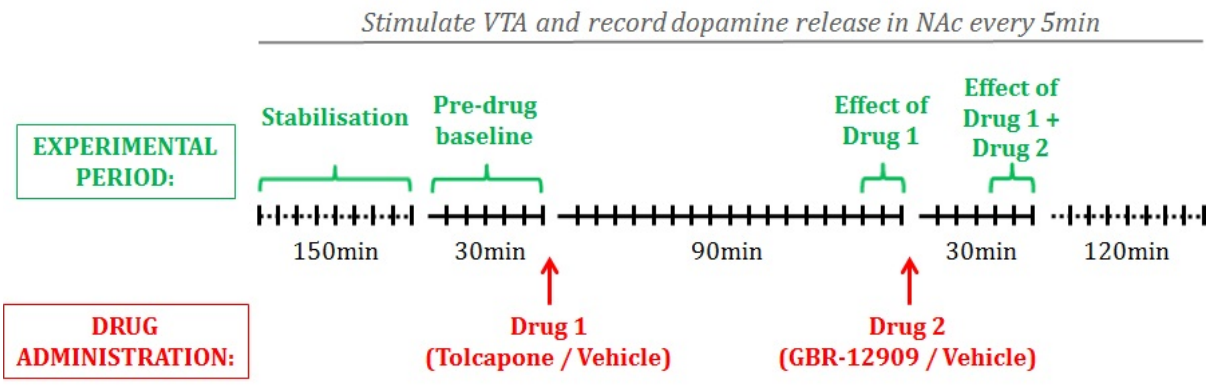


Figure 3: Schematic of experiment structure. Drug injections are indicated in red and time points of interest in green.

4.2.9 Analysis

FCV recordings were analysed using software written in LabVIEW (one version customised by Scott Ng-Evans and one version customised by Richard Keithley), Matlab R2012a, and Microsoft Excel 2010. The data were plotted using GraphPad Prism version 5.04. Statistical analyses were performed in SPSS version 20 with significance set at $\alpha = 0.05$. For repeated-measures ANOVAs, Greenhouse-Geisser corrections were applied where data failed Mauchley's test of sphericity; degrees of freedom are reported to two decimal places in such cases. Simple main effects analyses were conducted as necessary when significant interactions were found. Least square difference pairwise comparison tests were used to assess which groups were driving any significant main or interactive effects.

The first step of the analysis procedure consisted of building training sets for each animal to allow extraction of the dopaminergic component of the signal using a

chemometric approach; see Chapter 3 for a detailed description of this process. Signals were low-pass filtered at 2kHz and background subtracted: for each recording, the average data in a 0.5sec window prior to stimulation were subtracted from the 5sec preceding and the 20sec following the stimulation to remove changes in the signal attributable to the electrode's capacitance current. Following chemometric analysis, the recorded current attributable to dopamine was extracted at the time of the peak signal size, smoothed using a 5-point moving average, and then re-baselined to 0.1sec before the start of stimulation. Parameters were then extracted to quantify drug effects on recorded signals. These included:

(1) **peak height**, defined as the difference between the maximum signal size within 3sec of stimulation and the signal size at the time of stimulation

(2) **area under the curve (AUC)**, which was summed during the 5sec following stimulation

(3) **decay constant**, obtained by fitting a first order exponential curve to the signal over the 3sec following the signal's peak

Peak height and AUC were normalised to the averaged pre-drug baseline signal, binned, and then averaged across subjects within each drug group. Decay constants were calculated from un-normalised data: signals were first binned at the three time points of interest shown in Figure 3 (6 recordings binned to obtain the pre-drug baseline signal; 3 recordings binned to assess the effect of drug 1; and 3 recordings binned to assess the combined effects of drug 1 and drug 2), then the exponential fit was performed on the binned data, and finally decay constants were averaged across animals within each drug

group. Statistical comparisons were performed at the selected time points described in section 4.2.8 above.

4.3 Results

4.3.1 Characterisation of evoked dopamine release in NAc

We were able to reliably record evoked dopamine release in the NAc core. The pre-drug signal size was highly variable across animals (Figure 4). At the start of pre-drug baseline recordings made during the 30min preceding the first injection, signal peak height was between 0.36 and 53.64 nA (mean ± standard deviation: 11.05 ± 11.94). This corresponded to a dopamine concentration ranging from 9.13 to 4611.40 nM (mean ± standard deviation: 729.92 ± 1165.78), depending on each electrode's sensitivity.

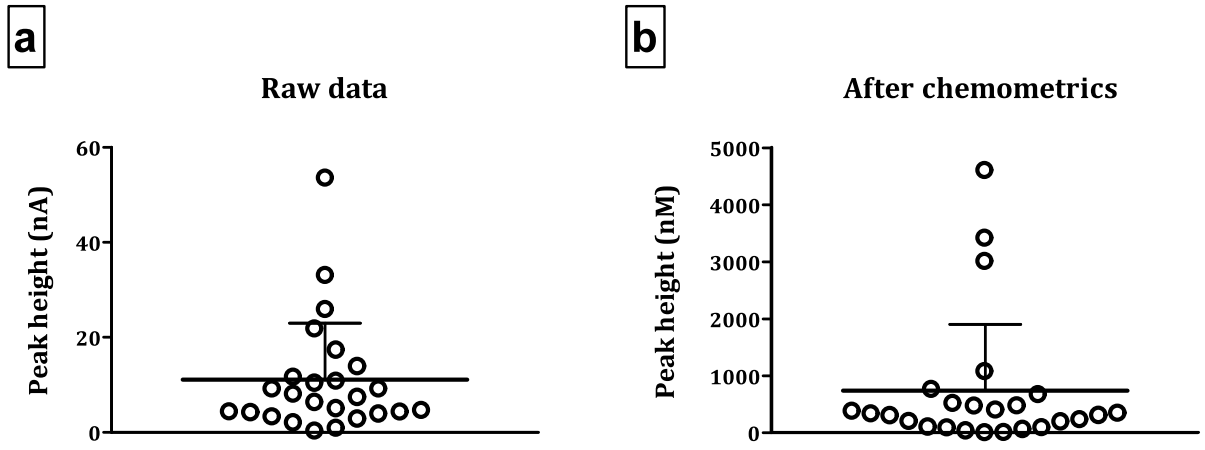


Figure 4: The range of signal sizes observed in the NAc at the start of the pre-drug baseline recording period. a) Peak height of raw signals, expressed in units of current. b) Peak height of signals after chemometric analysis, expressed in units of concentration. Graphs show data points from individual animals, with mean + standard deviation overlaid.

The kinetics of NAc signals were somewhat variable: at the start of pre-drug baseline recordings, signals peaked between 1.0 and 2.6 sec (mean ± standard deviation:

1.70 ± 0.45) after the time of stimulation. (Signals that peaked later than 3sec after stimulation were excluded from analysis, as described in Table 1 and explained further in section 4.4.1 below). Once the peak was reached, NAc signals gradually declined back towards baseline, although not all made a complete return to baseline by the end of the recording (Figure 5b). Cyclic voltammograms confirmed the identity of the released chemical in NAc signals as dopamine (Figure 5a).

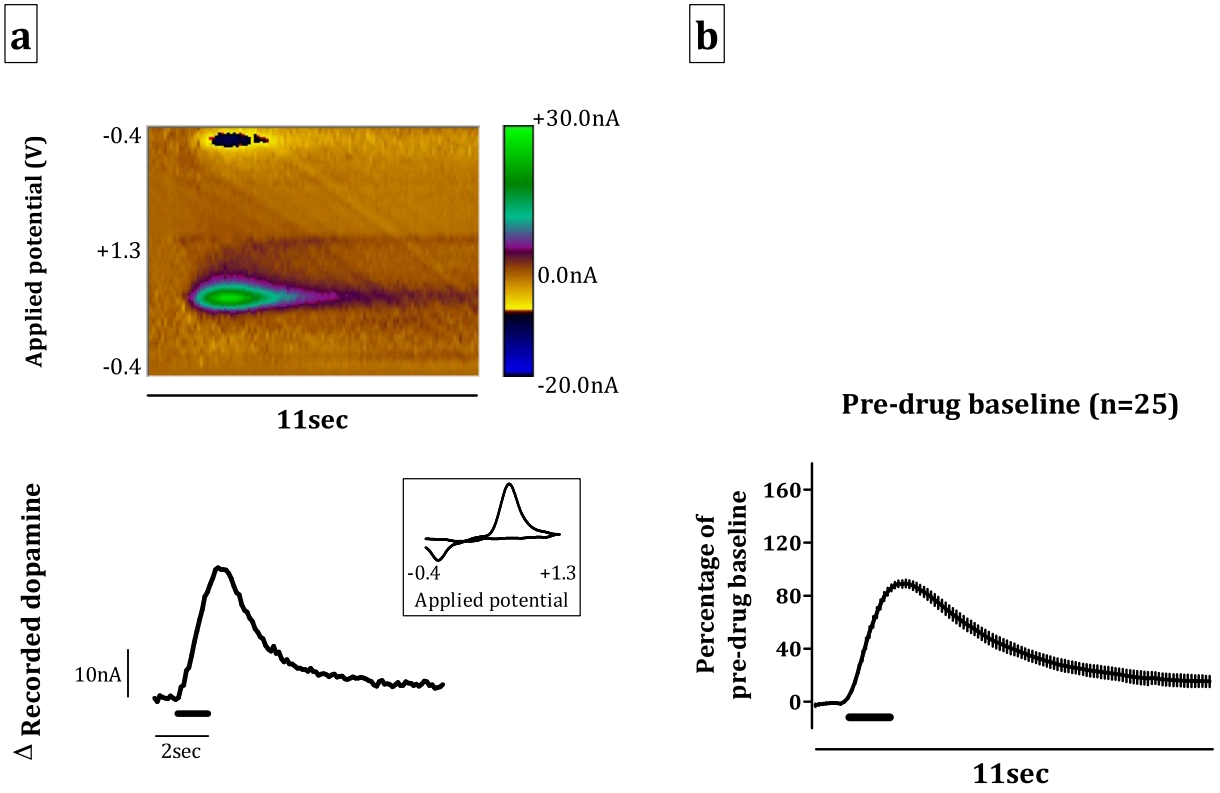


Figure 5: Recording evoked dopamine release in the NAc. a) An example recording made in the NAc. The pseudocolour plot shows the recorded current as a function of the applied potential of the triangular waveform (y axis) over an 11sec period (x axis). The trace below the pseudocolour plot shows the extracted current attributable to dopamine release as a function of time; timing and duration of stimulation indicated by thick black bar. The cyclic voltammogram (current versus applied voltage; boxed inset) during the peak of the signal, 1.5sec after the time of stimulation, is consistent with dopamine release. b) The average of 6 pre-drug baseline recordings (6 stimulations over 30min in each mouse), normalized to the average pre-drug baseline peak height (equivalent to 100% on the y axis) and presented as mean +/- SEM across all animals. Timing and duration of stimulation is indicated by the thick black bar.

4.3.2 Effects of COMT inhibition and DAT blockade on evoked dopamine in NAc

The effects of the COMT inhibitor tolcapone and the DAT blocker GBR-12909 on NAc dopamine release are shown in Figure 6 and quantified in Figures 7 and 8. We assessed drug effects by extracting and running statistical analyses on several signal parameters, including the **peak height**, the **AUC** in a 5sec window after the time of stimulation, and the **decay constant** of an exponential decay function fit to the signal over the 3sec following the peak. Peak height and AUC data are presented as a percentage of the pre-drug baseline signal; in these figures, therefore, 100% represents the size of the averaged pre-drug baseline recordings. The decay constant data is not normalised.

No discernible differences in NAc dopamine release were observed between mice that received tolcapone and those that received vehicle as drug 1 (Figure 6a). In both groups, signal size decreased to approximately 60% of pre-drug baseline levels by 85min after the first injection. We found no significant main effect of drug 1 in repeated-measures ANOVA analyses of either the peak height or the AUC (F's < 0.06, p's > 0.82; Figure 7).

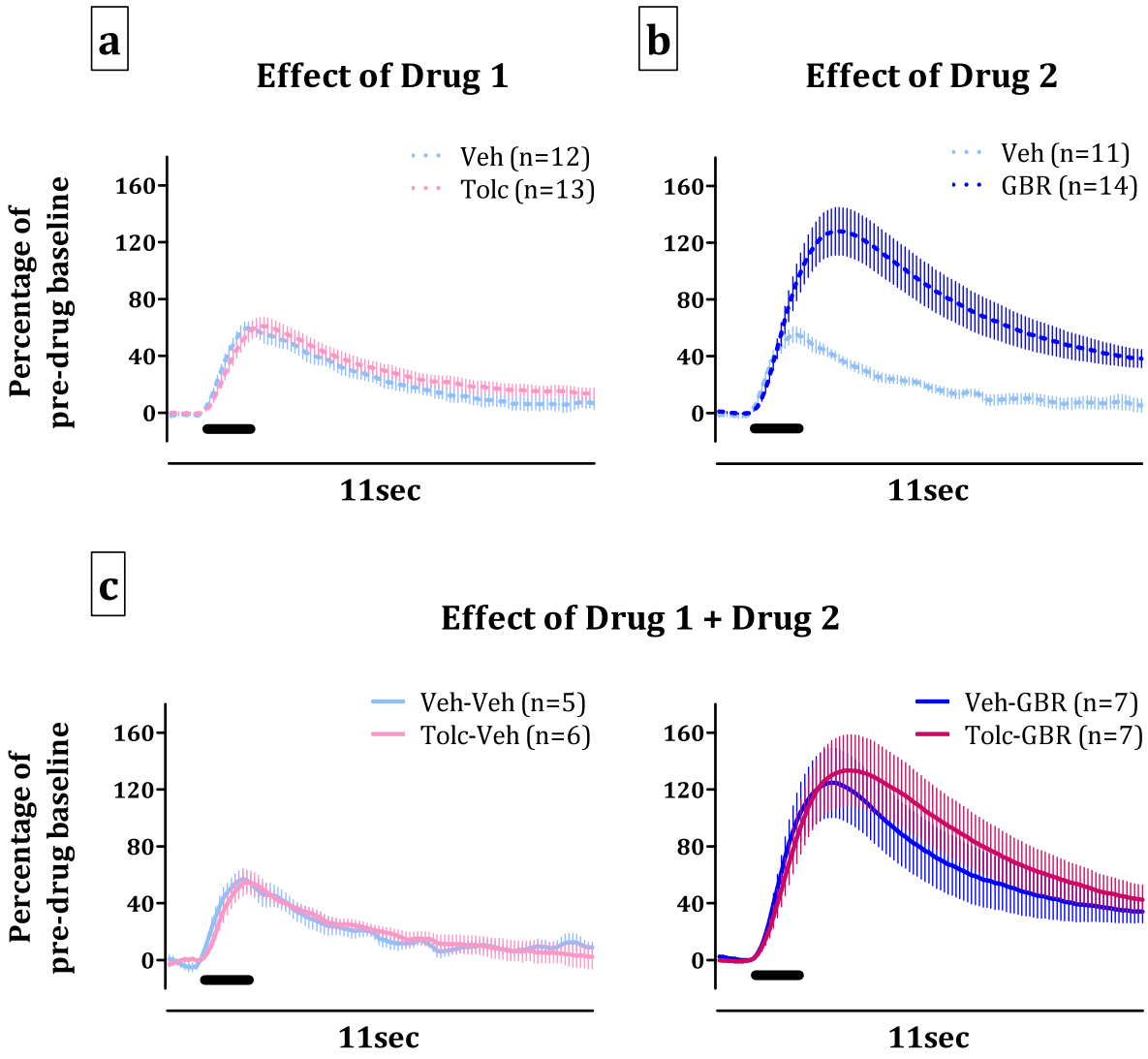


Figure 6: Evoked dopamine release in the NAc. The signals are normalized to the average pre-drug baseline peak height (equivalent to 100% on the y axis) and are presented as mean +/- SEM across animals within each drug group. Timing and duration of stimulation is indicated by the thick black bars. a) Release in animals given vehicle as drug 1 (light blue) compared to release in animals given tolcapone as drug 1 (pink) 85min after the first injection. b) Release in animals given GBR-12909 as drug 2 (dark blue) compared to release in those given vehicle as drug 2 (light blue) 25min after the second injection. c) Release 25min after the second injection, as in (b), but separated according to the four possible combinations of drug 1 and drug 2: vehicle plus vehicle (light blue, left), tolcapone plus vehicle (light pink, left), vehicle plus GBR-12909 (dark blue, right), and tolcapone plus GBR-12909 (dark pink, right).

In contrast to the lack of effect seen with tolcapone, GBR-12909 dramatically increased evoked dopamine release in NAc. By 25min after the second injection, signals in

animals that had received GBR-12909 were clearly larger than signals in those that had received vehicle as drug 2 (Figure 6b). Indeed, repeated-measures ANOVAs found a significant main effect of drug 2 on both peak height ($F_{1,21} = 10.4$, $p = 0.004$) (Figure 7a) and AUC ($F_{1,21} = 10.8$, $p = 0.004$) (Figure 7b). There was a significant main effect of time on both parameters (peak height: $F_{1,21} = 11.2$, $p = 0.003$; AUC: $F_{1,21} = 12.8$, $p = 0.002$) as well as a significant interaction between drug 2 and time (peak height: $F_{1,21} = 18.3$, $p < 0.001$; AUC: $F_{1,21} = 13.9$, $p = 0.001$). Least square difference posthoc tests confirmed that there was a significant difference in signal size between the first and second time points in animals that received GBR-12909 but not in those that received vehicle as drug 2 (peak height: $p < 0.001$; AUC: $p < 0.001$).

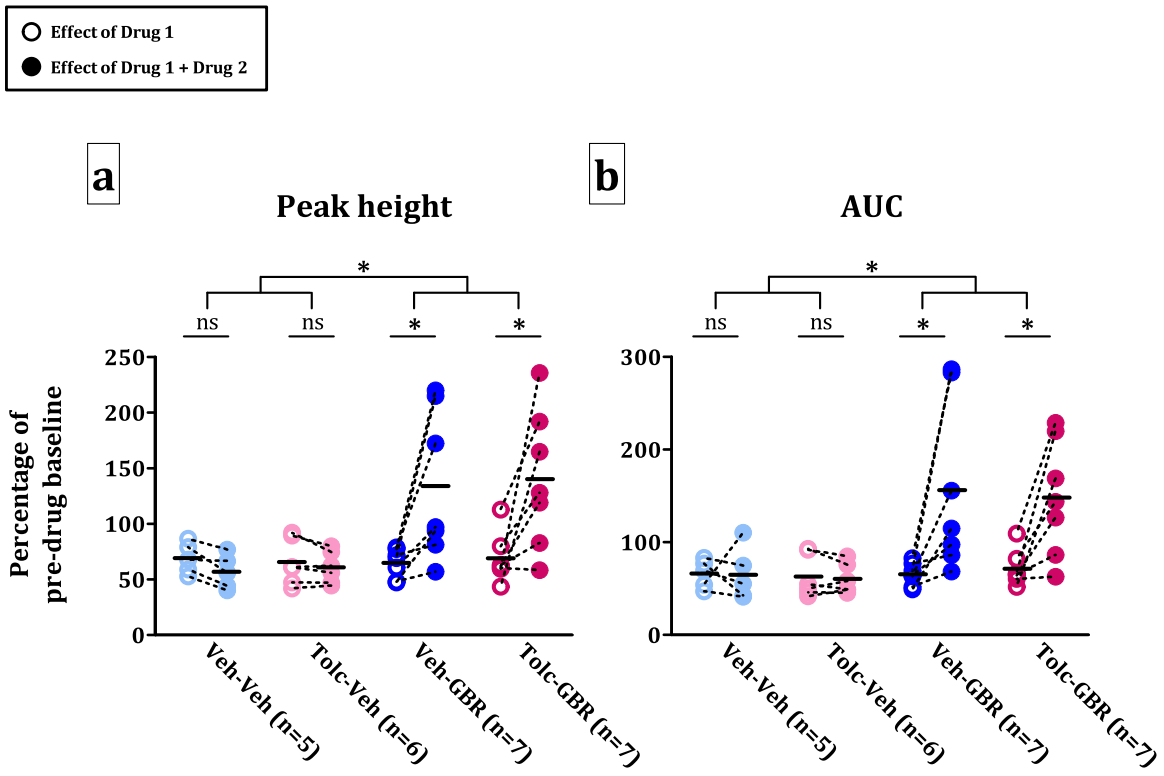


Figure 7: Quantification of a) the peak height and b) the area under the curve (AUC) of evoked dopamine release in NAc, normalised to the average pre-drug baseline peak height or AUC (equivalent to 100% on the y axis), respectively. Each animal's data is shown individually at two time points: open symbols show the effect of drug 1 on its own, while filled symbols show the combined effects of drug 1 and drug 2. Horizontal black bars indicate the means for each drug group at each time point. ns = not significant; * = $p < 0.05$

When the data at the later time point (25min after the second injection) are separated according to the four possible combinations of drug 1 and drug 2, tolcapone appears to potentiate the effect of GBR-12909 on signal size when the two drugs are given together (Figure 6c). This apparent potentiation does not emerge as a statistically significant effect, however, as there were no significant interactions involving drug 1 in either the peak height or AUC analyses ($F's < 0.2$, $p's > 0.74$).

It is possible that an evaluation of signal decay kinetics might reveal effects of GBR-12909 or tolcapone not captured by the AUC measurement, which reflects not only the

kinetics but also the peak height of the signal and therefore may not give a clear picture of the kinetics. We therefore assessed the decay from the peak of the signal using exponential curve fitting (Figure 8). In this analysis, a lower decay constant reflects a shallower slope, which indicates a slower rate of dopamine clearance. Note that because data were not normalised to pre-drug baseline signals for this analysis, the pre-drug baseline data were included in the statistical comparison, so the repeated-measures ANOVA was performed on data from three time points rather than the two used in the peak height and AUC analyses. Assessment of the signal decay did not reveal an influence of tolcapone, as there was no main effect of drug 1 on the decay constant (repeated-measures ANOVA: $F_{1,21} = 0.4$, $p = 0.513$). There were no significant interactions involving drug 1 ($F's < 0.9$, $p's > 0.40$).

Inspection of the data suggests that GBR-12909 slows the rate of decay, as the decay constant appears to consistently decrease following administration of GBR-12909 but to vary more randomly in animals that did not receive GBR-12909. A repeated-measures ANOVA revealed that the main effects of both drug 2 and time on the decay constant approached significance (drug 2: $F_{1,21} = 4.1$, $p = 0.055$; time: $F_{1.52,31.86} = 3.5$, $p = 0.054$). However, there was no significant interaction between drug 2 and time ($F_{1.52,31.86} = 0.8$, $p = 0.431$). This lack of interaction was unexpected, as blockade of DAT recycling by GBR-12909 should slow the rate of dopamine clearance, which should result in a lower decay constant in the groups that received GBR-12909 at the final time point. We therefore performed planned least square difference posthoc tests and found that the decay constant in GBR-12909-treated animals was significantly lower at the third time point compared to the first time point ($p = 0.009$) but not compared to the second time point ($p = 0.192$); there was no significant difference between the first and second time points ($p = 0.746$).

By contrast, posthoc tests found no significant differences in decay constant between any of the time points in animals that received vehicle as drug 2 (p's > 0.09). Although these results must be interpreted with caution, they do suggest a subtle effect of GBR-12909 on signal decay kinetics.

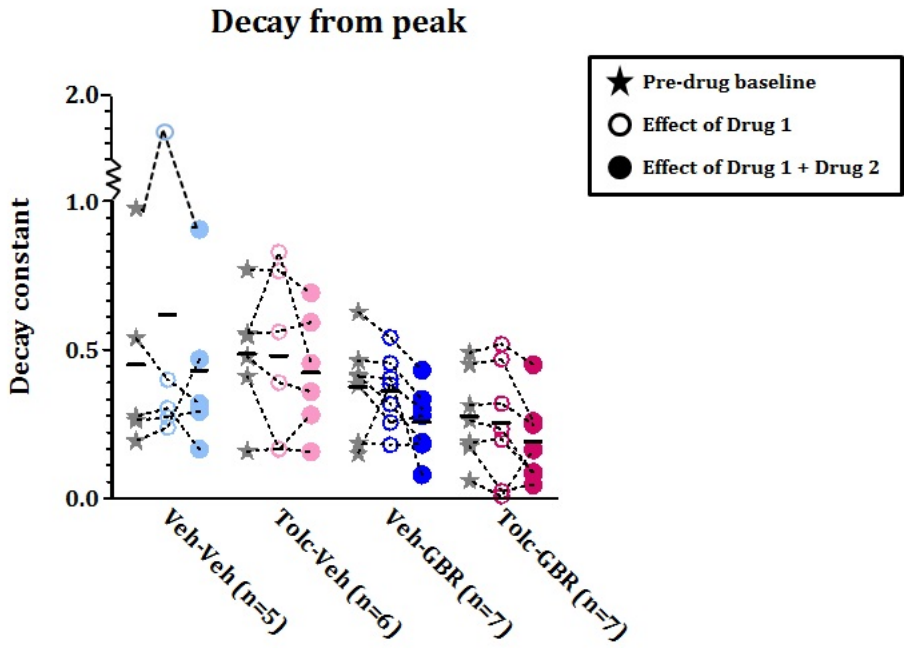


Figure 8: The kinetics of the decay from the peak of evoked dopamine release in NAc. Decay is quantified by the exponential decay constant, the value of which is presented on the y axis; a larger decay constant value indicates a faster rate of decay. Each animal's data is shown individually at three time points: stars show the rate of decay at baseline, 15min before the first drug injection; open circles show the effect of drug 1 on its own, 85min after the first injection; and filled circles show the combined effects of drug 1 and drug 2, 25min after the second injection. Horizontal black bars indicate the means for each drug group at each time point.

4.3.3 Histology

Brains in which the recording site was lesioned underwent histological processing. This revealed that recording electrodes were successfully targeted to the NAc core. The majority of lesion sites were found in the dorsomedial part of the NAc core, although some lesions straddled the core-shell border (Figure 9a and c). Only one lesion was clearly in the

medial shell (data not shown); however, this animal's data were excluded from analysis based on the peak timing criterion described in Table 1. No other data sets were excluded based on the placement of the recording electrode lesion. In the same set of brains in which the recording site was lesioned, we observed the stimulating electrode tract descending to the midbrain. The placement of the stimulating electrode varied somewhat in the medial-lateral plane: although many of the tracts terminated in the target region of the medial VTA, some ended at the border of the lateral VTA and substantia nigra (SN) (Figure 9b and c). Staining for tyrosine hydroxylase (the enzyme that synthesises dopamine) in a subset of brains revealed consistent placement of stimulating electrodes in the vicinity of midbrain dopaminergic nuclei (Figure 10).

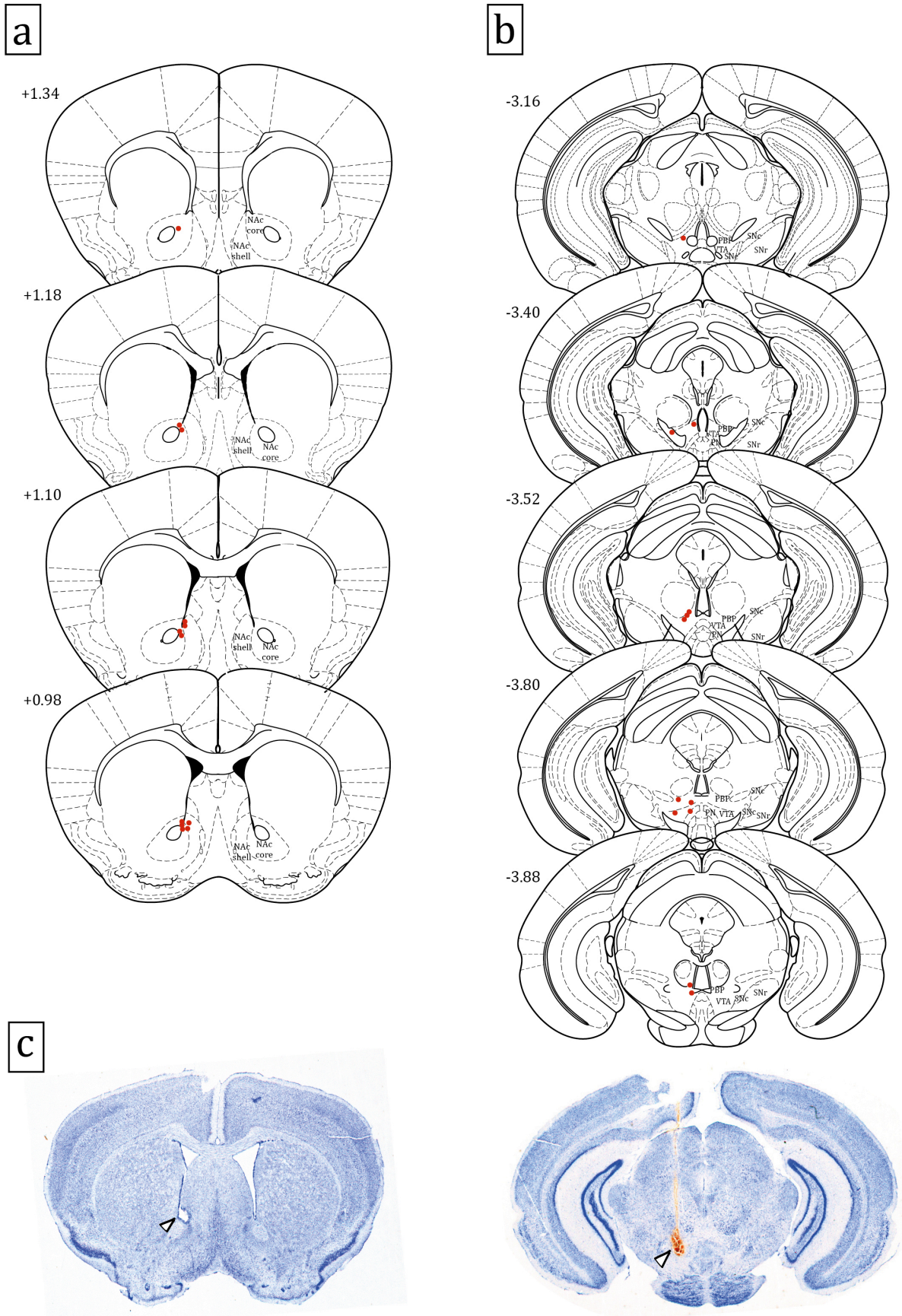

a
+1.34
NAc core
NAc shell
+1.18
NAc shell
NAc core
+1.10
NAc shell
NAc core
+0.98
NAc shell
NAc core
b
-3.16
PBP
SNc
VTA
SNr
-3.40
PBP
SNc
VTA
PN
SNr
-3.52
PBP
SNc
VTA
PN
SNr
-3.80
PBP
SNc
PN
VTA
SNr
-3.88
PBP
SNc
VTA
SNr
c

Figure 9: Electrode placements. a) Locations of electrolytic lesions made at recording electrode sites. b) Locations of the ends of stimulating electrode tracts. c) Examples of a lesion in the NAc (left) and an electrode tract terminating in the VTA (right), indicated by white arrowheads. Numbers to the left of coronal sections in (a) and (b) indicate distance from bregma (mm). NAc: nucleus accumbens; PBP: parabrachial pigmented nucleus; PN: paranigral nucleus; SNc: substantia nigra pars compacta; SNr: substantia nigra pars reticulata; VTA: ventral tegmental area.

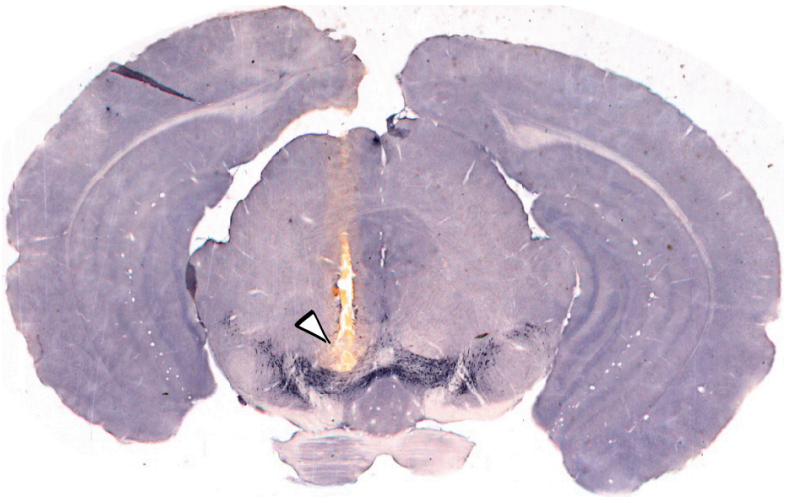


Figure 10: An example of a histological section stained for tyrosine hydroxylase, demonstrating the proximity of the stimulating electrode tract (white arrowhead) to dopaminergic cell bodies (black) in the midbrain.

4.4 Discussion

We observed clear effects of the DAT blocker GBR-12909 on evoked dopamine transients in NAc, which markedly increased the signal size compared to baseline. However, the COMT inhibitor tolcapone did not affect NAc signals, either on its own or in combination with GBR-12909. Our results therefore confirm that DAT recycling is the predominant clearance mechanism in NAc and suggest that, using the stimulation parameters we chose, COMT degradation makes little or no contribution to regulation of dopamine transmission in this region, at least at the sub-second timescale assessed by FCV.

4.4.1 Recording striatal dopamine with FCV

The NAc is an ideal region in which to record dopamine transmission using FCV, as it is densely innervated by dopamine fibres and contains only low levels of noradrenaline, which has an identical cyclic voltammogram to dopamine and can therefore complicate identification of the neurochemical being measured (Robinson et al., 2003; Wickens et al., 2007). We were indeed able to reliably record evoked dopamine release in NAc. Baseline transients exhibited a large range of signal sizes. This variability is likely due to a combination of factors, including the placement of the stimulating electrode, the placement of the recording electrode, and the relationship between the two. It also suggests that the distribution of dopamine terminals in NAc may be uneven (Wightman et al., 2007), with some areas receiving dense input and others only sparsely innervated.

The histological analyses show that recording electrodes were consistently targeted to the dorsomedial portion of the NAc core (Figure 9a and c). In some cases, electrode placement was on the border of the NAc core and medial shell. However, because electrode placement is difficult to precisely determine, due to the size of the electrolytic lesion compared to that of the electrode tip, we did not exclude animals from analysis based on histology. Notably, the one animal in which the recording electrode lesion was clearly within the shell rather than the core had already been excluded from analysis because its signals peaked beyond our criterion window (see Table 1), which aligns with previous findings showing that the kinetics of dopamine transmission in NAc shell are slower than they are in the core owing to lower density of DAT in the shell compared to the core (Nirenberg et al., 1997; Wickens et al., 2007).

Stimulating electrodes were consistently targeted to the midbrain (Figure 9b and c), in close proximity to dopaminergic cell bodies (Figure 10), although their exact placement varied in both the medial-lateral and dorsal-ventral plane. However, given the size of the stimulating electrodes, it is likely that stimulation reached VTA dopamine neurons despite this variability, and we indeed observed evoked dopamine release in all animals used in this study.

It should be noted that the stimulation parameters we used to evoke dopamine release are supraphysiological – whereas natural burst firing in dopamine neurons typically occurs at a frequency of about 20Hz, we used a 50Hz stimulus in order to reliably evoke discernible dopamine signals. Higher intensity stimulation may be necessary to produce clear signals under anaesthesia, which can reduce the size of electrically evoked dopamine transients (Avelar et al., 2013). This may be particularly true when recording dopamine release in the MFC, as dopamine levels are substantially lower in cortex than in striatum (Moghaddam et al., 1990). In order to maintain consistency across the NAc and MFC recordings reported here and in Chapter 5, respectively, we used the same stimulation parameters in both experiments. However, although they are supraphysiological, our stimulation parameters are similar to those previously shown to induce reproducible dopamine release and to be behaviourally reinforcing and are substantially less intense than parameters known to deplete dopamine stores (Avelar et al., 2013; Robinson et al., 2003).

4.4.2 Effects of DAT blockade on NAc dopamine

GBR-12909 dramatically increased the size of evoked dopamine transients in NAc by 25min after its injection (Figure 6b), reflected as increases in both peak height and AUC

following GBR-12909 administration (Figure 7a and b). Inspection of individual animal data in Figure 7 reveals that, although GBR-12909 consistently induced increases across subjects, the proportional magnitude of the effect varied considerably. This may be due to the route of administration of the drug: it was delivered intraperitoneally, which can lead to variability in the efficacy of a drug's actions and the timing of its effects. In addition, the dose of GBR-12909 that we used was lower than those typically used in previous studies (Budygin et al., 1999; Heikkila and Manzino, 1984; Huotari et al., 2002a; Nakachi et al., 1995; Raevskii et al., 2002; Salahpour et al., 2008). We selected this dose following preliminary experiments that demonstrated that it was behaviourally relevant but did not produce stereotypy (see Chapter 2) and found that it produced clear group-level effects on both dopamine transmission, as seen in this chapter, and behaviour, as seen in Chapters 6 and 7. Nevertheless, the dose we used may exhibit variability in its effects when examined on an animal-by-animal basis.

Assessment of the kinetics of signal decay suggested that GBR-12909 also slowed the decay from the peak, as a DAT blocker would be expected to do (Figure 8). Inspection of the data reveals that decay constants were smaller following GBR-12909 than they were before its administration in almost all cases, indicating that signals had broader peaks and decreased more slowly after GBR-12909. The initial statistical analysis indicated that this effect was not significant, as there was no interactive effect of drug 2 and time. However, because blockade of the recycling mechanism should slow the rate of dopamine clearance in the striatum, and because GBR-12909 has previously been shown to affect the decay kinetics of striatal dopamine transients measured with FCV (Budygin et al., 1999), we performed planned posthoc comparisons in order to ensure that our method of assessing

decay kinetics was sensitive to the effects of this drug. Posthoc testing showed that decay constants were lower following GBR-12909 administration than they were at baseline, whereas decay constants were not altered by vehicle treatment, suggesting that DAT blockade did subtly slow the rate of dopamine clearance.

Several factors may have prevented this effect from emerging robustly in the statistical analysis, including the observed variability in the starting value of decay constants, the presence of an outlier in the vehicle-vehicle treatment group, and two animals in the tolcapone-GBR-12909 treatment group that had very small decay constants before GBR-12909 was administered and therefore may exhibit a floor effect. A larger sample size in each drug group might therefore bring out the effect of GBR-12909 on the kinetics of signal decay more clearly. Alternatively, a different curve-fitting approach may be needed to better capture kinetic changes in our signals. Michaelis-Menten modelling is often used to assess the kinetics of electrochemical signals (Daberkow et al., 2013; Singer et al., 2016; Yorgason et al., 2011b), and several recent papers have developed modified versions of this approach specifically for FCV data (Harun et al., 2015; Hoffman et al., 2016). We intend to implement one of these more sophisticated curve-fitting methods in future analyses of this dataset, but this will involve a significant time investment, making it beyond the scope of this thesis.

Our GBR-12909 data are in agreement with many previous studies that have examined this drug's effects on striatal dopamine transmission. Numerous microdialysis studies have reported GBR-12909-induced increases in dorsal striatal dopamine, following both local infusion and systemic administration, in awake rats (Budygin et al., 1999; Huotari et al., 1999; Nakachi et al., 1995; Nomikos et al., 1990; Raevskii et al., 2002) as well

as after systemic administration in anaesthetised mice (Huotari et al., 2002a). In addition, studies using FCV to assess the effects of GBR-12909 have, in agreement with our findings, shown that systemic administration increases the size of evoked dopamine transients in both the dorsal striatum of anaesthetised rats (Budygin et al., 1999) and the NAc of awake rats (Owesson-White et al., 2012). These pharmacological experiments are supported by studies in transgenic DAT knockout mice, which exhibit dramatically slowed dopamine clearance and elevated extracellular dopamine levels in both NAc and dorsal striatum (Benoit-Marand et al., 2000; Giros et al., 1996; Jones et al., 1998a; Torres et al., 2003). Together, this literature and our results support the view that DAT recycling is a key mechanism for regulating striatal dopamine – both the tonic transmission measured by microdialysis and the phasic transients recorded by FCV.

One important factor to keep in mind when assessing the effects of GBR-12909 using FCV is that this drug is known to adsorb to carbon fibre electrodes and can reduce electrode sensitivity for dopamine, either by competition with dopamine molecules for adsorption sites at the electrode surface or by making the carbon surface more hydrophobic and therefore less able to adsorb dopamine (Davidson et al., 2000; Takmakov et al., 2010). However, the data on GBR-12909's effects on electrode sensitivity were collected in *ex vivo* slice preparations in which the drug was washed into the bath, and it is therefore not known whether similar effects occur when electrodes are implanted *in vivo* and GBR-12909 is systemically administered. Furthermore, if GBR-12909 does alter FCV electrode sensitivity *in vivo*, this should result in an underestimation of its effects (Davidson et al., 2000). Given that we observed clear positive effects of GBR-12909 administration on dopamine in our NAc recordings, this should affect neither our ability to

study regulation of striatal dopamine by DAT blockade nor our ability to assess how COMT degradation contributes to this regulation (although the electrode adsorption issue could potentially affect our MFC data set; see Chapter 5).

4.4.3 Effects of COMT inhibition on NAc dopamine

We found no evidence that tolcapone influences evoked dopamine transients in NAc on its own (Figure 6a). Tolcapone affected neither the peak height, the AUC, nor the decay constant of our signals, as seen in Figures 7 and 8.

This result is not entirely unexpected. The only demonstration of an effect of tolcapone on striatal dopamine levels comes from a study in which a high concentration of the drug (100 μM) was directly infused into the striatum via the microdialysis probe (Huotari et al., 2001). By contrast, numerous microdialysis studies in which tolcapone was systemically injected found drug-induced changes in the levels of the dopamine metabolites HVA and DOPAC in the absence of effects on dopamine itself (Acquas et al., 1992; Budygin et al., 1999; Huotari et al., 1999; Li et al., 1998; Raevskii et al., 2002). The absence of a tolcapone effect on dorsal striatal transmission has also been demonstrated using FCV (Budygin et al., 1999; Garris and Wightman, 1995). It is therefore likely that COMT has little influence on striatal dopamine transmission under baseline conditions, when the DAT is operating optimally. When DAT recycling is impeded, however, or when striatal dopamine levels are increased to unusually high levels by other means, COMT regulation may come into play, as discussed further below.

4.4.4 Combined effects of DAT blockade and COMT inhibition on NAc dopamine

Analysis of evoked dopamine transients following administration of both GBR-12909 and tolcapone found little indication that inhibition of COMT potentiates the effects

of DAT blockade. Administration of tolcapone together with GBR-12909 did not significantly enhance the effects that GBR-12909 had on its own on either the peak height, AUC, or decay kinetics (Figures 7 and 8). However, inspection of the data suggests that a potentiation of GBR-12909's effects by tolcapone should not be entirely ruled out: signals appear to undergo a greater increase in size and to display slower decay kinetics following administration of both drugs than following GBR-12909 alone (Figure 6c). This apparent potentiation is particularly apparent in the peak height data (Figure 7a). The range in the magnitude of GBR-12909's effects, as noted above, makes it difficult to determine whether the apparent potentiation by tolcapone is simply due to variability in the response to GBR-12909 or whether, conversely, this variability masks an influence of tolcapone. A larger sample size in this drug treatment group could be helpful for overcoming this uncertainty. In addition, alternative approaches to assessing signal kinetics, such as those discussed above, might better succeed in resolving any differences in GBR-12909's effects on dopamine transmission dynamics with and without concurrent administration of tolcapone.

Several previous studies have found a potentiation of GBR-12909-induced increases in dorsal striatal dopamine levels by tolcapone using microdialysis, and this potentiation of transmission has been shown to enhance stereotypy (repetitive behaviour) induced by high doses of GBR-12909 (Budygin et al., 1999; Huotari et al., 1999; Raevskii et al., 2002). Furthermore, tolcapone effects have been shown to emerge when it is administered together with other drugs that, like DAT blockers, increase striatal dopamine levels, such as the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) (Raevskii et al., 2002). However, studies using transgenic animals have failed to demonstrate interactive effects of

DAT and COMT. Tolcapone had no effect on dorsal striatal dopamine transmission, as measured by carbon fibre amperometry, in DAT knockout mice, despite the elevated striatal dopamine levels known to be present in these mice (Benoit-Marand et al., 2000). In COMT knockout mice, meanwhile, although GBR-12909 did alter dopamine tissue content in striatum and subtly changed behaviour in knockout animals compared to wildtypes, it did not differentially affect extracellular striatal dopamine measured by microdialysis in the two genotypes (Huotari et al., 2002a).

4.4.5 Potential factors influencing whether COMT inhibition affects NAc dopamine

Given the variability in the species used and the means of assessing dopamine transmission across these studies, as well as the differences between acute (pharmacological) and chronic (genetic) manipulations of COMT, it is difficult to reconcile these diverse findings. The literature indicates that pharmacological inhibition of COMT can affect tonic dopamine transmission in the striatum (although only when dopamine levels are already elevated by another drug), but there has as yet been no demonstration of an effect of COMT inhibition on phasic dopamine transmission in striatum, suggesting that COMT may only contribute to the regulation of tonic but not phasic transmission in this region. Inspection of our data suggests that an influence of COMT on phasic transients should not be completely ruled out, as discussed above, but does not provide a clear demonstration of a role for COMT in regulating phasic striatal dopamine transmission. It is also possible that our use of supraphysiological stimulation to evoke dopamine release prevented us from seeing an effect of COMT inhibition. Finally, it may be that we were unable to reproduce the results of microdialysis studies that found that tolcapone potentiated the effects of GBR-12909 because these studies made their measurements in

dorsal striatum – whereas we recorded dopamine release in the NAc – and there may be regional differences in the degree to which COMT contributes to dopamine clearance in the striatum.

To the extent that COMT degradation of dopamine does contribute to regulation of striatal dopamine transmission, there are several mechanisms by which this could occur. COMT could influence dopamine in the striatum locally (Matsumoto et al., 2003) or via the circuits linking the MFC to the NAc and VTA (Carr and Sesack, 2000; Moore et al., 1999; Sulzer et al., 2016). Indeed, given the evidence that manipulations of dopamine transmission in the MFC can alter NAc dopamine (Del Arco and Mora, 2005; Doherty and Gratton, 1996; Thompson and Moss, 1995) and COMT's known importance in regulating cortical dopamine transmission (Tunbridge et al., 2006), it seems likely that COMT activity in the cortex should have knock-on effects on striatal transmission. However, limited knowledge of the details of cortical dopamine transmission and of how exactly cortical and striatal dopamine are related makes it difficult to predict under what circumstances such COMT effects might emerge.

As noted in the introduction to this chapter and discussed in detail in Chapter 1, a leading theory about the cortical-striatal relationship posits that higher cortical dopamine levels should increase tonic transmission in the striatum and that this in turn should decrease phasic release (Grace, 1991). This idea has been extended to a hypothesis about how variability in the activity of the COMT enzyme might affect striatal transmission: lower COMT activity is proposed to increase tonic transmission in the striatum by increasing dopamine levels both locally and in the cortex, which then has a dampening effect on phasic transmission in striatum (Bilder et al., 2004). The previous evidence of tolcapone's ability

to potentiate the effects of GBR-12909, reviewed above, aligns with these ideas – systemic administration of a COMT inhibitor, which should increase cortical dopamine and perhaps also have subtle effects locally within the striatum, was found to increase dopamine levels measured by microdialysis, which samples dopamine on a tonic timescale. Following on from this, one might have expected tolcapone to *reduce* the effect of GBR-12909 on the signals we recorded with FCV, which makes measurements on a phasic timescale – but if anything, we found that tolcapone appeared to slightly *enhance* GBR-12909's effects, although this was not statistically significant. Although this indicates that cortical dopamine and striatal phasic transmission are not in fact inversely related, we cannot make such a conclusion definitely without simultaneous monitoring of drug effects on both tonic and phasic transmission. Evidence of how GBR-12909 and tolcapone affect cortical dopamine transmission could provide further insights into the relationship between cortical and striatal dopamine, however, as will be discussed further in the next chapter.

Chapter 5:
Effects of DAT blockade and COMT inhibition on dopaminergic transmission in cortex

5.1 Introduction

Dopamine is a key neuromodulator not only in striatum but also in cortex. Cortical dopamine is implicated in a variety of cognitive processes, particularly in executive functions like working memory and attention as well as in various forms of learning and decision making (O'Doherty et al., 2017; Robbins, 2005; Tunbridge et al., 2006). As discussed in Chapter 1, cortical dopamine transmission appears to involve slower and longer lasting transmission over larger areas than occurs in the striatum (Mundorf et al., 2001; Sesack et al., 1998; Sulzer et al., 2016). However, the detailed nature of cortical dopamine transmission is less well characterised than it is in striatum. For example, whereas striatal transmission is typically classified as either phasic (large but short-lived increases in dopamine release that often accompany behaviourally relevant events) or tonic (slow, steady release that maintains baseline dopamine levels) (Robinson et al., 2003; Willuhn et al., 2010), it is unknown whether a similar dichotomy exists in cortex.

The mechanisms regulating dopamine's clearance from the synapse in cortex are better understood. Whereas most clearance in the striatum occurs via the dopamine transporter (DAT), clearance in cortex relies on enzymatic degradation, most prominently by catechol-*O*-methyltransferase (COMT) (Sulzer et al., 2016; Tunbridge et al., 2006). As reviewed in Chapter 1, COMT has been extensively studied, as both the human Val^{158}Met polymorphism in the COMT gene and relevant animal models have implicated COMT

function in working memory and other executive processes as well as, potentially, in several psychiatric disorders (Barkus et al., 2016; Diaz-Asper et al., 2008; Egan et al., 2001; Farrell et al., 2012; Goldberg et al., 2003; Tunbridge et al., 2004, 2006). Interestingly, several human imaging studies have found interactive effects of COMT and DAT genotype on fMRI BOLD activity in the MFC during cognitive tasks (Bertolino, 2006; Caldú et al., 2007; Prata et al., 2009). It remains unclear, however, exactly how COMT affects dopaminergic transmission in cortex – particularly at sub-second timescales – and to what extent COMT interacts with DAT in regulating cortical transmission.

5.1.1 Aim and approach of this experiment

Therefore, in parallel to the nucleus accumbens (NAc) experiments described in Chapter 4, we also assessed the separate and combined effects of the COMT inhibitor tolcapone and DAT blocker GBR-12909 on cortical dopamine transmission. We evoked dopamine release by stimulating the ventral tegmental area (VTA) and recorded the resulting dopamine transients in the medial frontal cortex (MFC) of anaesthetized mice using fast scan cyclic voltammetry (FCV). Based on microdialysis studies that found that tolcapone increased cortical dopamine concentration (Lapish et al., 2009; Tammimaki et al., 2016; Tunbridge et al., 2004), we expected that tolcapone administration would also boost FCV-recorded signals in our study. Although DAT expression levels are lower in cortex than in striatum (Sesack et al., 1998), several studies, again using microdialysis, have shown GBR-12909-induced increases in cortical dopamine levels (Carboni et al., 2006; Tanda et al., 1997; Valentini et al., 2004). We therefore hypothesised that GBR-12909 would increase dopamine transients in our FCV recordings. We additionally hypothesised

that tolcapone and GBR-12909 might have an additive effect on cortical dopamine levels, as we initially expected they would on striatal dopamine.

We targeted our MFC recordings to the prelimbic cortex. The rodent prelimbic cortex is thought to be homologous to parts of the primate ventromedial prefrontal cortex (Balleine and O'Doherty, 2009). It has been implicated in many aspects of learning and decision making and is in particular thought to be involved in operant behaviour and goal-directed decision making (Balleine and Dickinson, 1998; Corbit and Balleine, 2003; Moscarello et al., 2010; Naneix et al., 2009; Uylings et al., 2003). As the behavioural tasks that we investigated in the experiments reported in Chapters 6 and 7 involve these processes, the prelimbic cortex was the obvious choice for our voltammetric measurements of cortical dopamine. In addition, cognitive processes such as attentional set shifting that COMT is known to influence also depend on the integrity of this region (Birrell and Brown, 2000; Tunbridge et al., 2004), adding interest to the question of what effects COMT inhibition would have on dopamine transmission in this area. Finally, the prelimbic cortex is, along with other subregions of the MFC, both anatomically and functionally linked to the NAc (Carr and Sesack, 2000; Del Arco and Mora, 2005; Del Arco et al., 2008; Doherty and Gratton, 1996; Jackson et al., 2001; Karreman and Moghaddam, 1996; Murase et al., 1993; Sulzer et al., 2016; Taber et al., 1995). We anticipated that our cortical recordings might therefore provide insight into the interaction between these two regions as well as into dopamine dynamics in the MFC itself.

5.1.2 Dopamine versus noradrenaline

Unlike the striatum, the cortex is innervated by significant noradrenergic as well as dopaminergic fibres (Lindvall et al., 1978; Slopsema et al., 1982). Dopamine and

noradrenaline cannot be distinguished by FCV because their cyclic voltammograms are virtually identical (Adams, 1976; Michael and Wightman, 1999), and FCV recordings made in MFC can therefore only be identified as catecholaminergic, not as definitively dopaminergic. Previous studies, however, have shown that VTA stimulation primarily evokes dopamine rather than noradrenaline release in MFC (Shnitko and Robinson, 2014). Therefore, for the sake of simplicity, evoked signals in the MFC will be termed 'dopamine' even though a contribution of noradrenaline to these signals cannot be fully ruled out.

5.2 Methods

The methods for FCV recordings made in the MFC are largely identical to those for recordings made in the NAc and are reviewed in brief here. Refer to the methods section of Chapter 4 for further details.

5.2.1 Subjects

A total of 46 male C57BL/6 mice, aged 10-16 weeks at the time of testing, were used for FCV recordings in the MFC. Data from 28 of these animals were included in the final analysis. We excluded 5 animals from analysis due either to death prior to completion of the experiment (n = 1) or ineffective drug injection (n = 4). In addition, we excluded 13 animals in which we were unable to record consistent trial-by-trial dopamine release: 3 from the vehicle-vehicle group, 2 from the tolcapone-vehicle group, 5 from the vehicle-GBR-12909 group, and 3 from the tolcapone-GBR-12909 group (see section 5.2.2 for more information about drug groups).

5.2.2 Drugs

Drugs were dissolved in 20% hydroxypropyl-beta-cyclodextrin in 0.9% saline, which also served as the vehicle control. As in NAc recording experiments, the COMT inhibitor tolcapone was used at a dose of 30mg/kg, and the DAT blocker GBR-12909 dihydrochloride was used at a dose of 6mg/kg. The dihydrochloride was not taken into account when calculating the dosage, so GBR-12909 injections contained 6mg/kg of GBR-12909 dihydrochloride. In MFC recording experiments, the effects of D-amphetamine sulfate (Tocris), used at a dose of 4mg/kg, were also tested. The sulphate *was* taken into account when calculating the dosage so that amphetamine injections contained 4mg/kg of amphetamine itself. All drugs were delivered by intraperitoneal injection (injection volume = 5mL/kg).

As described in Table 1, the first drug injection (vehicle or tolcapone) was given 1.5 hours before the second injection (vehicle or GBR-12909) to account for differences in the drugs' time courses of action (Acquas et al., 1992; Männistö and Kaakkola, 1999; Nakachi et al., 1995; Raevskii et al., 2002). Amphetamine injections were administered at the end of the recording session in 6 animals that had received vehicle and/or tolcapone, but not GBR-12909, injections.

Group	**Drug 1** **at Time 1**	**Drug 2** **at Time 2 (Time 1 + 1.5hrs)**
Veh-Veh	Vehicle	Vehicle
Tolc-Veh	Tolcapone	Vehicle
Veh-GBR	Vehicle	GBR-12909
Tolc-GBR	Tolcapone	GBR-12909

Table 1: Drug treatment groups.

5.2.3 Electrode making and calibration

Carbon fibre recording electrodes and Ag/AgCl reference electrodes were constructed in-house and bipolar, stainless steel stimulating electrodes obtained as described in Chapter 4. Recording electrodes were pre-calibrated in 1000nM dopamine in a flow cell, and calibration factors were used to convert recorded signals from current (nA) into concentration (nM). Details of the calibration procedure can be found in Chapter 4.

5.2.4 Surgery

Recordings were made under urethane anaesthesia as described in Chapter 4. Recording electrodes were targeted to the MFC and stimulating electrodes to the dopaminergic cell bodies in the VTA in the left hemisphere. The Ag/AgCl reference electrode and anchoring screw were implanted contralaterally to the recording and stimulating electrodes. Stereotaxic coordinates for the placement of the electrodes and screw during MFC recordings are listed in Table 2.

	Anterior-posterior (mm)	**Medial-lateral (mm)**	**Dorsal-ventral (mm)**
MFC	+2.50	-0.50	-1.30 to -2.30 from skull
VTA	-3.50	-0.35	-4.00 to -4.80 from brain
Reference	+4.80	+1.00	N.A.
Screw	+2.80	+2.00	N.A.

Table 2: Coordinates of regions targeted for placement of recording (MFC), stimulating (VTA), and reference electrodes and of the stabilizing screw.

As in the NAc recordings, stimulating electrodes were targeted to the posterior-medial VTA. Although populations of MFC- and NAc core-projecting dopamine neurons are composed of separate cells – the same neurons do not send collaterals to both regions (Swanson, 1982) – tracing studies have shown that both populations are localised to the

posterior-medial VTA, which encompasses the paranigral and medial parabrachial nuclei (Lammel et al., 2008, 2011, 2014). Cortically-projecting dopamine neurons primarily terminate in the deep layers of the MFC (Tritsch and Sabatini, 2012). We selected our stimulating coordinates to maximize the likelihood of evoking detectable dopamine release in MFC, as dopamine levels are lower in this region than in striatum (Moghaddam et al., 1990); serendipitously, these coordinates were appropriate for evoking release in the NAc as well.

At the end of recording sessions, electrolytic lesions were used to mark the placement of the recording electrode in a subset of animals; alternatively, recording electrodes were removed for post-calibration. All animals were then overdosed with pentobarbital and their brains removed for histological processing.

5.2.5 Histology

Placement of recording and stimulating electrodes was visualised using cresyl violet staining, and a subset of sections were immunostained for tyrosine hydroxylase (TH) to assess the proximity of stimulating electrodes to midbrain dopaminergic nuclei. Details of the staining procedures can be found in Chapter 4.

5.2.6 Data acquisition

Dopamine release in the MFC was evoked by stimulating the VTA via a DS3 Stimulator (stimulation parameters: pulse number = 60 pulses, frequency = 50Hz, amplitude = 300μA, pulse width = 2ms, pulse phase = biphasic). Release was recorded by applying a triangular waveform (voltage step from –0.4V to +1.3V and back to –0.4V [vs. Ag/AgCl], sweep rate = 400 V/sec, scan rate frequency = 10Hz) to the recording electrode. This voltage step transiently oxidises electroactive molecules at the electrode surface, and

the resultant current can be used to identify the chemicals that are present in the surrounding tissue. Dopamine was identified by its cyclic voltammogram (a plot of the current as a function of the applied potential of the voltage step), which is distinct from the voltammograms of all other chemical species that can be detected by FCV except noradrenaline (see introduction and discussion sections of this chapter for further information). Stimuli were generated and recordings collected using Tarheel CV. Refer to Chapter 3 and to the methods section of Chapter 4 for additional details on FCV data acquisition.

5.2.7 Experiment structure

As in NAc experiments, we observed a decrease in the size of signals recorded in the MFC over the recording session. Signal rundown in the MFC, however, was much more gradual than in the NAc (Figure 1; compare to Figure 2 in Chapter 4). We therefore allowed the signal to stabilize for approximately 1.0hr (rather than 2.5hrs), while stimulating every 5-10min, before beginning to record pre-drug baseline signals.

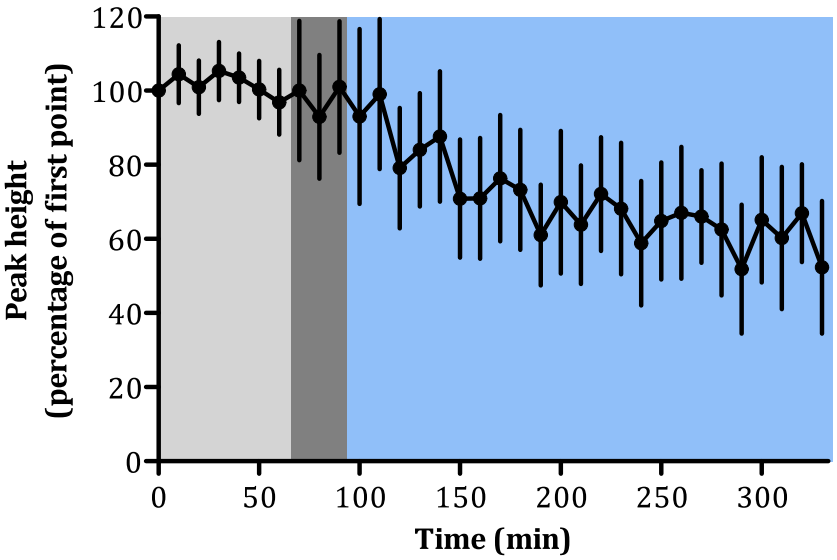


Figure 1: Rundown of signal size across the session in MFC recordings. Peak height at each time point is expressed as a percentage of the peak height of the recording made at time 0 and then averaged across animals. The light grey box frames the stabilisation period (n=41), the dark grey box frames the pre-drug baseline recordings (n=6), and the blue box frames recordings made following drug administration in animals that received only vehicle injections (n=6). Refer to Figure 3 for further details on the structure of the experiment. Note that here peak height is calculated from raw data that has not undergone chemometric analysis.

After stabilisation, recordings proceeded as described in Chapter 4 and as shown in Figure 2. Stimulation was induced and recordings made every 5min. Six pre-drug baseline recordings were made over the 30min prior to the injection of drug 1 (tolcapone or vehicle). We then recorded for 90min before injecting drug 2 (GBR-12909 or vehicle). Recordings continued for an additional 2.5hrs following the second injection. Data were averaged into 15min bins for analysis, with each bin covering 3 stimulations, and two time points were assessed for drug effects. The first time point, labelled 'Effect of Drug 1' in Figure 2, is an average of 3 recordings made approximately 85min after administration of drug 1 (i.e. the last bin before the second injection). The second time point, labelled 'Effect

of Drug 1 + Drug 2' in Figure 2, is an average of 3 recordings made approximately 25min after administration of drug 2.

At the end of 6 experiments in which animals received either two vehicle injections or a tolcapone and a vehicle injection, amphetamine was injected approximately 150min after administration of drug 2, after completion of data collection for the main drug study. Two time points are presented to illustrate the effect of amphetamine: the pre-amphetamine time point is an average of 3 recordings made approximately 10min before the injection, and the post-amphetamine time point is an average of 3 recordings made approximately 25min after the injection.

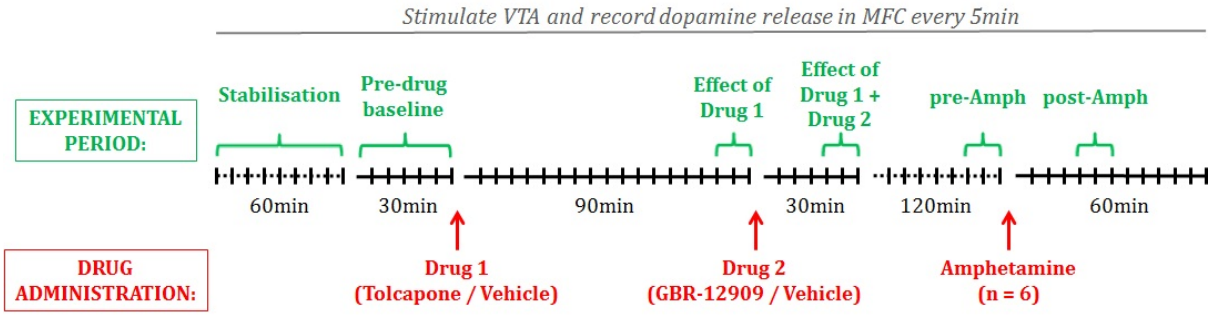


Figure 2: Schematic of experiment structure. Drug injections are indicated in red and time points of interest in green.

5.2.8 Analysis

FCV recordings were analysed using CV Analysis (one version customised by Scott Ng-Evans and one customised by Richard Keithley), Matlab R2012a, Microsoft Excel 2010, GraphPad Prism version 5.04, and SPSS version 20 as described in Chapter 4. Significance was set at $\alpha = 0.05$. For repeated-measures ANOVAs, Greenhouse-Geisser corrections were

applied where data failed Mauchley's test of sphericity; degrees of freedom are reported to two decimal places in such cases. Simple main effects analyses were conducted as necessary when significant interactions were found. Least square difference pairwise comparison tests were used to assess which groups were driving significant main and interactive effects.

The dopaminergic component of recorded signals was extracted using chemometrics, with individual training sets constructed for each animal as described in detail in Chapter 3. Signals were low-pass filtered at 2kHz and were background subtracted: for each recording, the average data in a 0.5sec window prior to stimulation were subtracted from the 5sec preceding and the 40sec following the stimulation to remove changes in the signal attributable to the electrode's capacitance current. After chemometric analysis, the recorded current was extracted at the time of the signal's peak, smoothed using a 5-point moving average, and re-baselined to 0.1sec before the start of stimulation. Drug effects on dopamine release were assessed using several parameters, including:

(1) **peak height**, defined as the difference between the maximum signal size within 3sec of stimulation and the signal size at the time of stimulation

(2) **area under the curve (AUC)**, which was summed during the 5sec following stimulation

(3) **decay constant**, obtained by fitting a first order exponential curve to the signal over the 3sec following the signal's peak

Peak height and AUC were normalized to the averaged pre-drug baseline signal, binned, and then averaged across subjects within each drug group. Decay constants were

calculated from un-normalized data: signals were first binned at the time points of interest shown in Figure 2 (6 recordings binned to obtain the pre-drug baseline signal; 3 recordings binned to assess the effect of drug 1; 3 recordings binned to assess the combined effects of drug 1 and drug 2; 3 recordings binned to assess the signal just before amphetamine administration; and 3 recordings binned to assess the effect of amphetamine), then the exponential fit was performed on the binned data, and finally decay constants were averaged across animals with each drug group. Statistical comparisons were performed at the selected time points shown in Figure 2.

5.3 Results

5.3.1 Characterisation of evoked dopamine release in MFC

Although signals in the cortex were substantially smaller than those seen in the striatum, we were able to record discernible evoked dopamine release in MFC in most of the animals studied. Signal size in the MFC at the start of pre-drug baseline recordings ranged from 0.71 to 5.03 nA (mean ± standard deviation: 2.15 ± 1.02), which corresponded to a dopamine concentration of 28.25 to 254.40 nM (mean ± standard deviation: 109.82 ± 66.54), depending on the sensitivity of the electrode (Figure 3).

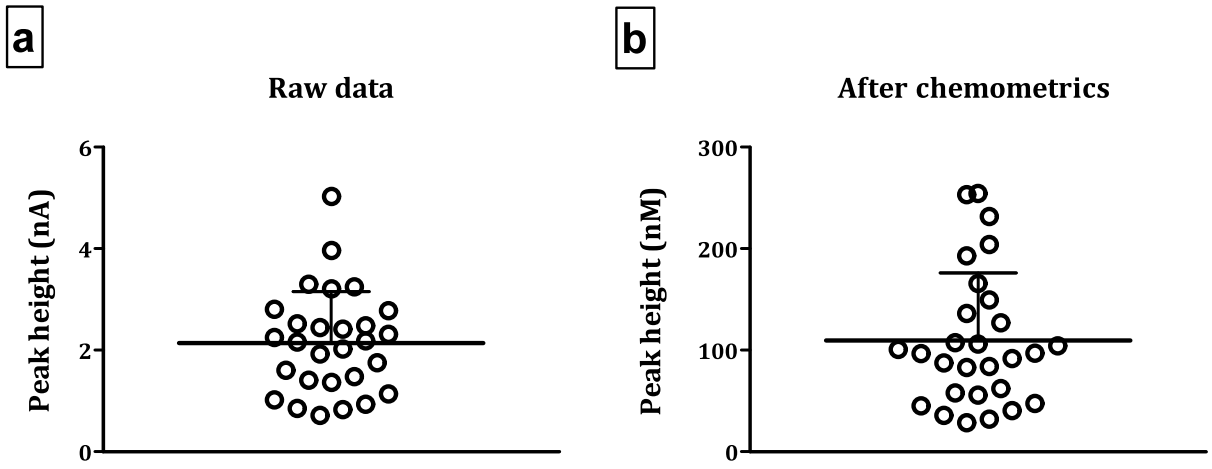


Figure 3: The range of signal sizes observed in the PFC at the start of the pre-drug baseline recording period. a) Peak height of raw signals, expressed in units of current. b) Peak height of signals after chemometric analysis, expressed in units of concentration. Graphs show data points from individual animals, with mean + standard deviation overlaid.

At the start of pre-drug baseline recordings, MFC signals peaked between 1.1 and 3.0sec (mean ± standard deviation: 1.69 ± 0.44) after the time of stimulation and then decreased back towards baseline (although in many cases the signal failed to make a complete return to baseline) (Figure 4b). Inspection of cyclic voltammograms identified the released chemical as putative dopamine (Figure 4a).

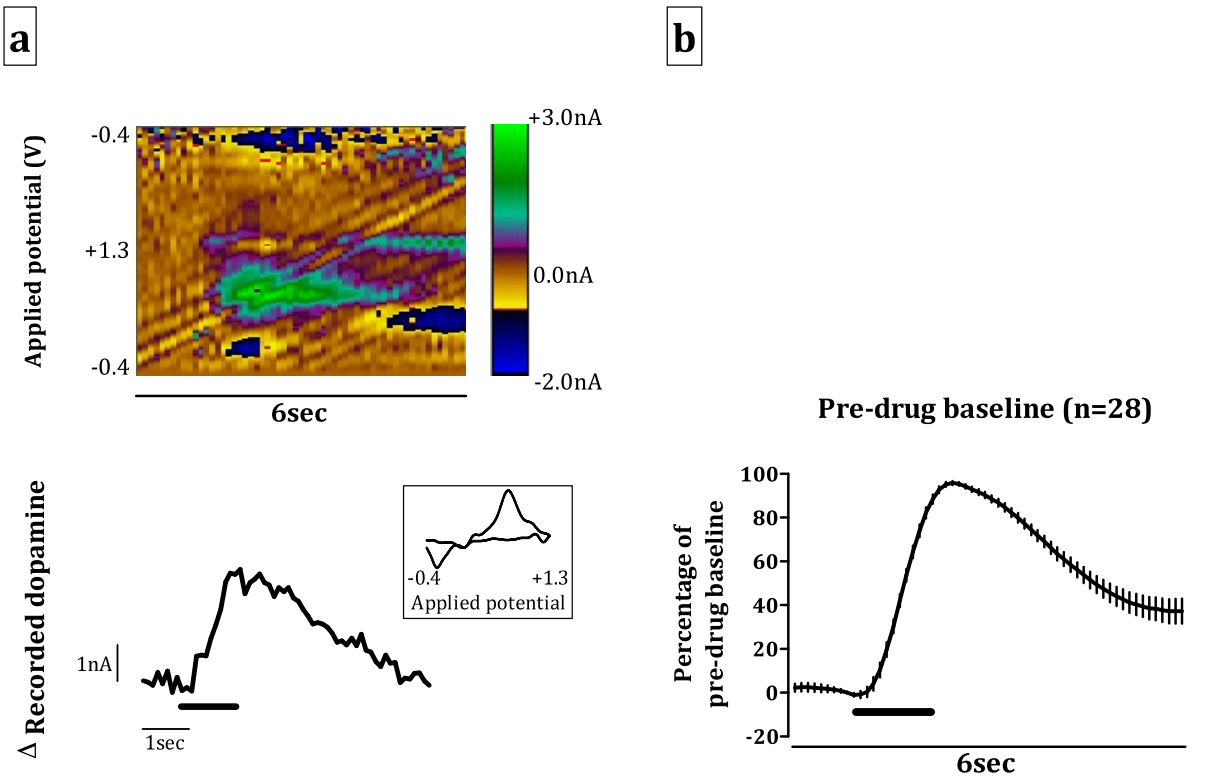


Figure 4: Recording evoked dopamine release in the MFC. a) An example recording made in the MFC. The pseudocolour plot shows the recorded current as a function of the applied potential of the triangular waveform (y axis) over a 6sec period (x axis). The trace below the pseudocolour plot shows the extracted current attributable to dopamine release as a function of time; timing and duration of stimulation indicated by thick black bar. The cyclic voltammogram (current versus applied voltage; boxed inset) during the peak of the signal, 1.4sec after the time of stimulation, is consistent with catecholamine release. b) The average of 6 pre-drug baseline recordings (6 stimulations over 30min in each mouse), normalized to the average pre-drug baseline peak height (equivalent to 100% on the y axis) and presented as mean +/- SEM across all animals. Timing and duration of stimulation is indicated by the thick black bar.

5.3.2 Effects of COMT inhibition and DAT blockade on evoked dopamine in MFC

The effects of the COMT inhibitor tolcapone and DAT blocker GBR-12909 on dopamine release in the MFC are shown in Figure 5 and quantified in Figures 6 and 7. Drug effects were assessed by extracting and running statistical analyses on several signal parameters, including the **peak height**, the **AUC** in a 5sec window after the time of stimulation, and the **decay constant** of an exponential decay fit to the signal over the 3sec

following the peak. Peak height and AUC data are presented as a percentage of the pre-drug baseline signal; in these figures, therefore, 100% represents the size of the averaged pre-drug baseline recordings. The decay constant data is not normalized.

As can be seen in Figure 5a, there was no discernible difference in MFC dopamine release between animals that received tolcapone and those that received vehicle as the first injection. Signals in both groups diminished in size to approximately 65% of pre-drug baseline recordings by 85min after the first injection. Repeated-measures ANOVAs found no significant main effect of drug 1 on either the peak height or the AUC (F's < 0.02, p's > 0.91) (Figure 6).

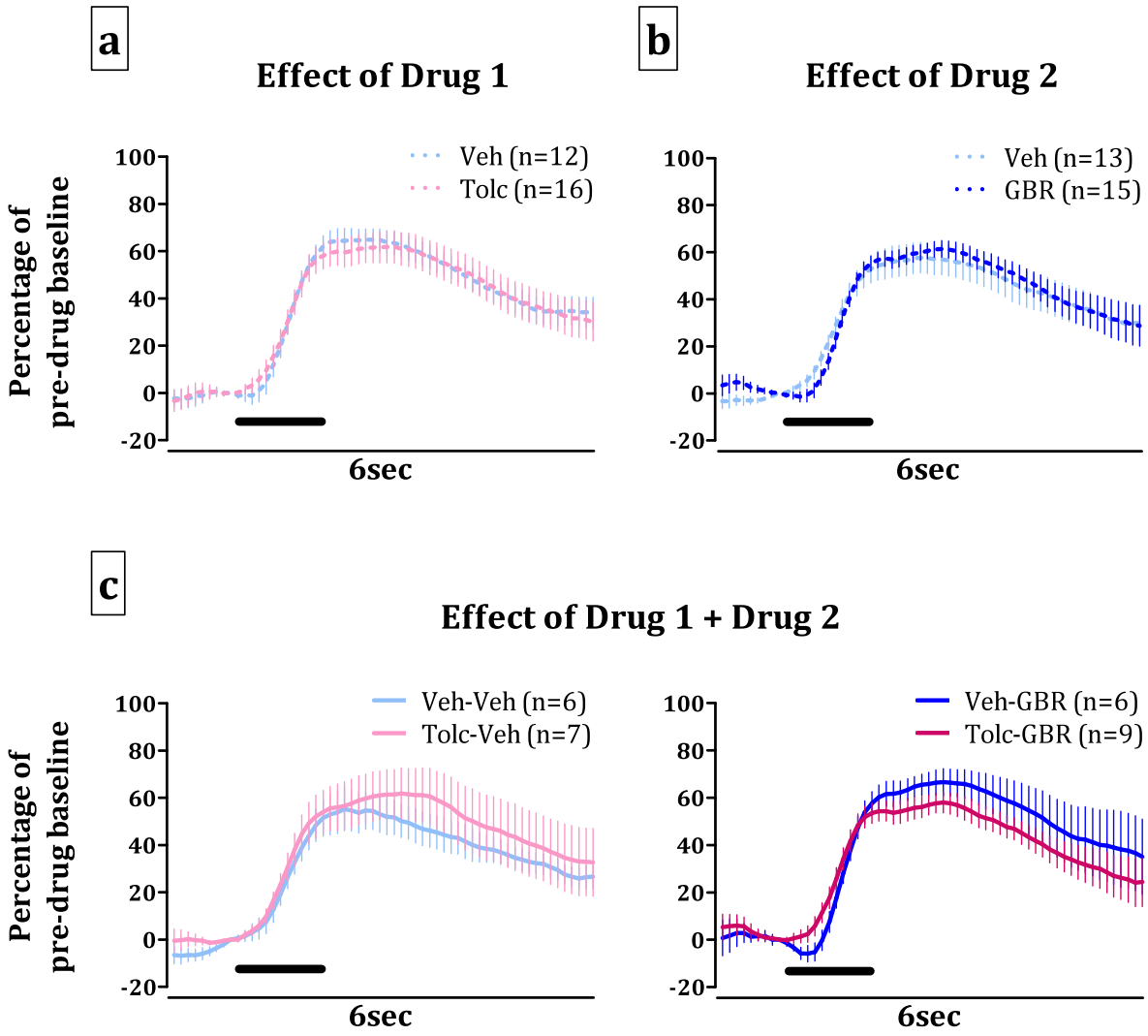


Figure 5: Evoked dopamine release in the MFC. The signals are normalized to the average pre-drug baseline peak height (equivalent to 100% on the y axis) and are presented as mean +/- SEM across animals within each drug group. Timing and duration of stimulation is indicated by the thick black bars. a) Release in animals given vehicle as drug 1 (light blue) compared to release in animals given tolcapone as drug 1 (light pink) 85min after the first injection. b) Release in animals given GBR-12909 as drug 2 (dark blue) compared to release in those given vehicle as drug 2 (light blue) 25min after the second injection. c) Release 25min after the second injection, as in (b), but separated according to the four possible combinations of drug 1 and drug 2: vehicle plus vehicle (light blue, left), tolcapone plus vehicle (light pink, left), vehicle plus GBR-12909 (dark blue, right), and tolcapone plus GBR-12909 (dark pink, right).

In contrast to its effect in the NAc, GBR-12909 had little effect on MFC dopamine. At 25min after the second injection – a time at which the effects of GBR-12909 should start to

become apparent (Carboni et al., 2006; Tanda et al., 1997; Valentini et al., 2004) and at which we observed a significant effect of GBR-12909 on NAc dopamine – there were no differences between evoked release in animals that received GBR-12909 and release in those that received vehicle as drug 2 (Figure 5b). When the data at this time point are separated according to the four possible combinations of drug 1 and drug 2, some variability across the four drug groups becomes apparent (Figure 5c). However, this variability did not emerge as a main effect of drug 2, which was not significant in either the peak height or the AUC analyses (repeated-measures ANOVAs, F's < 0.7, p's > 0.43) (Figure 6).

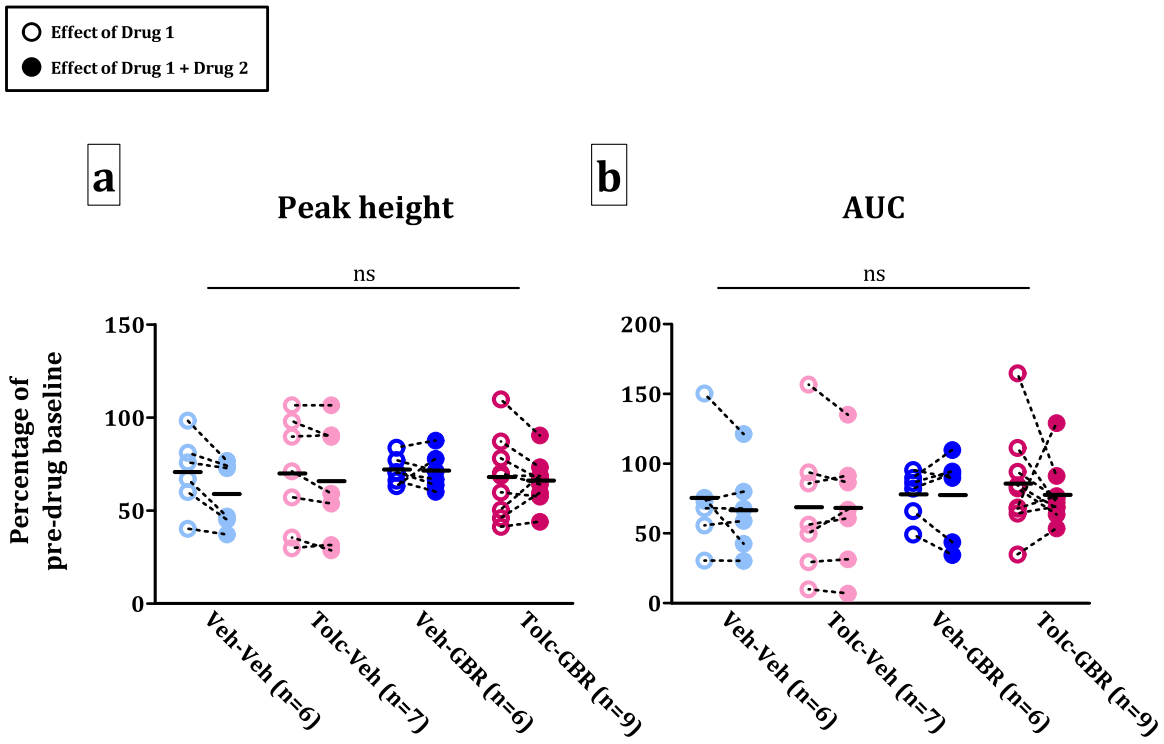


Figure 6: Quantification of a) the peak height and b) the area under the curve (AUC) of evoked dopamine release in MFC, normalized to the average pre-drug baseline peak height or AUC (equivalent to 100% on the y axis), respectively. Each animal's data is shown individually at two time points: open symbols show the effect of drug 1 on its own, 85min after the first injection, while filled symbols show the combined effects of drug 1 and drug 2, 25min after the second injection. Horizontal black bars indicate the means for each drug group at each time point. ns = not significant

The repeated-measures ANOVA of peak height revealed a significant main effect of time ($F_{1,24} = 6.6$, $p = 0.017$) such that the peak was significantly smaller at the later time point, 25min after the second injection, than it was at the earlier time point, 85min after the first injection. The interaction between time and drug 2 in the peak height analysis approached but did not reach significance ($F_{1,24} = 3.3$, $p = 0.082$). In contrast to the peak height analysis, there was no significant main effect of time on the AUC ($F_{1,24} = 0.9$, $p = 0.362$). No other interactions approached significance in either the peak height or AUC analyses (F's < 1.6, p's > 0.21).

To further investigate the kinetics of signal decay under different drug treatments, we performed exponential curve fitting and compared the decay constants of signals at baseline, under the influence of drug 1 alone, and under the influence of both drug 1 and drug 2 (Figure 7). Smaller decay constants reflect shallower slopes and, thus, slower clearance of dopamine. (Note that because these data were not normalised to the pre-drug baseline signal, baseline data were included as a third time point in the analysis.) Tolcapone had no effect on the decay constant, as a repeated-measures ANOVA found no significant main effect of drug 1 on this parameter ($F_{1,24} = 0.004$, $p = 0.950$). GBR-12909 also had little effect on decay kinetics, as there was neither a significant main effect of drug 2 nor a significant main effect of time on the decay constant (drug 2: $F_{1,24} = 0.013$, $p = 0.909$; time: $F_{1.45,34.89} = 1.2$, $p = 0.313$), and the interaction between drug 2 and time neared but did not reach significance ($F_{1.45,34.89} = 3.5$, $p = 0.055$). The analysis revealed no other significant interactions (F's < 1.7, p's > 0.20).

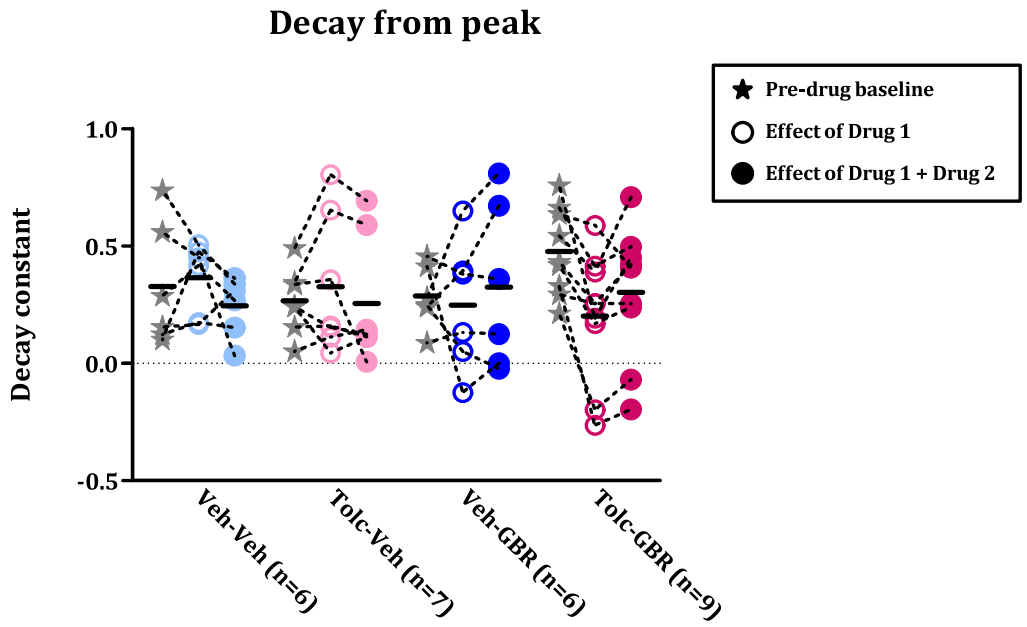


Figure 7: The kinetics of the decay from the peak of evoked dopamine release in MFC. Decay is quantified by the exponential decay constant, the value of which is presented on the y axis; a larger decay constant value indicates a faster rate of decay. Each animal's data is shown individually at three time points: grey stars show the rate of decay at baseline, 15min before the first drug injection; open circles show the effect of drug 1 on its own, 85min after the first injection; and filled circles show the combined effects of drug 1 and drug 2, 25min after the second injection. Horizontal black bars indicate the means for each drug group at each time point.

5.3.3 Effects of amphetamine on evoked dopamine release in MFC

Given the lack of any substantial effect of either tolcapone or GBR-12909 on MFC dopamine release, we also tested the effects of amphetamine as a positive control in 6 animals that had received vehicle and tolcapone, but not GBR-12909, injections. Within 25min of injection, amphetamine boosted signals from approximately 50% of the pre-drug baseline – the level to which signals had fallen after 5.5hrs of recording – to over 80% of pre-drug baseline signal size (Figure 8a). The amphetamine effect can be clearly seen in both a significant increase in the peak height (paired-samples t-test: $t(5) = -3.0$, $p = 0.029$) (Figure 8b) and a significant increase in the AUC (paired-samples t-test: $t(5) = -7.0$, $p = 0.001$) (Figure 8c).

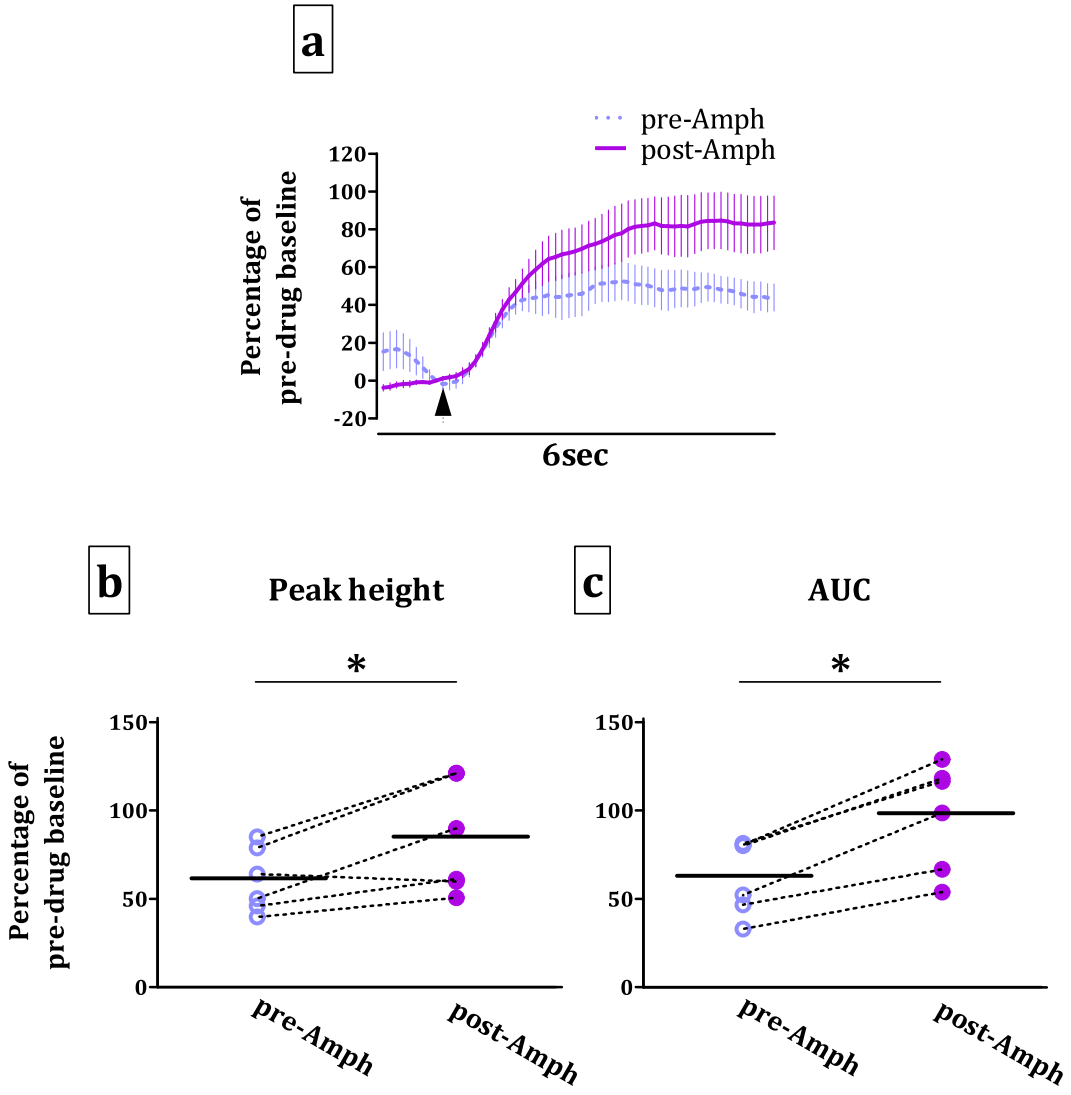


Figure 8: The effect of amphetamine on evoked dopamine release in MFC. Animals injected with amphetamine had previously received either two vehicle injections (n=3) or a tolcapone and a vehicle injection (n=3). Signals are normalized to the average pre-drug baseline peak height (a and b) or area under the curve (AUC) (c) (equivalent to 100% on the y axis). a) Release (mean +/- SEM) across animals 10min before (dotted light purple line) and 25min after (solid dark purple line) injection of amphetamine. Time of stimulation indicated by black arrowhead. b) The peak height before (open light purple symbols) and after (filled dark purple symbols) administration of amphetamine. Horizontal black bars indicate the mean peak height at each time point. c) As in (b) but for AUC. * = $p < 0.05$

Assessment of amphetamine's effects on signal decay kinetics revealed a significant main effect of time on the decay constant (repeated-measures ANOVA, $F_{2,10} = 5.6$, p =

0.023) (Figure 9). (Note that three time points are included in this analysis – the pre-drug baseline, the pre-amphetamine time point, and the post-amphetamine time point – because the data is not normalised to pre-drug baseline signals.) Least square difference posthoc tests revealed that decay constants following amphetamine administration were significantly smaller than at baseline ($p = 0.032$) and nearly significantly smaller than at the pre-amphetamine time point ($p = 0.063$).

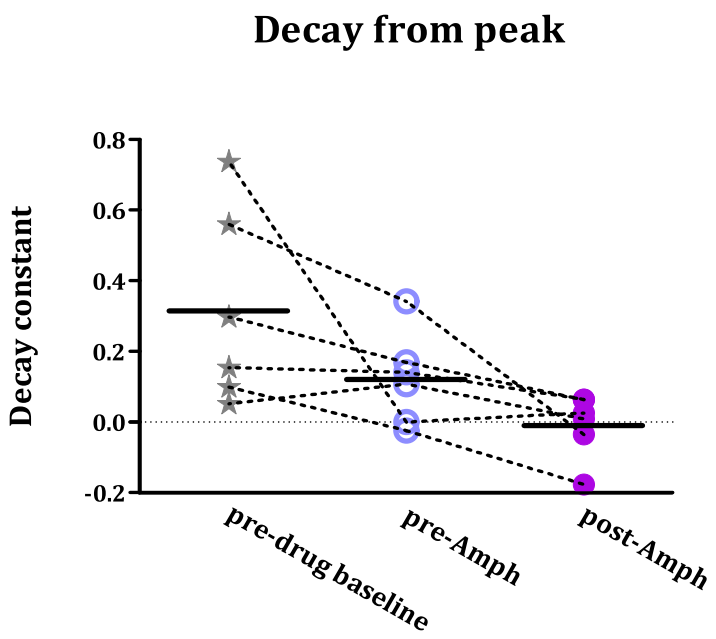
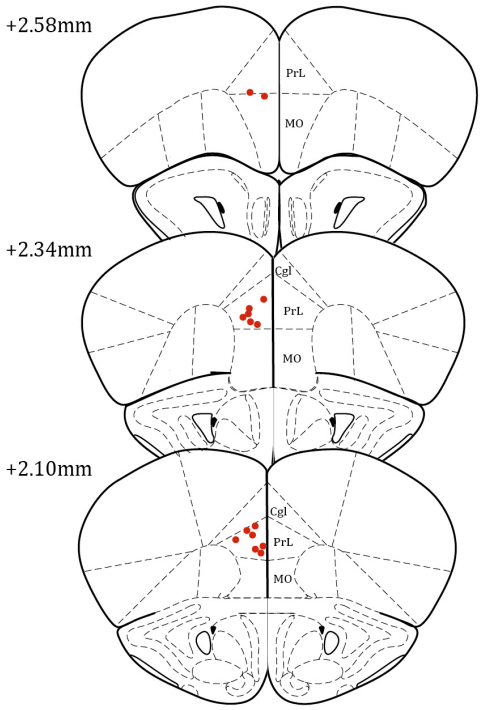


Figure 9: The effect of amphetamine on the decay kinetics of evoked dopamine release in MFC. Animals injected with amphetamine had previously received either two vehicle injections (n=3) or a tolcapone and a vehicle injection (n=3). Decay is quantified by the exponential decay constant, the value of which is presented on the y axis; a larger decay constant value indicates a faster rate of decay. Each animal's data is shown individually at three time points: grey stars show the baseline rate of decay at the beginning of the session, before any vehicle or tolcapone injections had been administered; light purple open circles show the rate of decay 10min before the amphetamine injection; and dark purple filled circles show the rate of decay 25min after the amphetamine injection. Horizontal black bars indicate the mean decay constant at each time point.

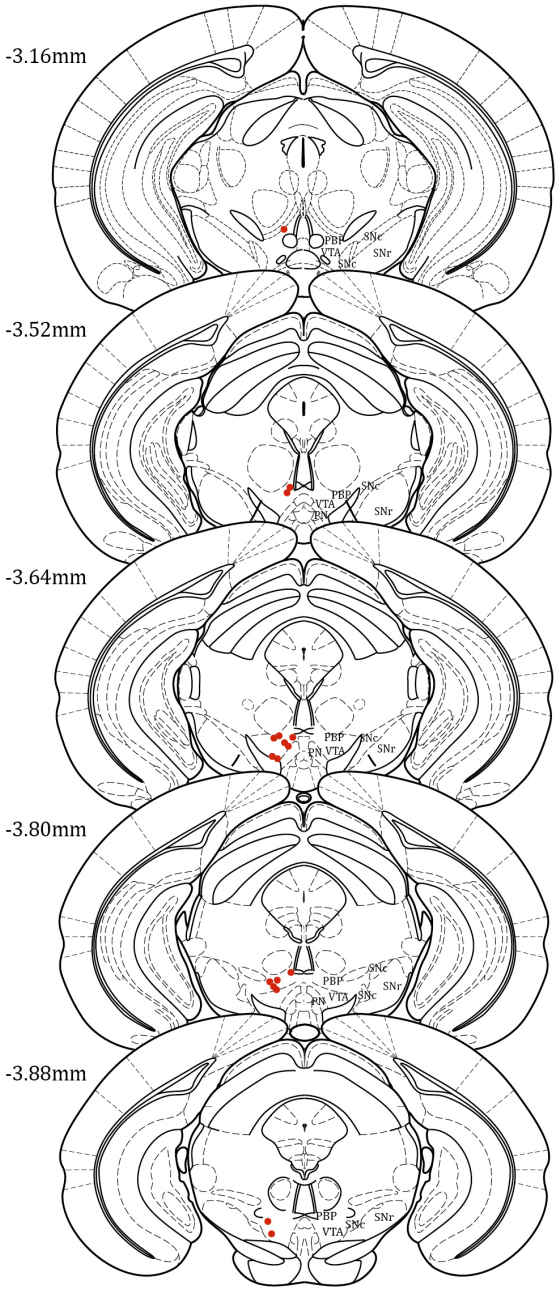
5.3.4 Histology

Histological assessment of those brains in which the recording site was lesioned confirmed that recording electrodes were successfully targeted to the prelimbic region of the MFC; lesion sites were typically found near the ventral border of this region (Figure 10a and c). In the same set of brains, the stimulating electrode tract was observed descending to the midbrain. The tract usually terminated at the site of the medial VTA, although in some cases the placement was more lateral, so that the tract terminated at the border of the VTA and substantia nigra (SN) (Figure 10b and c). A subset of brains stained for tyrosine hydroxylase, the enzyme that produces dopamine, confirmed that the placement of stimulating electrodes put them in the vicinity of dopaminergic cell bodies in the midbrain (Figure 11).

a


+2.58mm
PrL
MO
+2.34mm
Cgl
PrL
MO
+2.10mm
Cgl
PrL
MO

b


-3.16mm
PBP
VTA
SNc
SNr
-3.52mm
PBP
VTA
PN
SNc
SNr
-3.64mm
PBP
PN
VTA
SNc
SNr
-3.80mm
PBP
PN
VTA
SNc
SNr
-3.88mm
PBP
VTA
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c

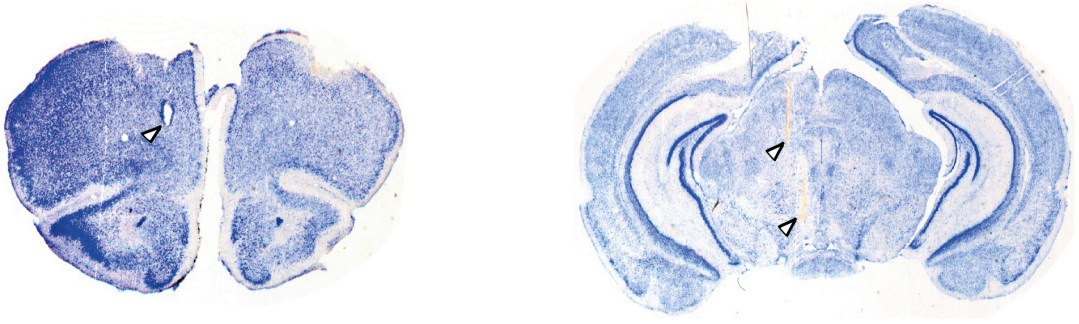

Figure 10: Electrode placements. a) Locations of electrolytic lesions made at recording electrode sites. b) Locations of the ends of stimulating electrode tracts. c) Examples of a lesion in the MFC (left) and a pair of electrode tracts descending to the VTA (right), indicated by white arrowheads. Numbers to the left of coronal sections in (a) and (b) indicate distance from bregma (mm). Cgl: cingulated cortex; MO: medial orbital cortex; PBP: parabrachial pigmented nucleus; PN: paranigral nucleus; PrL: prelimbic cortex; SNc: substantia nigra pars compacta; SNr: substantia nigra pars reticulata; VTA: ventral tegmental area.

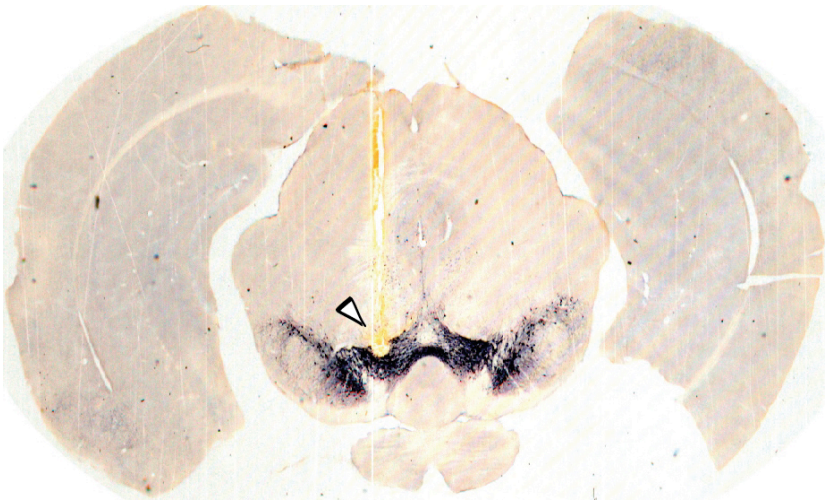

Figure 11: An example of a histological section stained for tyrosine hydroxylase, demonstrating the proximity of the stimulating electrode tract (white arrowhead) to dopaminergic cell bodies (black) in the midbrain.

5.4 Discussion

Although we were able to reliably record evoked dopamine transients in MFC, we found no effects of the COMT inhibitor tolcapone, and only hints of an effect of the DAT blocker GBR-12909, on the size or kinetics of these transients. By contrast, systemic administration of amphetamine significantly increased the size of evoked release, confirming that we were able to detect drug-induced changes in cortical dopamine signals with our testing paradigm. Our results therefore indicate that, at the sub-second timescale

at which FCV operates, neither COMT inhibition nor DAT blockade exert a substantial influence on dopaminergic transmission in MFC.

5.4.1 Recording cortical dopamine with FCV

Before turning to the results of the drug study, it is important to consider several points about FCV recordings of cortical dopamine. As noted in the introduction, the MFC is innervated by both dopaminergic and noradrenergic fibres (Lindvall et al., 1978; Slopsema et al., 1982). Although it is not possible to distinguish dopamine from noradrenaline using their cyclic voltammograms, the signals we detected in MFC are likely attributable to dopamine. We targeted our stimulating electrodes specifically to the dopaminergic nuclei of the midbrain instead of to the medial forebrain bundle, a fibre tract often used as a site of stimulation to evoke dopamine release but that contains noradrenergic as well as dopaminergic axons (Nieuwenhuys et al., 1982). Our VTA-targeted stimulation decreases the likelihood that we evoked noradrenaline release in addition to, or instead of, dopamine release. Furthermore, a previous FCV study that used comparable coordinates for VTA stimulation in rats found that cutting cortically-projecting noradrenergic fibres had little effect on evoked cortical signals, indicating that these signals were attributable to dopamine (Shnitko and Robinson, 2014). We plan to confirm that the cortical signals recorded in our study are dopaminergic in future control experiments, either by using a similar approach to the Shnitko study or by chemically lesioning noradrenergic cells (Michael and Wightman, 1999).

Cortical dopamine is more difficult to detect than striatal dopamine due to the much lower levels of this neuromodulator that are found in the cortex (Moghaddam et al., 1990). Only a few studies have reported successful recordings of cortical dopamine using FCV, and

in all of those instances dopamine release was artificially stimulated in anaesthetised animals (Garris and Wightman, 1994; Garris et al., 1993; Shnitko and Robinson, 2014; Shnitko et al., 2014; Yavich et al., 2007). As seen in our NAc recordings (Chapter 4), stimulated release in striatum generally ranges from 10-30nA and is sometimes considerably larger, whereas behaviourally evoked signals in NAc are typically on the order of 1-5nA. Given that stimulated release in cortex was consistently less than 10nA in size, it would likely be challenging to detect behaviourally evoked cortical dopamine signals. Indeed, even when microdialysis is used, researchers may resort to chemical or electrical stimulation to boost cortical dopamine to measurable levels (Tunbridge et al., 2004). These technical limitations led us to use recordings of stimulated dopamine release in anaesthetised animals, rather than recordings of behaviourally-evoked release in awake animals, to study the effects of COMT inhibition and DAT blockade in both striatum and cortex and to compare transmission in these two regions.

Despite these challenges, we were able to reliably record evoked dopamine release in the MFC. The signals we recorded were quite consistent in their size and kinetics across animals. The size and time course of our MFC signals were broadly similar to those of cortical dopamine transients recorded in previous studies (Garris and Wightman, 1994; Garris et al., 1993; Shnitko and Robinson, 2014; Shnitko et al., 2014; Yavich et al., 2007).

Histological analysis revealed consistent placement of the recording electrodes in the prelimbic cortex (Figure 10a). There was more variability in the placement of the stimulating electrodes (Figure 10b). Although many terminated in or near the posterior-medial VTA, some terminated more laterally, along the VTA-SN border. This variability in the locus of stimulation may have contributed to variability in signal size across animals.

Nevertheless, given the large size of the stimulating electrodes relative to the midbrain dopaminergic nuclei, it is likely that some stimulation reached the cortically-projecting dopamine neurons even when the electrode placement was slightly off-target; indeed, we detected dopamine release in MFC in all animals tested.

5.4.2 Comparing dopamine release in NAc and MFC

We observed a more gradual rundown of the signal size in MFC over the course of the recording session than we did in NAc, despite the similarity in the placement of the stimulating electrode, in the stimulation parameters, and in the recording electrodes used to assess dopamine transmission in these two regions. Whereas NAc signals were highly variable during the first few hours of recording and displayed a rapid drop-off approximately 2.5hrs into the session before stabilising at less than 50% of their original size (see Figure 2 in Chapter 4), MFC signals underwent a slower and more gradual decline (Figure 1). The difference in the initial rundown between the NAc and MFC indicates that the circuitry of these two areas responds differently to repeated VTA stimulation – the larger rundown in the NAc suggests that release in this region is down-regulated in response to the stimulation, whereas adaptations in MFC appear to be subtler.

5.4.3 Effects of COMT inhibition on MFC dopamine

We found no effect of tolcapone on evoked dopamine release in MFC (Figure 5a). At the first time point, 85min after the first injection, there were no significant differences in any of the signal parameters analysed (peak height, AUC, or decay constant; Figures 6 and 7) between animals that received vehicle and those that received tolcapone as the first injection. An effect of tolcapone failed to emerge over time, as there were also no differences in the size or kinetics of dopamine transients between vehicle- and tolcapone-

treated animals at the second time point, 25min after the second injection (Figures 5c, 6, and 7).

The lack of effect of tolcapone was unexpected, as previous research suggested that this drug would alter cortical dopamine levels. Several microdialysis studies have demonstrated tolcapone-induced increases in cortical dopamine (Lapish et al., 2009; Tammimaki et al., 2016; Tunbridge et al., 2004) – although tissue homogenate studies have only observed an effect of tolcapone on levels of the dopamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), but not on dopamine itself (Laatikainen et al., 2013; Maj et al., 1990). Furthermore, COMT knockout mice have been shown to have elevated cortical dopamine (Gogos et al., 1998; Huotari et al., 2002b; Käenmäki et al., 2010), and the Val^{158}Met polymorphism is known to influence cortical dopamine transmission in humans (Slifstein et al., 2008). One might therefore expect that administration of tolcapone in our study would have increased cortical dopamine levels. Notably, however, the microdialysis studies that found an effect of tolcapone on dopamine did so only under conditions in which dopamine levels were elevated – by local chemical stimulation or systemic administration of a dopamine-increasing drug (Tammimaki et al., 2016; Tunbridge et al., 2004) – or in conjunction with a behavioural event such as presentation and consumption of food (Lapish et al., 2009). This suggests that COMT may become particularly important for regulating cortical dopamine in situations where this system is already activated, such as during heightened stress or engagement with a task (Thierry et al., 1976; Tunbridge et al., 2006). It also indicates that any influence of tolcapone on MFC dopamine transmission in our study might only emerge when the drug is administered together with GBR-12909; this is discussed further below.

5.4.4 Effects of DAT blockade on MFC dopamine

In contrast to its clear effects on evoked dopamine release in NAc recordings (see Chapter 4), GBR-12909 had no clear effects on MFC dopamine transients 25min after injection (Figure 5b). There were no significant main effects of GBR-12909 on the peak height, AUC, or decay constant. The analyses of peak height and decay constant suggest that GBR-12909 may have had subtle effects on cortical dopamine transmission (Figures 6 and 7), as both analyses found trend level interactions between drug 2 and time. Because these interactions did not reach significance, however, overall our data indicate that DAT blockade has little influence on dopamine clearance in the cortex, at least at the sub-second timescale assessed by FCV.

Previous findings on GBR-12909's effects in cortex are mixed. GBR-12909 is known to be a more effective uptake blocker in the striatum than in the cortex (Cass and Gerhardt, 1995; Izenwasser et al., 1990; Mundorf et al., 2001), as this drug is highly specific to the DAT (Andersen, 1989), and DAT expression levels are low in cortex (Sesack et al., 1998). Indeed, DAT knockout mice show little change in cortical dopamine levels (Gainetdinov and Caron, 2003). Nevertheless, several microdialysis studies have found that GBR-12909 produces a moderate but significant increase in dopamine levels in cortex (Carboni et al., 2006; Tanda et al., 1997; Valentini et al., 2004). However, other studies using a similar approach have found no such effect (Mazei et al., 2002; Pozzi et al., 1994; Weikop et al., 2007). Variability in the cortical region considered, the route of drug administration, the drug dosage, and the species used make it difficult to interpret these disparities.

The situation is further complicated by the presence of noradrenaline and its transporter, the NET, in cortex. The NET is known to take up dopamine as well as

noradrenaline (Raiteri et al., 1977) – indeed, NET may account for more dopamine reuptake than DAT within the cortex (Morón et al., 2002; Sulzer et al., 2016; Williams and Steketee, 2004). Pharmacological blockade of NET may thus affect cortical dopamine levels more significantly than DAT blockade. Selective NET blockers have been shown to increase cortical dopamine levels (Carboni et al., 1990; Mazei et al., 2002; Valentini et al., 2004; Yamamoto and Novotney, 1998), and several studies have found that the effects of DAT blockers like GBR-12909 are enhanced – or even only emerge – when they are administered together with NET blockers or when noradrenergic neurons are concurrently lesioned (Carboni et al., 2006; Pozzi et al., 1994).

An additional factor that may have contributed to the lack of a clear GBR-12909 effect on MFC dopamine in our recordings is that, as discussed in Chapter 4, this drug can adsorb to carbon fibre FCV electrodes and decrease their sensitivity, potentially resulting in a underestimation of GBR-12909's effects (Davidson et al., 2000). It is not clear whether this occurs *in vivo*, as the data on this effect have been gathered in *ex vivo* slice preparations, but if it does than reduced sensitivity at the electrode caused by GBR-12909 could have diminished the size of recorded dopamine transients and hampered our ability to detect drug-induced changes in the signal.

5.4.5 Combined effects of COMT inhibition and DAT blockade on MFC dopamine

The literature on tolcapone's and GBR-12909's effects in cortex discussed above suggests that the actions of these drugs may only become apparent in conjunction with additional manipulations. We anticipated, therefore, that tolcapone and GBR-12909 might together alter MFC dopamine transmission even if neither drug had a discernible effect on its own. However, no such additive effect was apparent in our data: there were no clear

differences in signal size or kinetics in GBR-12909-treated animals that had previously received tolcapone compared to those that had received vehicle (Figure 5c), and signals following Tolc-GBR treatment did not differ significantly on any of the parameters analysed from signals following other drug treatments (Figures 6 and 7). To our knowledge, the combined influence of tolcapone and GBR-12909 on cortical dopamine has not previously been assessed, although one study has examined the effects of GBR-12909 on dopamine tissue homogenate levels in the cortex of COMT-deficient transgenic mice; this study found no interaction of GBR-12909 with COMT genotype (Huotari et al., 2002a).

5.4.6 Effects of amphetamine on MFC dopamine

Given the lack of effect of either tolcapone or GBR-12909 in cortex, we tested an additional dopaminergic drug, amphetamine, in a subset of animals to ensure that we could detect drug-induced changes in MFC dopamine with our paradigm. As can be seen in Figures 8 and 9, amphetamine significantly increased signal size as assessed by both peak height and AUC and also appeared to slow decay kinetics. This finding is in line with previous demonstrations that amphetamine increases cortical dopamine levels (Mazei et al., 2002; Tanda et al., 1997). Amphetamine's clear effects in cortex may be due to its numerous mechanisms of action: it is known to inhibit uptake, induce reverse transport, and alter vesicular release of dopamine, among other things (Avelar et al., 2013; Daberkow et al., 2013; Jones et al., 1998b; Schmitz et al., 2001; Sulzer, 2011). Notably, amphetamine can also alter noradrenaline transmission and has affinity for NET (Sulzer, 2011), which, as mentioned above, may be more important than DAT for dopamine uptake in cortex.

5.4.6 Potential explanations for the lack of drug effects on MFC dopamine

One explanation for the lack of effect of either COMT inhibition or DAT blockade on cortical dopamine in our study, as well as for the variable findings in previous work, is that different types of transmission may be differently affected by these drugs. As discussed in the introduction, striatal dopaminergic transmission can either consist of large but brief surges in dopamine levels (phasic transmission) or steady, low-level release (tonic transmission), and FCV and microdialysis are best suited to assessing the former and the latter, respectively (Marinelli and McCutcheon, 2014; Robinson et al., 2003; Willuhn et al., 2010). While the distinction between phasic and tonic transmission is well characterised in striatum, it is not known whether cortical dopamine transmission can be similarly categorised. On the one hand, burst firing activity, which gives rise to phasic transmission (Goto et al., 2007; Wickham et al., 2013), does not appear to differ in dopamine neurons projecting to the cortex compared to the striatum (Marinelli and McCutcheon, 2014), suggesting that the phasic versus tonic transmission dichotomy is at least physiologically possible in both regions. However, the evidence gathered thus far highlights numerous differences between dopaminergic transmission in cortex and transmission in striatum – dopamine levels are substantially lower in cortex compared to striatum (Moghaddam et al., 1990), and cortical dopamine is believed to diffuse over longer distances and act over larger areas before it is cleared (Jones et al., 1998a; Mundorf et al., 2001; Sesack et al., 1998). Thus, the same phasic bursts of activity that cause large but short-lived transients in striatum might result in more prolonged release in cortex. Given the data currently available, it remains unclear whether the phasic-tonic distinction would make sense in a cortical context.

Theoretically, however, if the phasic versus tonic distinction applies in cortex, this could explain why tolcapone and GBR-12909 altered cortical dopamine in previous microdialysis studies but not in our FCV study. The effects of these drugs may become apparent only over the longer timescales assessed by microdialysis but not in the fast and short-lived phasic transients detected by FCV – which, as discussed in Chapter 3, requires background subtraction to resolve dopamine transients from the capacitative currents generated by the recording process and which is therefore ill-suited to detecting slower, more gradual changes in dopamine transmission. In our MFC recordings, we observed components of the evoked signal that emerged after, and lasted considerably longer than, the initial fast dopamine transient (Figure 12). Tolcapone or GBR-12909 may have affected this later component of the signal, perhaps even beyond the end of our 45sec recording window. Inspection of voltammograms in the later signal component indicated that it may have contained some dopamine but that several other compounds such as DOPAC, HVA, and other metabolites were likely present as well. Unfortunately, the chemical composition was too complex to allow us to identify exactly which compounds were contributing to this portion of the signal or to perform chemometric analysis on it.

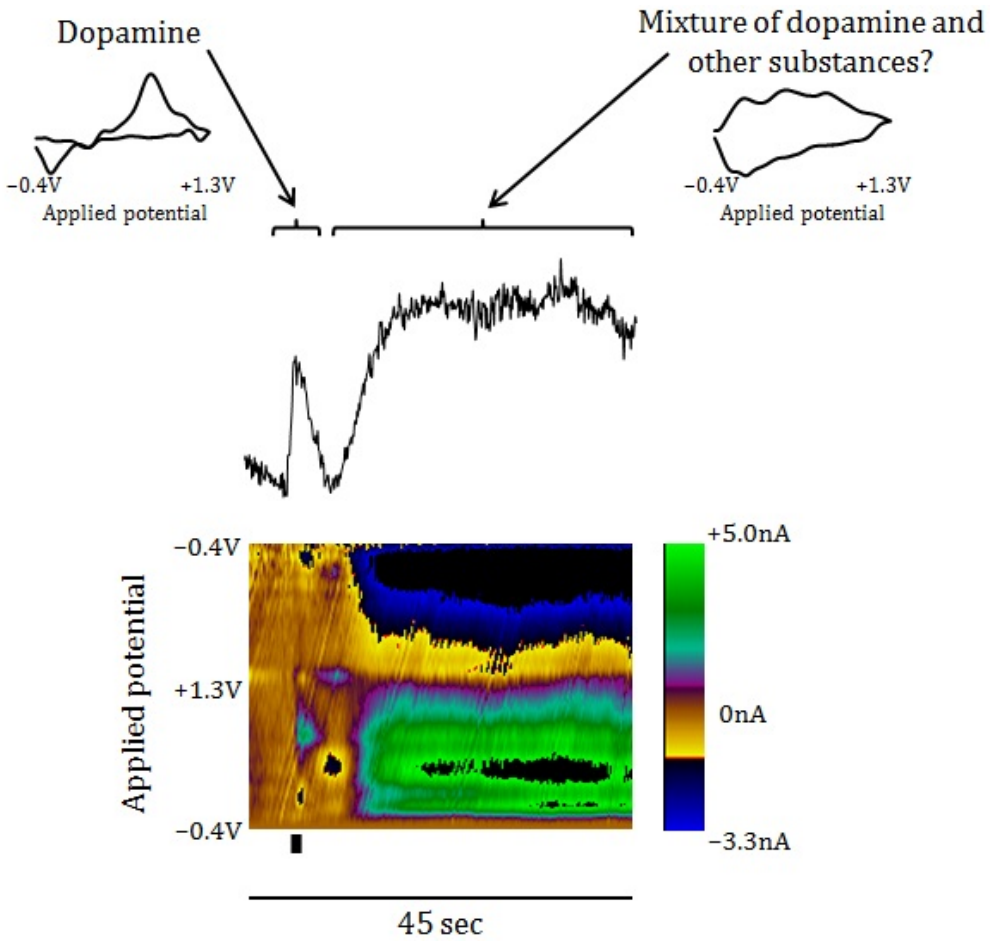

Figure 12: An example of a full length (45sec) MFC recording showing both the initial dopamine transient and the portion of the evoked signal that emerged after it. Voltammograms extracted during both the dopaminergic and later signal components are shown for comparison. Timing and duration of stimulation indicated by thick black bar below pseudocolour plot.

Chapter 6:
Effects of DAT blockade and COMT inhibition on the progressive ratio task

6.1 Introduction

Dopamine is implicated in multiple aspects of reward-guided behaviour. One of the leading theories about dopamine's role in such behaviour is that it mediates behavioural activation, motivational drive, and the exertion of effort in the pursuit of reward (Berridge, 2006; Robbins and Everitt, 2006; Salamone et al., 2007). Numerous studies have demonstrated the importance of striatal dopamine transmission, including its regulation by the dopamine transporter (DAT), to animals' ability to make cost/benefit decisions about exerting effort to obtain reward. In addition, there is some evidence that cortical dopamine also modulates motivation to work for reward. However, it is not known whether degradation of dopamine by catechol-*O*-methyltransferase (COMT) can also influence motivational drive. We therefore assessed the contributions of DAT and COMT to reward-guided behaviour on a classic test of motivation called the progressive ratio (PR) task.

6.1.1 Dopamine's role in motivational drive and exertion of effort for reward

Much of the evidence for dopamine's importance in mediating motivation to work for reward comes from studies showing that dopamine antagonists affect animals' willingness to exert effort for reward. Dopamine antagonists cause animals to choose rewards that require little work, are immediately accessible, or are freely available over options that require more work or are only available after a delay, even when the less effortful or immediate option yields a smaller or less palatable reward (Denk et al., 2005;

Salamone et al., 2007). This effect appears to arise from alterations in cost/benefit calculations about how to allocate effort in the pursuit of reward, and dopamine is thought to be particularly important for overcoming the costs of an effortful option in order to allow that option to be chosen (Phillips et al., 2007). Striatal dopamine, particularly in the nucleus accumbens (NAc), is central to mediation of these processes (Phillips et al., 2007; Salamone et al., 2007).

DAT-mediated regulation of dopamine transmission can impact motivation to work for reward. Both pharmacological blockade and genetic knockdown of DAT increase the amount of effort animals are willing to exert for a given reward and also shift choices to high-effort/high-palatability options over low-effort/low-palatability options (Cagniard et al., 2006a, 2006b; Young and Geyer, 2010). Given that DAT's most prominent influence is on striatal (rather than cortical) dopamine transmission – as seen in our voltammetry recording experiments in Chapters 4 and 5, as well as in elevated striatal dopamine levels in DAT knockdown mice (Cagniard et al., 2006a, 2006b; Zhuang et al., 2001) – the behavioural effects of DAT blockade and knockdown provide further evidence of the importance of striatal dopamine for mediating motivational drive and exertion of effort.

A number of studies indicate that the frontal cortex and cortical dopamine transmission also contribute to motivational and effort-related aspects of reward-guided behaviour. Lesions of medial frontal cortex (MFC) in rats shift choices away from high-effort/high-reward options and towards low-effort/low-reward options (Walton et al., 2002). There appears to be variability in the effect of lesioning different regions within the MFC. For example, anterior cingulate cortex (ACC) lesions affect rats' ability to expend effort for reward but not to tolerate a delay to obtain a high reward option (Walton et al.,

2003, 2007). Lesions of the prelimbic cortex, meanwhile, result in the opposite effect on effort expenditure compared to lesions of the medial orbital frontal cortex, with the former reducing and the latter increasing the amount of effort animals are willing to exert for reward (Gourley et al., 2010).

Dopamine in the MFC contributes to cortical mediation of motivation and effort expenditure. Infusion of a D1 dopamine receptor antagonist into the ACC reduces preference for a high-effort/high-reward option in rats (Schweimer and Hauber, 2006), as does lesion of dopamine terminals within the ACC (Schweimer et al., 2005) – although findings are mixed (Walton et al., 2005). Furthermore, infusion of a mixed D1/D2 dopamine receptor antagonist into the prelimbic cortex in rats reduces exertion of effort to obtain reward under conditions of food deprivation (Moscarello et al., 2010).

6.1.2 Aim and approach of this experiment

There is thus substantial evidence that cortical as well as striatal dopamine can influence motivation to work for reward, and DAT is clearly implicated in motivation and the exertion of effort. Previous studies have not investigated whether COMT contributes to these processes. However, given the importance of COMT in regulating cortical dopamine (at least under some circumstances; see Chapter 5 for discussion), it seems likely that COMT could affect motivational drive and effort expenditure, either on its own or in combination with DAT. Indeed, COMT inhibition has been shown to potentiate the effects of DAT blockade on behaviour in tests of motor function (Budygin et al., 1999; Raevskii et al., 2002). Interactive effects of DAT and COMT on reward-guided behaviour could be mediated either by the low levels of COMT expressed in striatum (Matsumoto et al., 2003) or via mesocorticolimbic circuitry, as reviewed in detail in Chapter 1.

We therefore examined how DAT and COMT contribute to motivation to work for reward using the PR task. This task is a self-initiated operant task that is standardly believed to probe motivational aspects of reward-guided behaviour (Cagniard et al., 2006a; Richardson and Roberts, 1996; Roane, 2008). Animals are initially trained to work for reward – in this study, by pressing a lever – on a fixed ratio (FR) schedule of reinforcement, such that a particular number of lever presses are always required to earn one reward within a given session. During the PR test session, however, the number of lever presses required to earn each reward increases over the course of the session, so that more and more work is required to obtain each subsequent reward. The point at which animals cease to lever press is taken as a measure of their maximum motivational drive to work for the reward.

We used a DAT blocker and COMT inhibitor to interfere with dopamine reuptake and degradation, respectively, in wildtype mice. Based on the previous work reviewed above, we predicted that the DAT blocker would exert clear effects on behaviour in the PR task and hypothesized that the COMT inhibitor would potentiate the DAT blocker's effects.

6.2 Methods

6.2.1 Subjects

Male C57BL/6 mice were obtained from Envigo (formerly Harlan). Mice were aged 10-23 weeks (Cohort 1: 18-23 weeks, Cohort 2: 10-15 weeks – see below for information aobut cohorts) during training and testing. Animals were housed on a 12/12-hr light/dark cycle (lights on at 0700hr) in groups of 4 mice per cage and were given water *ad libitum*. Mice were food deprived to 85-90% free feeding weight throughout the experiment to

encourage task engagement. Testing was conducted during the light phase. Care and testing of all animals was conducted under the auspices of the UK Home Office laws and guidelines for the treatment of animals under scientific procedures and of the local ethical review board at the University of Oxford.

A total of 24 mice were used for this experiment. Of these, 3 mice were excluded from the analysis: 2 due to ineffective injections and 1 that was unable to learn the complete task.

The mice were tested in two cohorts, with 12 animals per cohort. Cohort 1 had previously undergone the LMA-stereotypy and sucrose preference tests reported in Chapter 2, and each mouse in this cohort had thus already received 4 drug or vehicle injections; group assignment for this cohort was counterbalanced for previous drug exposure. Cohort 2, which was tested 21 months after the first cohort, had not undergone any previous testing. Mice in this cohort therefore received a vehicle injection 3 days prior to the first PR drug testing day (see below) so that the stress associated with the first injection an animal receives would not confound the experiment's findings. All mice were habituated to handling – including the restraint position used during injections – prior to testing.

6.2.2 Drugs

Drugs were dissolved in 20% hydroxypropyl-beta-cyclodextrin (Acros Organics) in 0.9% saline (AquPharm). This served as a vehicle control. The COMT inhibitor tolcapone (Ro 40-7592; 4'-methyl-3,4-dihydroxy-5-nitro-benzophenone) (TRC Inc) was used at a dose of 30mg/kg. The DAT blocker GBR-12909 (1-[2-(bis[4-fluorophenyl]methoxy)ethyl]-4-[3-phenylpropyl]piperazine) dihydrochloride (Tocris) was used at a dose of 6mg/kg.

The dihydrochloride was not taken into account when calculating the dosage, so GBR-12909 injections contained 6mg/kg of GBR-12909 dihydrochloride. See Chapter 2 for further information on tolcapone and GBR-12909 and on how these dosages were selected. Drugs and vehicle were delivered by intraperitoneal injection, with an injection volume of 5mL/kg.

6.2.3 Apparatus

The task was conducted in standard operant chambers (Med Associates Inc) measuring 22 x 20 cm. Boxes were equipped with: two levers, on the left and right sides of one wall; a receptacle located between the levers to which the reward was delivered by a syringe pump (Med Associates Inc); a cue light above the receptacle; and a house light placed on the roof of the box. In each box, one lever was assigned to be 'active' – pressing it resulted in delivery of reward to the receptacle in conjunction with illumination of the cue light – and one was 'inactive' – pressing it had no effect. The position of active and inactive levers was counterbalanced across mice.

6.2.4 Training schedule

Testing was conducted between 0800hr and 1600hr, with all mice tested each day except where otherwise noted. An individual mouse was always tested in the same operant box. Rewards consisted of 60μL of 10% weight/volume sucrose solution (Sigma Aldrich).

The experiment consisted of habituation, training, and testing phases completed over either 34 days (for Cohort 1) or 36 days (for Cohort 2). These phases are described in Table 1; the session length, size of a single reward, and criterion required to move on to the next phase are specified. Mice that reached criterion early were subsequently tested only

on alternate days until all mice had achieved criterion, at which point all animals were moved to the next phase of training.

On PR testing days, the number of active lever presses required to obtain each subsequent reward was increased according to the following equation (Richardson and Roberts, 1996; Sharma et al., 2012):

$$\text{Number of required lever presses} = 5*e^{i*0.16} - 5$$

where 'i' is the trial number and 'e' is the base of the natural logarithm. (Note that one trial consists of the lever presses required by a given level of the PR schedule to obtain a single reward).

Phase (duration)	Details	Session length	Reward size	Criterion
Sucrose exposure (1 day)	Sucrose provided in home cage	Overnight	*ad libitum*	N.A.
Habituation (1 day)	Sucrose provided in operant box receptacle	20min	*ad libitum*	N.A.
Magazine training (3 days)	Sucrose delivered on variable interval schedule	30min	20-60uL	N.A.
FR1 (10-11 days)	1 lever press resulted in delivery of 1 reward	30min	40-60uL	≥ 20 rewards, ≥ 3:1 active:inactive lever presses, on 2 consecutive days
FR2 (3 days)	2 lever presses resulted in delivery of 1 reward	30min	60uL	≥ 15 rewards, ≥ 3:1 active:inactive lever presses, on 2 consecutive days
FR3 (1 day)	3 lever presses resulted in delivery of 1 reward	30min	60uL	≥ 15 rewards, ≥ 3:1 active:inactive lever presses
FR5 (3 days)	5 lever presses resulted in delivery of 1 reward	30min	60uL	≥ 15 rewards, ≥ 3:1 active:inactive lever presses, on 2 consecutive days
PR (3-4 days)	Number of lever presses resulting in delivery of 1 reward increased for each subsequent reward	45min	60uL	Session terminated early if 5min elapses without lever press
Drug testing (10 days)	3-day cycle: PR with drug, FR5 without drug, PR without drug; repeated 3x	45, 30, or 30 min	60uL	N.A.

Table 1: Phases of training and testing during the progressive ratio task. FR: fixed ratio schedule of reinforcement. PR: progressive ratio schedule of reinforcement.

6.2.5 Drug testing

On drug testing days, mice received 2 injections prior to starting the task. Drugs were administered according to a within-subjects design, so that each mouse received all drug treatments (Table 2) in rotation. The order of the rotation was pseudorandomised across mice and was counterbalanced for home cage, testing box, time of day of testing, and, for Cohort 1, previous drug treatment.

Treatment	1st injection	2nd injection
Veh-Veh	Vehicle	Vehicle
Tolc-Veh	Tolcapone	Vehicle
Veh-GBR	Vehicle	GBR-12909
Tolc-GBR	Tolcapone	GBR-12909

Table 2: Drug treatments.

Drug testing days were interleaved with two washout days during which no injections were given: one day of testing on an FR5 schedule and one day of testing on a PR schedule. This testing sequence is illustrated in Figure 1.

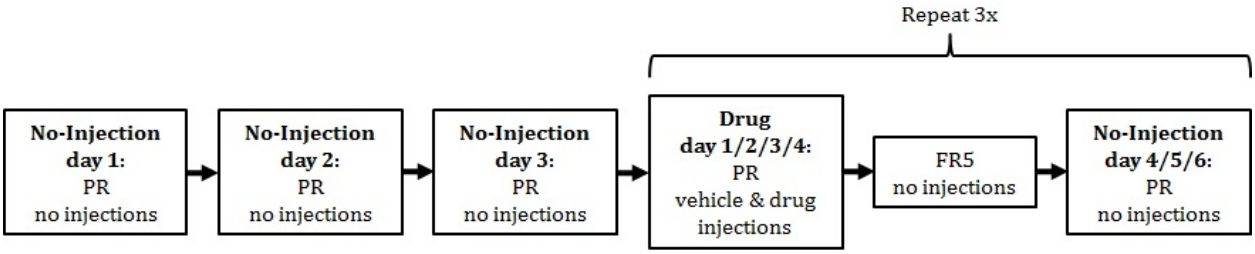

Figure 1: Testing sequence during the final two phases of the PR task (see Table 1).

The first injection (vehicle or tolcapone) was given prior to the second injection (vehicle or GBR-12909) to account for differences in the drugs' time courses of action. The timing of the injections differed slightly between the two cohorts, as shown in Figure 2. Cohort 1 received the first injection 120min, and the second injection 60min, before testing began. Cohort 2 received the first injection 105min, and the second injection 15min, prior to testing. The timing of drug administration was adjusted in Cohort 2 to more closely match the timing used in the anaesthetized voltammetry recordings presented in Chapters 4 and 5.

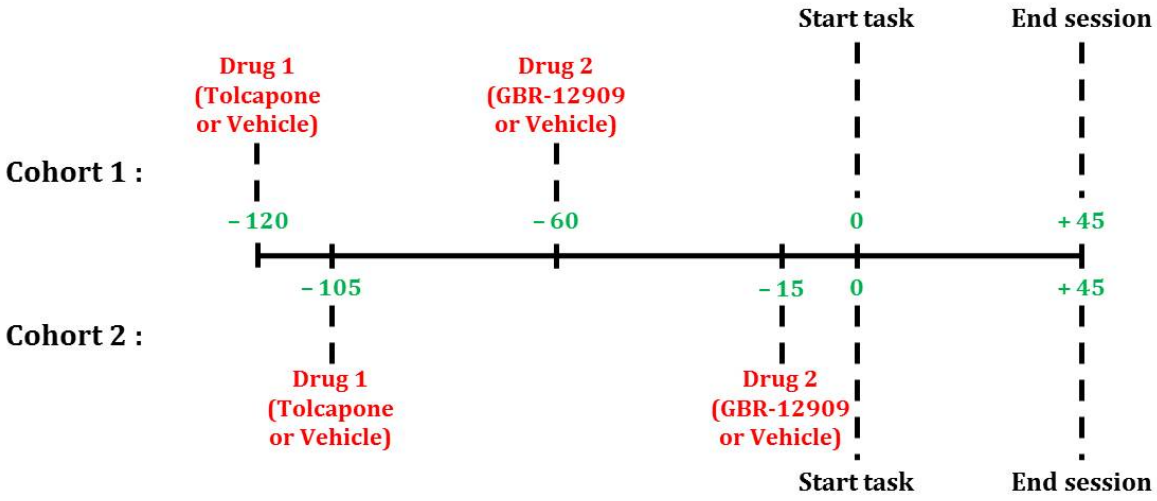

Figure 2: Timing of drug injections relative to the testing session in Cohort 1 (above timeline) versus Cohort 2 (below timeline). Time, in minutes, is indicated in green and is measured relative to the start of the task (i.e. time 0). Drug injections are indicated in red.

6.2.6 Analysis

Data were analysed using Microsoft Excel 2010 and Matlab R2012a and plotted using GraphPad Prism version 5.04. Statistical comparisons were performed using SPSS version 20 with significance set at $\alpha = 0.05$. For repeated-measures ANOVAs, Greenhouse-Geisser corrections were applied where data failed Mauchley's test of sphericity; degrees of freedom are reported to two decimal places in such cases. Simple main effects analyses were conducted as necessary when significant interactions were found. Least square difference pairwise comparison tests were used to assess which groups were driving significant main and interactive effects.

Four parameters were used to assess the effects of drug treatment on task performance. The number of **active lever presses** served as a measure of motivation to work for reward, while the number of **inactive lever presses** provided information on general activity levels. We also examined the effects of drug treatment on two additional

measures of motivation. The **reward collection latency** was defined as the time between illumination of the magazine light, which indicated reward delivery, and the animal's subsequent head entry into the magazine. The **re-engagement latency** was defined as the interval between the time the animal exited the magazine after consuming a reward and the time it made its next lever press (on either the active or inactive lever).

For analysis of lever pressing, we calculated the total number of active or inactive lever presses for each animal within each testing session and then averaged across animals within each treatment condition. For the analyses of reward collection and re-engagement latencies, we calculated the mean latency for each animal within each testing session and then averaged across animals based on treatment condition.

We performed two types of repeated-measures ANOVAs on each performance parameter. The first took the same approach that was used to analyse the voltammetry recording data presented in Chapters 4 and 5: this ANOVA included Drug 1 and Drug 2 as separate within-subjects factors. However, in order to be able to compare performance following drug and vehicle injections to performance on No-Injection PR testing days (see Figure 1), we also performed an ANOVA with a single within-subjects factor, Drug Group, that encompassed all 5 possible treatment conditions (including the No-Injection condition). Testing cohort was included as a between-subjects factor in both analyses. Main and interactive effects of testing cohort are reported in the results section only when they approached or reached significance.

In order to compare the drug and vehicle treatments to the No-Injection condition in the second ANOVA, we used averaged data from the six No-Injection PR sessions as a measure of No-Injection performance. However, because three of the No-Injection PR

sessions were interleaved with drug testing days (refer to Table 1 and Figure 1), we first checked that there were no lingering effects of either tolcapone or GBR-12909 treatment on subsequent No-Injection days that should be taken into account in our analyses. We assessed whether Drug 1 (vehicle or tolcapone) affected subsequent No-Injection PR sessions by comparing active lever pressing following drug days when mice received tolcapone as Drug 1 to active lever pressing following drug days when mice received vehicle as Drug 1. Similarly, to assess whether Drug 2 (vehicle or GBR-12909) affected subsequent No-Injection PR performance, we compared active lever pressing following drug days when animals received GBR-12909 as Drug 2 to active lever pressing following drug days when they received vehicle as Drug 2.

6.3 Results

6.3.1 No-Injection PR performance

We found no indication that either tolcapone or GBR-12909 affected performance on subsequent No-Injection PR sessions (Figure 3). There was no significant difference in active lever pressing on No-Injection days following tolcapone versus vehicle Drug 1 injections (paired t-test: $t(20) = -0.3$, $p = 0.749$), nor was there a significant difference in active lever pressing on No-injection days following GBR-12909 versus vehicle Drug 2 injections (paired t-test: $t(20) = 0.3$, $p = 0.756$). When examining each of the parameters discussed below, therefore, we averaged performance on that parameter across the six No-Injection PR testing days for each animal and then compared its performance under each drug treatment to this average, in addition to comparing performance across drug treatments.

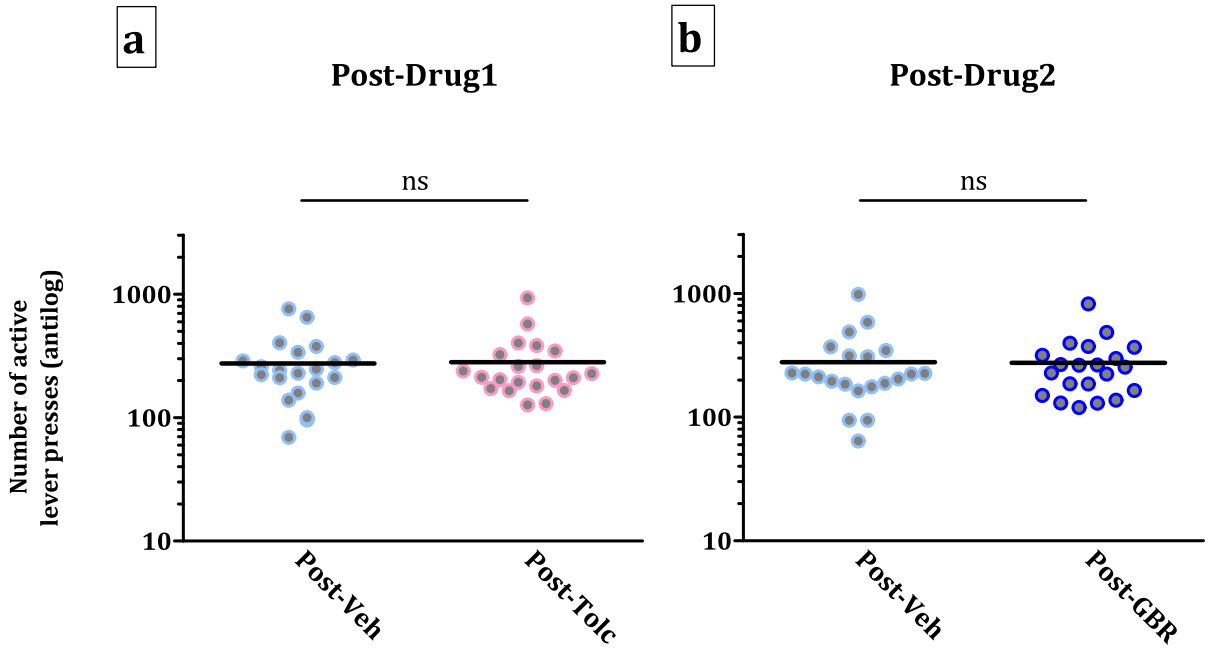

Figure 3: Active lever pressing on No-Injection PR sessions following drug testing days. a) Lever pressing following drug days on which animals received vehicle as Drug 1 (light blue) compared to those on which they received tolcapone as Drug 1 (pink). b) Lever pressing following drug days on which animals received vehicle as Drug 2 (light blue) compared to those on which they received GBR-12909 as Drug 2 (dark blue). Each animal's data is shown individually (total n = 21) and displayed on an antilog (base 10) scale for clarity. Horizontal black bars indicate the means for each condition. ns = not significant

6.3.2 Lever presses

GBR-12909 increased the number of active lever presses mice performed (Figure 4a and Table 3). We assessed the significance of this effect using a repeated-measures ANOVA in which Drug 1 (vehicle or tolcapone) and Drug 2 (vehicle or GBR-12909) were defined as separate within-subjects factors. The ANOVA revealed a significant main effect of Drug 2 on the number of active lever presses ($F_{1,19} = 26.4$, $p < 0.001$). The interaction between Drug 2 and the testing cohort approached significance ($F_{1,19} = 3.5$, $p = 0.076$), suggesting that the degree of the GBR-12909 effect may have differed between the two cohorts.

Contrary to predictions, however, tolcapone had no effect on active lever pressing, either on its own or when administered together with GBR-12909. There was no significant main effect of Drug 1 and no significant interaction of Drug 1 and Drug 2 on active lever pressing (F's < 1.1, p's > 0.31).

Neither GBR-12909 nor tolcapone affected the number of inactive lever presses (Figure 4b). A repeated-measures ANOVA found no main or interactive effects of Drug 1 or Drug 2 on inactive lever pressing (repeated-measures ANOVA, F's < 2.7, p's > 0.11).

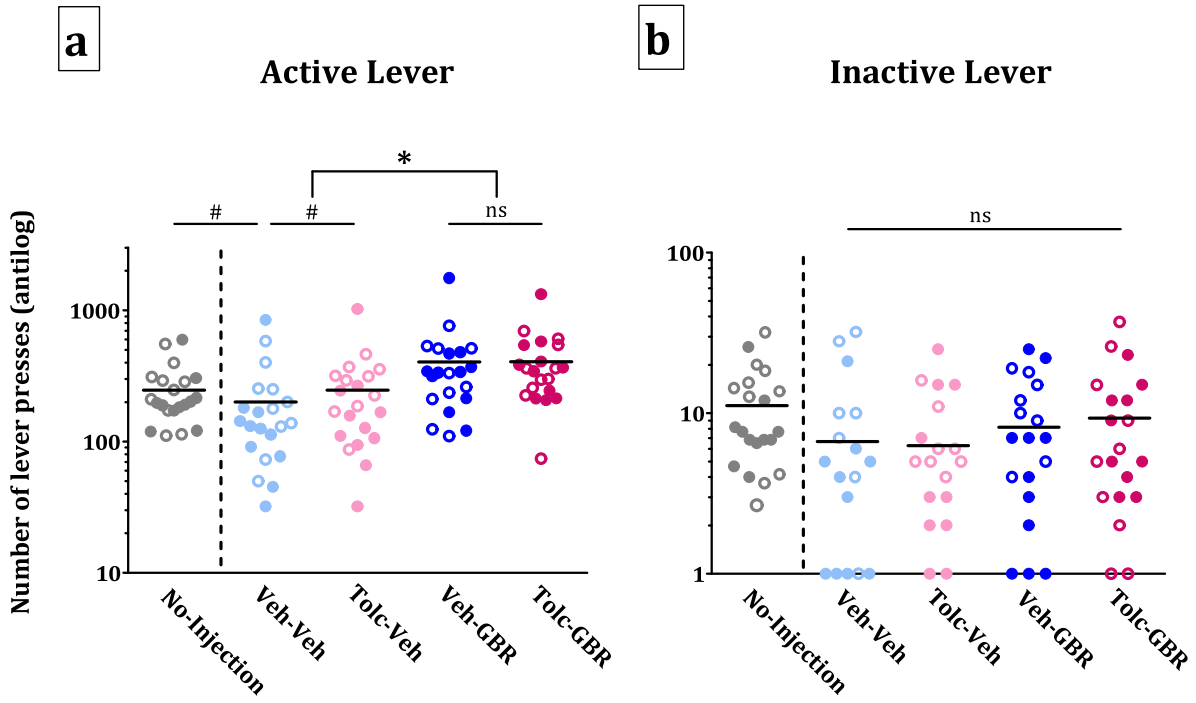

Figure 4: Drug effects on active (a) and inactive (b) lever pressing, displayed on an antilog (base 10) scale for clarity. Each animal's data is shown individually and colour-coded by drug treatment (blue and pink symbols). The average number of lever presses made by each animal on PR testing days on which it received neither drug nor vehicle injections is included for comparison (grey symbols). Data from both Cohort 1 (closed symbols) and Cohort 2 (open symbols) are presented (total n = 21). Horizontal black bars indicate the means for each drug treatment condition. ns = not significant, * = p < 0.05 in Drug 1/Drug 2 ANOVA, # = p < 0.05 in Drug Group ANOVA

Cohort	Active lever presses				
	No-Injection	**Veh-Veh**	**Tolc-Veh**	**Veh-GBR**	**Tolc-GBR**
1	225.89 ± 39.84	177.09 ± 68.15	217.36 ± 83.19	446.27 ± 135.82	437.55 ± 96.05
2	269.13 ± 42.49	225.60 ± 51.00	277.80 ± 35.13	359.20 ± 66.99	371.60 ± 59.94
Cohort	**Inactive lever presses**				
	No-Injection	**Veh-Veh**	**Tolc-Veh**	**Veh-GBR**	**Tolc-GBR**
1	8.82 ± 1.81	4.36 ± 1.77	6.64 ± 2.46	7.18 ± 2.58	8.27 ± 2.03
2	13.68 ± 2.81	9.20 ± 3.69	5.90 ± 1.46	9.30 ± 2.14	10.50 ± 3.84

Table 3: Average number of active (top) and inactive (bottom) lever presses, displayed as mean ± SEM, for each drug treatment in each cohort.

Our lab has previously observed that tolcapone can counter the effects of injection stress, and we hypothesised that this phenomenon might produce a difference in performance on the PR task following administration of tolcapone versus vehicle alone in comparison to performance on No-Injection PR sessions. Indeed, inspection of the data (Figure 4a) indicates that the Veh-Veh treatment decreased lever pressing compared to the No-Injection sessions, whereas no such decrease is apparent following Tolc-Veh treatment, which appears to have induced similar levels of active lever pressing to those seen on No-Injection days. In order to assess this effect, we performed a second repeated-measures ANOVA using a single within-subjects factor, Drug Group, with 5 levels: the 4 drug groups from above and a No-Injection condition consisting of average performance on the six PR sessions when animals received neither vehicle nor drug injections.

This second ANOVA found a significant main effect of Drug Group ($F_{2.07,39.40} = 11.8$, $p < 0.001$). Posthoc tests confirmed the findings of the Drug 1/Drug 2 ANOVA, as both the Veh-GBR and Tolc-GBR treatments significantly increased active lever pressing compared to all other treatment conditions, including No-Injection sessions (p's < 0.02). The Drug Group ANOVA, however, revealed an additional effect: active lever pressing was

significantly reduced by Veh-Veh treatment compared to both the No-Injection condition ($p = 0.046$) and to Tolc-Veh treatment ($p= 0.033$). By contrast, there was no difference between the Tolc-Veh and No-Injection days ($p = 0.998$). In addition, there was no difference between the Veh-GBR and Tolc-GBR treatment conditions ($p = 0.965$).

The second ANOVA analysis confirmed that there were no drug effects on inactive lever pressing, as the main effect of Drug Group approached but did not reach significance ($F_{2.64,50.16} = 2.7$, $p = 0.065$).

6.3.3 Latencies

We found that GBR-12909 had minimal effects on the speed with which mice collected rewards (Figure 5a and Table 4). A repeated-measures ANOVA with Drug 1 and Drug 2 included as separate within-subjects factors showed that Drug 2 had a borderline significant main effect on reward collection latency ($F_{1,19} = 4.1$, $p = 0.056$), suggesting that animals may have collected their rewards slightly faster following GBR-12909 compared to vehicle treatment.

By contrast, there was no indication of an effect of tolcapone on reward collection latency. Drug 1 had no significant main effect on the collection latency ($F_{1,19} = 2.0$, $p = 0.175$), and the interaction between Drug 1 and cohort approached but did not reach significance ($F_{1,19} = 3.2$, $p = 0.089$). Furthermore, there was no significant interaction between Drug 1 and Drug 2 ($F_{1,19} = 2.4$, $p = 0.142$).

The Drug1/Drug2 repeated-measures ANOVA did reveal a significant main effect of testing cohort on reward collection latency ($F_{1,19} = 7.0$, $p = 0.016$). In addition, there was a borderline significant three-way interaction between Drug 1, Drug 2, and cohort ($F_{1,19} =$

4.2, p = 0.055). Posthoc testing suggested that this interaction was driven by Cohort 1, in which there was a significant difference in collection latency in animals that received vehicle versus tolcapone as Drug 1 (p = 0.019) as well as in animals that received vehicle versus GBR-12909 as Drug 2 (p = 0.009). No other posthoc comparisons were significant (p's > 0.43).

As in the analysis of the lever press data, we performed a second repeated-measures ANOVA with a single within-subjects factor, Drug Group, in order to be able to compare performance on No-Injection testing days to performance under each of the drug treatments. The results of this second ANOVA were largely in agreement with the Drug1/Drug2 analysis. The second ANOVA found a trend level effect of Drug Group, ($F_{1.96,37.18}$ = 3.0, p = 0.065). There was again a significant main effect of testing cohort ($F_{1,19}$ = 7.9, p = 0.011) but only a trend level interaction between Drug Group and testing cohort ($F_{1.96,37.18}$ = 2.8, p = 0.075).

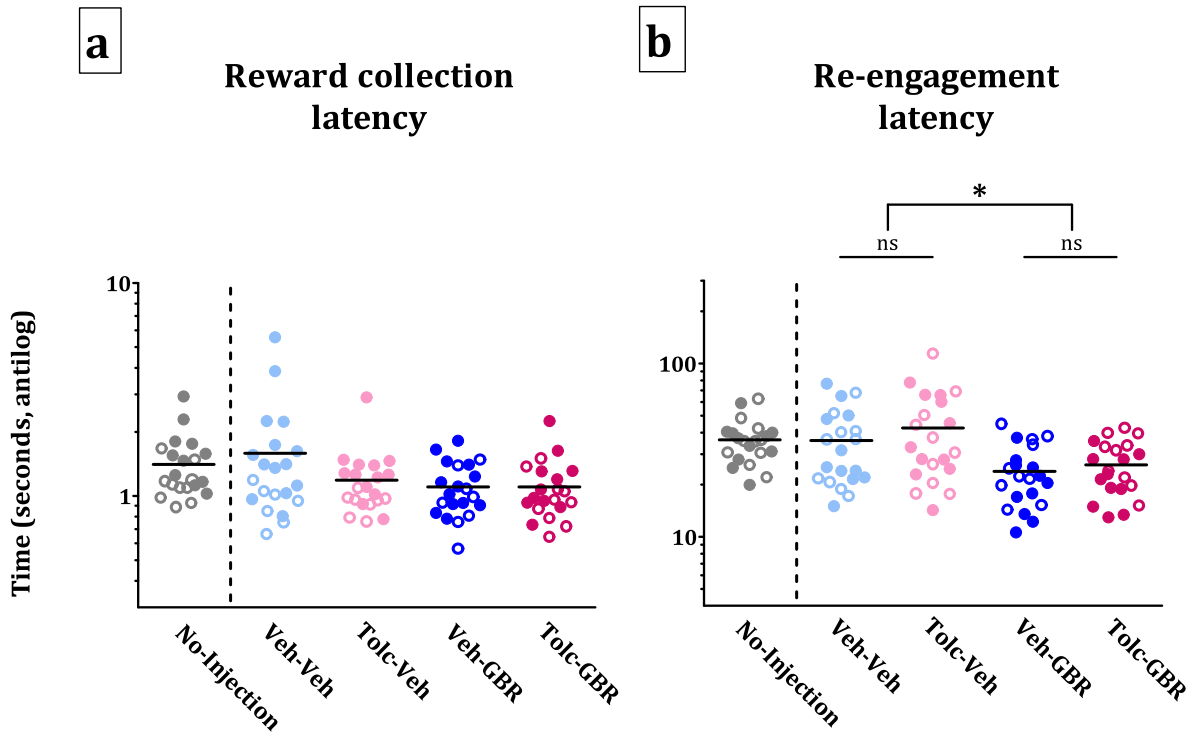

Figure 5: Drug effects on the latency to collect the reward after its delivery (a) and the latency to recommence lever pressing following reward consumption (b), displayed on an antilog (base 10) scale for clarity. Each animal's data is shown individually and colour-coded by drug treatment (blue and pink symbols). The average latency for each animal on PR testing days on which it received neither drug nor vehicle injections is included for comparison (grey symbols). Data from both Cohort 1 (closed symbols) and Cohort 2 (open symbols) are presented (total n = 21). Horizontal black bars indicate the means for each drug treatment condition. ns = not significant, * = $p < 0.05$

Cohort	Reward collection latency (sec)				
	No drugs	Veh-Veh	Tolc-Veh	Veh-GBR	Tolc-GBR
1	1.63 ± 0.17	2.10 ± 0.43	1.22 ± 0.07	1.20 ± 0.10	1.20 ± 0.13
2	1.16 ± 0.08	1.03 ± 0.09	1.14 ± 0.20	1.00 ± 0.09	0.99 ± 0.09
Cohort	**Re-engagement latency (sec)**				
	No drugs	**Veh-Veh**	**Tolc-Veh**	**Veh-GBR**	**Tolc-GBR**
1	35.53 ± 3.10	36.56 ± 6.08	42.22 ± 6.49	20.87 ± 2.37	22.29 ± 2.23
2	36.92 ± 3.71	35.12 ± 5.12	42.72 ± 9.43	27.13 ± 3.31	30.07 ± 2.97

Table 4: Average reward collection latency (top) and re-engagement latency (bottom), displayed as mean ± SEM, for each drug treatment in each cohort.

The final measure of performance that we examined was the latency to re-engage with the task following consumption of a reward (Figure 5b and Table 4), defined as the time that elapsed between an animal removing its head from the magazine after receiving a reward and the first lever press that it subsequently made. A repeated-measures ANOVA with Drug 1 and Drug 2 as separate within-subjects factors found that GBR-12909 significantly shortened the re-engagement latency, as there was a significant main effect of Drug 2 on this parameter ($F_{1,19} = 15.3$, $p = 0.001$). The main effect of Drug 1 approached but did not reach significance ($F_{1,19} = 3.0$, $p = 0.099$), and there was no significant interaction of Drug 1and Drug 2 ($F_{1,19} = 0.6$, $p = 0.443$), indicating that tolcapone did not influence the re-engagement latency.

A repeated-measures ANOVA with a single within-subjects factor confirmed these results. There was a significant main effect of Drug Group ($F_{2.13,40.46} = 7.6$, $p = 0.001$) that posthoc tests revealed was driven by significantly shorter re-engagement latencies following the Veh-GBR and Tolc-GBR treatments compared to all other treatment conditions (p's < 0.02). There was no difference in re-engagement latency between the Veh-GBR and Tolc-GBR conditions ($p = 0.205$) and no significant differences between the No-Injection, Veh-Veh, and Tolc-Veh conditions (p's > 0.21).

6.4 Discussion

We found that administration of the DAT blocker GBR-12909 increased motivation to work for reward on the PR task. GBR-12909 increased active lever pressing and caused animals to resume lever pressing more quickly after completing a trial. Effects of the COMT inhibitor tolcapone were much less prominent, and it did not potentiate the effects of GBR-

12909 as we had predicted. However, tolcapone did appear to ameliorate injection-induced decreases in active lever pressing, suggesting that tolcapone may counteract the effects of injection stress.

6.4.1 Effects of DAT blockade on PR performance

GBR-12909 selectively increased pressing on the active, but not on the inactive, lever (Figure 4). This specificity indicates that the drug's effect on lever pressing was not simply a consequence of the increased activity levels induced by GBR-12909 (see locomotor activity experiment results in Chapter 2) and suggests that DAT blockade modulated motivation to work for reward. However, caution is required in interpreting the results of PR experiments due to several caveats about the task itself. The PR task, although widely used, is a relatively simplistic means of assessing motivation, and its results cannot always be clearly interpreted. Specifically, it is difficult to dissociate goal-directed action from the arousal and vigor necessary to exert effort – both of which are key components of motivation to pursue reward (Bailey et al., 2015a).

Because of this quandary, some researchers have developed alternatives to the PR task that disentangle the various components of motivated behaviour. One option is the 'concurrent choice' task developed by Cagniard and colleagues, which requires animals to choose between a high-effort, high-reward option – lever pressing for a highly palatable reward such as chocolate pellets – and a low-effort, low-reward option – standard rodent chow that is freely available on the cage floor (Cagniard et al., 2006a). This task more clearly dissociates motivated behaviour from locomotor activity levels but still does not distinguish arousal/vigor to exert effort from goal-directed action. By contrast, Bailey and colleagues have recently designed a clever variation on the PR task called the 'progressive

hold down' task in which animals are trained to hold down a lever for increasingly long periods of time in order to obtain each subsequent reward (Bailey et al., 2015a, 2015b). This task not only eliminates the locomotor activity confound but also differentiates between effects on vigor to work for reward and effects on goal-directed action.

We do not predict that testing GBR-12909 (or tolcapone) on either of these alternative tasks would yield different results to our PR experiment. Nevertheless, it is important to keep in mind that, although we interpret GBR-12909's effects on the PR task as increased motivation to work for reward, a definitive conclusion about the specific component of motivated behaviour that is affected by GBR-12909 cannot be drawn from the PR paradigm.

The analysis of active lever pressing found a trend level interaction between the effect of Drug 2 and testing cohort, indicating that the strength of the GBR-12909 effect differed between the cohorts. This is likely due to the difference in the timing of drug administration in the two cohorts (see Figure 2). Indeed, inspection of the mean number of active lever presses in each of the two cohorts (Table 3) reveals that GBR-12909 induced more active lever presses in Cohort 1, in which the drug had had an hour to act before the session began, than in Cohort 2, in which it had had only 15 minutes to act.

GBR-12909 also affected re-engagement latency by causing animals to return to lever pressing more quickly following consumption of rewards (Figure 5b). This effect could be interpreted as a consequence of the locomotor effects of GBR-12909 rather than of its motivating properties. However, given the specificity of GBR-12909's effects on the active compared to the inactive lever, we believe that the effect on re-engagement latency

is more likely indicative of increased motivation under DAT blockade to more quickly resume the task and start working to earn the next reward.

One might expect GBR-12909 to similarly speed reward collection latency (Figure 5a), but we found only hints of such an effect, as the effect of Drug 2 on this parameter was only borderline significant. It is possible that there is a floor effect on the collection latency measure that minimises any influence of GBR-12909. Given that it took animals only 1-2sec to enter the magazine following reward delivery in the No-Injection condition (i.e. in the absence of any drug or vehicle injections; see Table 4), there may not have been much scope for the drug to speed this process. The reward collection latency analysis indicated that the speed of reward collection differed between the two cohorts. A three-way interaction amongst testing cohort, Drug 1, and Drug 2 suggested that this difference might be due to a difference in the effects of tolcapone and GBR-12909 in the two cohorts. However, the interaction is more likely driven by several outlier data points in the Veh-Veh treatment condition in Cohort 1 (see Figure 5a), which resulted in a slower mean Veh-Veh collection latency in Cohort 1 compared to Cohort 2 (Table 4), rather than to effects of either of the experimental drugs.

Our findings on the effects of GBR-12909 confirm numerous previous studies in rodents that have found that manipulation of dopamine levels, particularly in the striatum, alters motivation on the PR task. Both systemic pharmacological blockade and genetic knockdown of DAT have been shown to increase responding during PR sessions in mice (Cagniard et al., 2006a, 2006b; Young and Geyer, 2010). In addition, infusion of amphetamine (which increases extracellular dopamine levels by a number of mechanisms, including DAT blockade (Avelar et al., 2013; Jones et al., 1998b)) directly into NAc induced

increased lever pressing on the PR task in rats (Zhang et al., 2003). Conversely, dopamine receptor antagonists, whether systemically administered or locally infused into NAc, decrease PR lever pressing, as does permanent lesion of NAc dopamine terminals using 6-hydoxydopamine (6-OHDA) (Aberman et al., 1998; Hamill et al., 1999; Moscarello et al., 2010). As in our experiment, these manipulations appeared to specifically affect motivation to work for reward rather than other aspects of reward-guided behaviour: only active, but not inactive, lever pressing was increased by DAT blockade and knockdown; and dopamine antagonists and 6-OHDA lesions impaired responding only on PR sessions, but not when FR or variable interval schedules of reinforcement were used.

Of these previous investigations, one study used GBR-12909 in mice, as we did, to examine the influence of DAT on the PR task. This study reported effects of GBR-12909 on exertion of effort for reward at a 16mg/kg dose but not at lower (10mg/kg) or higher (28mg/kg) doses (Young and Geyer, 2010). One might therefore expect the 6mg/kg dose used in our experiments not to produce an effect. However, we found that 6mg/kg GBR-12909 reliably increased the size of phasic dopamine transients in the NAc in our voltammetry recording experiments (Chapter 4). Given that we observed stereotypic effects of GBR-12909 with an 8mg/kg dose during locomotor activity testing (see Chapter 2), it is possible that stereotypic behaviours affected performance in the Young and Geyer study, which did not report whether any motor effects of the drug were observed. In addition, the wildtype mice used in this previous study were littermate controls of a transgenic line, whereas mice used in our experiments were purebred C57BL/6 mice; this difference may also explain the discrepancy.

6.4.2 Effects of COMT inhibition on PR performance

Unlike GBR-12909, tolcapone did not dramatically alter behaviour in the PR experiment. We initially predicted that COMT inhibition might impact PR performance, either on its own or in combination with DAT blockade, because of evidence from previous work that the MFC and dopamine transmission in this region influence motivational and effort related aspects of reward-guided behaviour. Lesions of the prelimbic cortex and ACC reduce the amount of effort animals will exert to obtain reward (Gourley et al., 2010; Walton et al., 2003), and dopamine transmission within these regions is critical for cortical mediation of motivation to work for reward, including on the PR task (Moscarello et al., 2010; Schweimer and Hauber, 2006; Schweimer et al., 2005). Despite these findings, it remains unclear to what extent COMT regulates the influence of cortical dopamine on reward-guided behaviour. Our voltammetry recording data suggest that not all types of dopaminergic transmission in MFC are subject to regulation by COMT (see Chapter 5). Therefore, although cortical dopamine may contribute to the mediation of PR responding, the administration of a COMT inhibitor like tolcapone may not alter this contribution.

Although it did not impact motivation to work for reward, tolcapone did have a subtle effect on active lever pressing on the PR task. This effect emerged in one of the two types of statistical analyses that we performed on the data. The first analysis, in which Drug 1 and Drug 2 are included as separate within-subjects factors, is advantageous for detecting potential interactive effects between the two experimental drugs. The second, however, usefully allows the inclusion of data from No-Injection PR sessions in the analysis, as this approach uses a single within-subjects factor, Drug Group, that can encompass data from all PR sessions. While we found no effects of tolcapone on any of the performance

parameters using the Drug 1/Drug 2 approach, the second Drug Group approach revealed an influence of tolcapone on behaviour. Specifically, this second analysis uncovered a dampening effect of the Veh-Veh treatment on performance in comparison to No-Injection performance, seen in a decrease in active lever pressing (Figure 4a). The Tolc-Veh treatment reversed this dampening effect, as active lever pressing during Tolc-Veh sessions was comparable to that seen during No-Injection sessions.

It should be noted that we did not correct for multiple comparisons when performing posthoc tests in the Drug Group analysis and that the effect of tolcapone would likely be lost if we were to implement a multiple comparisons correction. Therefore, this finding should be interpreted with caution. Nevertheless, it suggests an interesting impact of COMT inhibition on behaviour, as discussed further below.

One potential explanation for this finding is that tolcapone may modulate the effects of injection stress. It is known that intraperitoneal injections activate the stress response in mice (Drude et al., 2011; Meijer et al., 2006). Whether increased stress levels are detrimental or beneficial to behaviour, however, is a complex question. According to the inverted-U hypothesis of the relationship between stress and behavioural performance (see Chapter 1 for details), increased arousal can improve performance up to a point but proves detrimental if stress levels become too high (Yerkes and Dodson, 1908). Depending on where animals sit on the inverted-U during testing, therefore, the stress of an injection may adversely affect their performance. Indeed, a previous study from our lab found that vehicle-injected rats performed worse than both non-injected controls and tolcapone-treated animals on several tasks used to assess memory (Laatikainen et al., 2012).

In line with this study, our PR data suggest that tolcapone can reverse the detrimental effects of stress caused by Veh-Veh injections. This effect is subtle, as it only appears when performance under the non-GBR-12909 treatments is compared to performance during No-Injection sessions, possibly because the increase in responding under GBR-12909 masks any auxiliary stress-induced changes in performance. Nevertheless, given the frequency with which we have observed an apparent reversal of injection stress-induced behavioural deficits by tolcapone in our lab, this effect merits further investigation.

Chapter 7:
Effects of DAT blockade and COMT inhibition on the 2-step decision making task

7.1 Introduction

Dopamine has long been thought to be a key component of reward-guided learning, but its precise function has remained elusive. One reason for this is that many studies have failed to distinguish different modes of learning. In the study of decision making, the dual-process theory proposes that choices about what action to take in a given situation can be influenced by two distinct behavioural modes: habitual and goal-directed (Daw et al., 2005; O'Doherty et al., 2017). These two types of behaviour are in turn linked with two types of reinforcement learning (RL) – the computational theory of how subjects learn about the reward (or punishment) contingencies associated with different actions and use this information to make decisions that will maximise positive outcomes (or minimise negative ones) (Daw et al., 2005). Model-free RL is thought to underlie habitual behaviour, while model-based RL is linked with goal-directed behaviour (O'Doherty et al., 2017).

Model-free RL uses reward prediction error (RPE) signals to track associations between actions and outcomes retrospectively and to update cached estimates of the value of these actions (Daw et al., 2005). Over time, integration of RPEs and value estimates across previous reinforcements causes actions that lead to positive outcomes to be reinforced. This process is relatively computationally efficient but lacks flexibility, as cached action values are dissociated from specific outcomes, and changes to the environment such as devaluation of a rewarding outcome therefore do not influence

behaviour (Daw et al., 2005; O'Doherty et al., 2017). Behaviour thus becomes habitual when only model-free RL is at play.

Model-free RL has long been linked with dopamine based on the classic finding that dopamine neurons code RPEs – a key input to model-free RL – by signalling the difference between the expected outcome and the outcome actually experienced (Schultz et al., 1997). RPE signals have been observed in dopamine release in the rodent nucleus accumbens (Clark et al., 2010; Day et al., 2007; Hart et al., 2014) and in the fMRI BOLD signal in both dorsal and ventral striatum in human imaging studies (Delgado et al., 2000; Gottfried et al., 2002; Knutson et al., 2001; O'Doherty et al., 2004, 2003; Schönberg et al., 2007). Studies have shown that BOLD signals in human ventral striatum (as well as in the prefrontal cortex) reflect model-free influences on learning (Daw et al., 2011; Deserno et al., 2015), which supports the idea that dopaminergic RPE signals in the striatum mediate model-free RL.

By contrast, model-based RL builds an internal model of the world that can be used to predict the future states likely to result from a particular action (Daw et al., 2005). One way that these predictions can be made is by simulating the consequences of an action in an action-outcome tree that maps the various potential outcomes, as well as intermediate steps leading to them. State prediction errors – an analogue of RPEs that signal the difference between predicted and experienced states rather than particular outcomes – may be used in the construction of such maps (Gläscher et al., 2010; O'Doherty et al., 2017). Model-based RL is far more computationally demanding than model-free RL but is advantageous because of its flexibility – subjects can more readily adapt their behaviour to changes in the environment by integrating new information into the model (Daw et al.,

2005). Behaviour driven by model-based RL is therefore goal-directed (O'Doherty et al., 2017).

There is increasing evidence that dopamine might play a role in model-based as well as model-free RL. For example, Parkinson's patients showed deficits in model-based learning that were restored by dopamine replacement therapy (Sharp et al., 2016). Similarly, model-based RL was enhanced by administration of the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) to healthy subjects (Wunderlich et al., 2012). One prominent idea is that these effects reflect the importance of cortical dopamine in shaping cognitive functions, particularly working memory (Brozoski et al., 1979; Phillips et al., 2004; Robbins, 2005; Sawaguchi and Goldman-Rakic, 1991), which are beneficial for representing transitions between different states during model-based learning (Eppinger et al., 2013; Otto et al., 2013a, 2013b; Smittenaar et al., 2013). In support of the idea that dopamine's influence on model-based processing is mediated in the cortex, a study using COMT genotype as an index of cortical dopamine function found evidence that cortical dopamine influences model-based RL (Doll et al., 2016). However, although these findings are suggestive, it is nonetheless possible that striatal dopamine may influence model-based RL, particularly as dopamine-rich striatal regions such as dorsomedial striatum have been implicated as key for implementing model-based strategies (Yin et al., 2005).

As shown by previous work (Budygin et al., 1999; Käenmäki et al., 2010; Lapish et al., 2009; Owesson-White et al., 2012; Raevskii et al., 2002; Tammimaki et al., 2016; Tunbridge et al., 2004) as well as by our voltammetry recording data (see Chapters 4 and 5), recycling by the dopamine transporter (DAT) regulates striatal dopamine but has little influence on cortical dopamine transmission, whereas degradation by catechol-*O*-

methyltransferase (COMT) regulates cortical dopamine tone (at least under some circumstances) but has minimal impact on striatal dopamine. We therefore assessed the impact of DAT blockade and COMT inhibition on a recently developed rodent analogue of a human task that assesses the relative contributions of model-free and model-based RL to behaviour in mice (Akam et al., 2016) to gain further insight into how manipulations of dopamine transmission in striatum and cortex, respectively, affect the balance between model-free and model-based RL strategies. We hypothesised that interference with DAT reuptake would preferentially impact model-free RL, whereas interference with COMT degradation would preferentially impact model-based learning.

7.2 Methods

7.2.1 Subjects

Male C57BL/6 mice were obtained from Envigo (formerly Harlan), housed on a 12/12-hr light/dark cycle (lights on at 0700hr) in groups of 4 animals per cage, and given water *ad libitum*. The mice were food deprived to 85-90% free feeding weight throughout the experiment to encourage task engagement. Testing was conducted during the light phase. Mice were aged 11-26 weeks during training and testing. Care and testing of all animals was conducted under the auspices of the UK Home Office laws and guidelines for the treatment of animals under scientific procedures and of the local ethical review board at the University of Oxford.

A total of 16 mice were used during this experiment. All animals were trained on the task for 6 days. Performance was then evaluated based on the number of trials

performed, and the 8 most active animals were selected for further training; these 8 mice comprised the cohort used for the remainder of the experiment, including drug testing.

7.2.2 Drugs

Drugs were dissolved in 20% hydroxypropyl-beta-cyclodextrin (Acros Organics) in 0.9% saline (AquPharm). This served as a vehicle control. The DAT blocker GBR-12909 (1-[2-(bis[4-fluorophenyl]methoxy)ethyl]-4-[3-phenylpropyl]piperazine) dihydrochloride (Tocris) was used at a dose of 6mg/kg. The dihydrochloride was not taken into account when calculating the dosage, so GBR-12909 injections contained 6mg/kg of GBR-12909 dihydrochloride. The COMT inhibitor tolcapone (Ro 40-7592; 4'-methyl-3,4-dihydroxy-5-nitro-benzophenone) (TRC Inc) was used at a dose of 30mg/kg. See Chapter 2 for further information on tolcapone and GBR-12909 and on how these dosages were selected. Drugs and vehicle were delivered by intraperitoneal injection, with an injection volume of 10mL/kg.

7.2.3 Apparatus

The task was conducted in custom-built boxes made of Plexiglas and measuring 14 x 14 x 14 cm. The back and side walls of the boxes were black, while the front wall (door), floor, and ceiling were clear. A strip of 3 LED lights, covered with a piece of black paper, was affixed to the top of the box to provide ambient lighting. Boxes were located in sound attenuating chambers (Med Associates Inc), with 3 boxes placed side-by-side within each chamber. Animal behaviour was monitored and filmed using 700TVL Bird Box Cameras (SpyCameraCCTV) and an SRD_876D digital video recorder (Samsung).

A panel containing 4 nose poke ports – 2 central ports (top and bottom) and 2 side ports (left and right) – was affixed to the back wall of each testing box. Each port was

surrounded by 4 LEDs, which were illuminated as appropriate during the task, as well as with an infrared beam to detect nose pokes. Each of the 2 side ports also contained a metal spout to which liquid reward (20% sucrose solution) could be delivered via HDI valve solenoids (Lee Products Ltd). The apparatus was controlled by pyboards (Jaltek Systems) running custom-written Python code.

Boxes were cleaned after each test session using Anistel High Level Surface Disinfectant (Tristel). Solenoids and attached tubing were flushed with hot distilled water after every session and with 0.6% v/v Milton Sterilising Fluid (Labaratoire Rivadis) in distilled water approximately every 2 weeks.

7.2.4 Task overview

The steps of the task are illustrated in Figure 1a. During the 1st step of each trial, both the top and bottom central nose poke ports are illuminated, and the mouse must choose one by nose poking in the port. This causes the central port lights to be switched off and leads to illumination of either the left or right side port. During the 2nd step, the mouse must nose poke in the illuminated port, which may result in reward delivery. Both the **transition probability** – the likelihood that a poke in a given central port will cause illumination of a given side port – and the **reward probability** – the likelihood that reward will be delivered to the illuminated side port in the 2nd step – fluctuate during the session. Animals must therefore learn and track both the transition and reward probabilities in order to select the central port (top or bottom) that is most likely to lead to the side port (left or right) with the highest probability of reward.

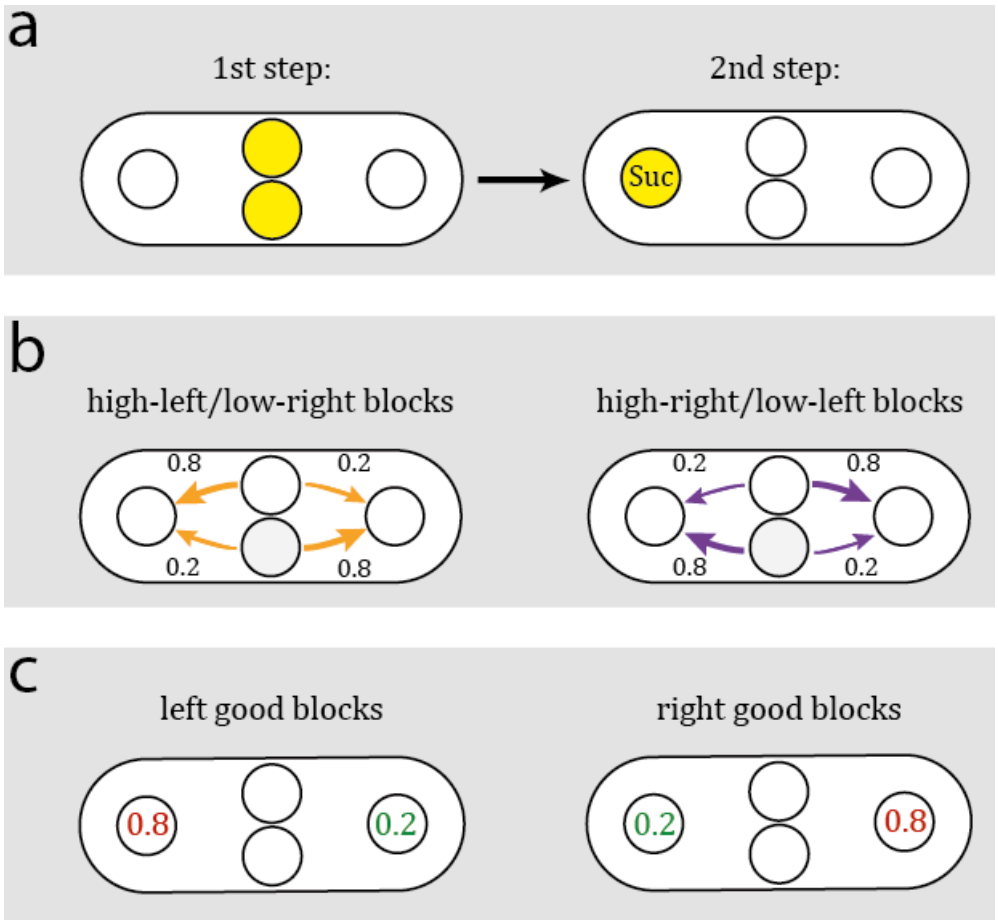

Figure 1: The 2-step task. a) In the 1st step of the task, the central ports illuminate, and the animal must nose poke in one of these ports; a side port will then illuminate, and a nose poke in this port results probabilistically in delivery of a sucrose reward. b) The two types of transition probability blocks. c) The two types of reward probability blocks.

During a session of the fully trained version of the task (see below for training procedure), either the transition or reward probability changes at the end of each block of trials. A block is defined by both its transition probabilities (Figure 1b) and reward probabilities (Figure 1c). The two possible transition probability states are: 'high-left/low-right' blocks, in which high pokes lead to left port illumination and low pokes to right port illumination with 0.8 probability; and 'high-right/low-left' blocks, in which high pokes lead to right port illumination and low pokes to left port illumination with 0.8 probability. The

two possible reward probability states are: 'left good', in which the left port has 0.8 probability of reward and the right port has 0.2 probability of reward; and 'right good', in which the probabilities are reversed. These probabilities lead to reward on 68% of trials if the correct central port is chosen during the 1st step, whereas only 32% of trials are rewarded if the incorrect central port is chosen.

Transitions between blocks are triggered when an animal reaches a performance criterion defined by an exponential moving average (calculated over 8 trials) of 1st step choices. When this moving average exceeds a threshold of 75% correct choices (selection of the central port most likely to lead to the side port with the highest reward probability), the block continues for an additional 20 trials in the current block, after which either the reward or transition probability changes. Block transitions were determined by a pseudo-random selection process such that, for every 3 block transitions, 1 involved a change in transition probability and 2 involved a change in reward probability. For a given animal, each session began with the same transition and reward probabilities that the previous session had ended with. The block transition process is illustrated in Figure 2.

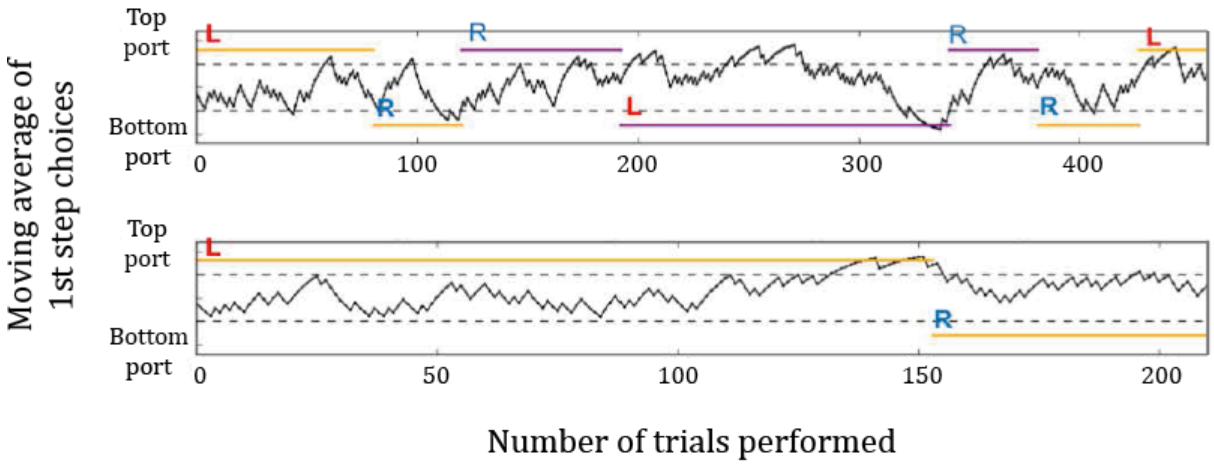

Figure 2: Examples of behaviour across block transitions in two different animals. The plots show a moving average of which central port (top or bottom) was chosen during the 1st step of each trial. The coloured lines indicate the type of transition block (gold for high-left/low-right blocks and purple for high-right/low-left blocks), and the coloured letters indicate which side port has the higher reward probability (red L for left-good blocks and blue R for right-good blocks). Dotted lines indicate the 75% correct criterion that the moving average must pass to trigger a block change. The animal whose behaviour is shown in the upper panel has learned the task well and rapidly adapts its behaviour to block changes, allowing it to perform many trials in the time span shown. By contrast, the animal whose behaviour is shown in the lower panel has not yet mastered the task and is slow to learn which central port should be selected to maximise chances of receiving a reward.

Trials were separated by a 1sec inter-trial interval (ITI). The task was self-paced such that animals could take as much (or as little) time as they chose between trials following the ITI and could perform as many trials as they liked within the session time. Animals were not penalised by nose poking in the unlit side port during the 2nd step – the lit side port simply remained illuminated until a nose poke was made at this port, which then resulted probabilistically in delivery of a reward and led to the end of the trial.

7.2.5 Training schedule

Testing was conducted between 0800hr and 1600hr, with all mice tested each day, 5-6 days per week during training and 7 days per week during drug testing. An individual mouse was always tested in the same box. Rewards consisted of 20% weight/volume

sucrose solution (Sigma Aldrich); the reward size varied over the course of training as described below.

The transition probabilities, reward probabilities, reward size, and session length were changed over the course of training as animals became progressively more engaged with the task and learned to perform it better. Training phases are described in Table 1. Animals were considered fully trained and ready to transition from phase 3 of training to the drug study when the group average met the following criteria:

(1) a 3-day average of >450 trials per session

(2) a 3-day average of >4 block changes (reversals) per session

(3) a 3-day average combined reversal learning speed (tau) of <30 trials

Phase (duration)	**Transition probability**	**Reward probability**	**Session length**	**Reward Size**
Phase 1 (18 days)	0.9/0.1 common/rare transitions	1.0/1.0 for 1st 40 trials, then 0.9/0.1 good side/bad side	1.5hrs for 1st 4 days, then 2.5hrs	Varied between either 4uL or 8uL
Phase 2 (5 days)	0.8/0.2 common/rare transitions	0.9/0.1 good side/bad side	2.5hrs	8uL (3 days) then 6.5uL (2 days)
Phase 3 (15 days)	0.8/0.2 common/rare transitions	0.8/0.2 good side/bad side	2.5hrs	6.5uL (3 days) then 4uL (12 days)
Drug testing (53 days)	0.8/0.2 common/rare transitions	0.8/0.2 good side/bad side	2.5hrs	4uL

Table 1: Phases of training and testing during the 2-step task.

7.2.6 Drug testing

Mice received a vehicle injection 5 days prior to the start of the drug study so that the stress associated with the first injection an animal receives would not confound the

experiment's findings. All mice were habituated to handling – including the restraint position used during injections – prior to drug testing.

On drug testing days, mice received an injection of either drug or vehicle before starting the task. GBR-12909 injections were administered 15min before testing began and tolcapone injections 90min before testing to account for differences in the drugs' time courses of action (Acquas et al., 1992; Männistö and Kaakkola, 1999; Nakachi et al., 1995; Raevskii et al., 2002). In order to account for this difference in the timing of drug administration, each animal received two types of vehicle control injections, one administered 15min before testing (to match the GBR-12909 injections) and one administered 90min before testing (to match the tolcapone injections). There were thus 4 possible drug treatment conditions, as illustrated in Table 2. Drug testing days were interleaved with testing days on which no injections were administered in order to allow for drug washout and to re-equilibrate behaviour.

Treatment	Time of injection
GBR-12909	15min before testing
Vehicle-15	15min before testing
Tolcapone	90min before testing
Vehicle-90	90min before testing

Table 2: Drug treatments.

Drugs were administered according to a within-subjects design, so each animal experienced all possible drug treatments in rotation. The order of rotation was pseudorandomised across mice and was counterbalanced for mice within the same home cage. In order to gather sufficient data to assess drug effects on behaviour, the drug

treatment rota was repeated for a total of 8 cycles. The first 6 cycles were 6 days long and included 1 GBR-12909 injection, 1 tolcapone injection, 1 vehicle injection (either 15min or 90min before testing), and 3 days with no injections. The final 2 cycles were 8 days long and included 1 GBR-12909 injection, 1 tolcapone injection, 1 vehicle injection 15min before testing, 1 vehicle injection 90min before testing, and 4 days with no injections. The structure of drug treatment cycles is illustrated in Figure 3.

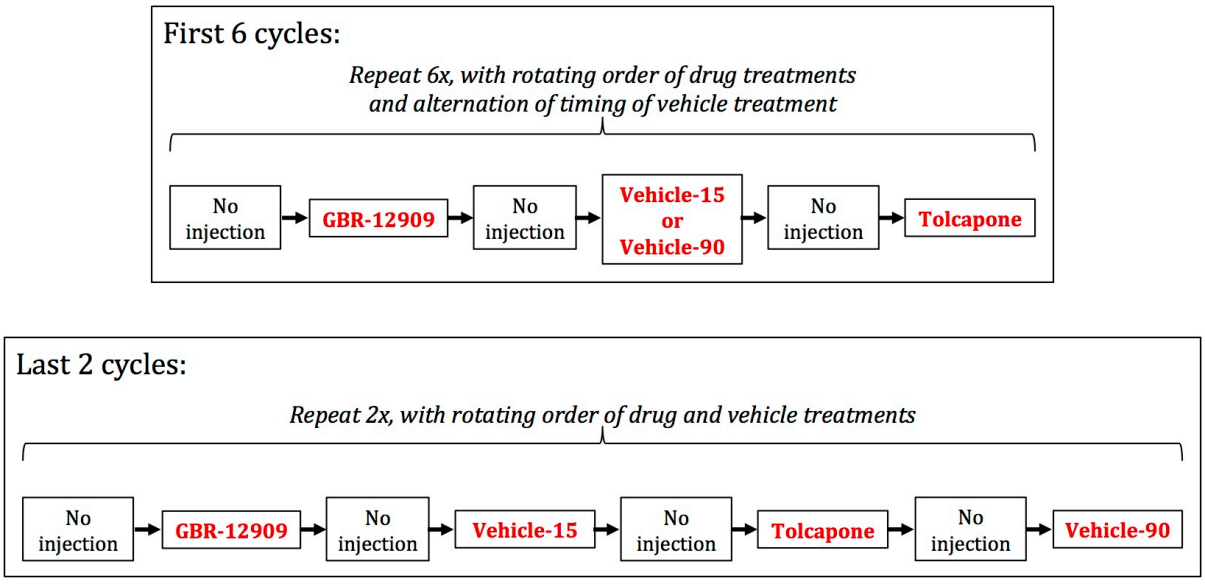

Figure 3: Testing sequence during the drug study. Drug and vehicle treatments are indicated in red. Note that the particular treatments shown in the figure are for illustrative purposes only – treatments were counterbalanced across animals, and the order in which each animal received the treatments was rotated across cycles.

7.2.7 Analysis

Data analysis was performed using custom-written code in the Anaconda package Python version 3.5 with NymPy (van der Walt et al., 2011) and Matplotlib (Hunter, 2007). Data were plotted in Python 3.5 and GraphPad Prism version 5.04.

Statistical comparisons between each drug treatment and its appropriate vehicle control were conducted using either permutation tests in Python or paired-samples t-tests in SPSS version 20, with significance set at $\alpha = 0.05$. Briefly, permutation testing involves evaluating the absolute difference in the value of a parameter between the drug and vehicle conditions and then permuting the dataset – randomly reassigning the data between the two conditions – to generate a P value. This value indicates in what fraction of the permuted datasets the absolute difference is larger between conditions than it is in the true dataset. The number of permutations was limited to 1000 re-samples, as unlimited permutations are computationally impractical, and permutations were randomly generated during each analysis. This process yields an approximation of the true P value.

In order to minimise the effect of behavioural changes arising from learning over the course of the drug study (which lasted 59 days and involved an average of 30,000 trials per mouse), permutations were performed within cycles. Sessions were divided into 5 cycles for analysis, with the first 3 cycles each being 12 days long (covering adjacent pairs of the 6 day long cycles described in section 7.2.6 and Figure 3 above) and the final 2 cycles each being 8 days long (covering one of the 8 day long cycles shown in Figure 3). These 12- and 8-day cycles provided a reasonable length of time over which to permute sessions to study drug effects on behaviour but were still short enough to limit the influence of long-term learning effects. A total of 3 sessions were excluded from analysis due to technical errors with the reward delivery system.

A number of parameters were used to assess drug effects on behaviour in the 2-step task, including:

(1) **trial rate**, quantified by the number of trials performed per minute

(2) **2nd step reaction time**, defined as the time between selection of a central nose poke port in the 1st step and the subsequent nose poke in the illuminated side port

(3) **fraction of correct choices**, defined as the number of 'correct' 1st step choices (i.e. nose pokes in the central port most likely to lead to the side port with the highest probability of reward) divided by the total number of trials in a session

(4) **reversal block end choice probability**, defined as the average probability of making a correct choice in the 15 trials preceding a block change, which was evaluated separately for reversals in reward probability and for reversals in transition probability as well as for all reversals combined

(5) **reversal tau (τ)**, quantified by the time constant of an exponential fit to the choice probability trajectory over the 40 trials following a block change, which was evaluated separately for reversals in reward probability and for reversals in transition probability as well as for all reversals combined

(6) **influence of model-based versus model-free control**, quantified by the mean absolute preference of each of these decision making systems in an RL model to which the data was fitted (see section 7.2.8 for details)

Although sessions were 2.5hrs long, analyses were conducted on only the first 90min of data from each session, as after this time drug effects may have started to wear off, particularly for GBR-12909 (Acquas et al., 1992; Männistö and Kaakkola, 1999; Nakachi et al., 1995; Raevskii et al., 2002).

7.2.8 Modeling

The RL model comprised a set of equations describing learning and decision making processes underlying animals' behaviour. These equations capture how variables such as action values are updated by new experiences and how these variables guide future choices. When the model is fit to actual behavioural data, the model parameters are adjusted to achieve the best fit – in other words, the form of the model most likely to generate the observed behaviour. We used a multilevel modelling approach (sometimes referred to as hierarchical or nested modelling). Parameters were assumed to be Gaussian-distributed across the population of sessions, and the mean and variance at the population level were fit using maximum likelihood methods.

Previous work (Akam et al., 2016; Daw et al., 2011) has determined that modelling of the 2-step task works best when the model includes both a model-free RL component and a model-based RL component . The model-free component learns and updates action values using RPEs that indicate the difference between the expected outcome of an action and the actual outcome. These RPEs occur both when a side port illuminates following selection of a central port in the 1st step of the task and when a reward is delivered (or not delivered) to the side port. The model-based component, by comparison, tracks the relationship between actions and states rather than actions and specific outcomes. This component learns and updates state values using RPEs that indicate the difference between the expected and actually experienced state.

The RL model combines the action values learned by the model-free and model-based systems in a weighted sum and uses the resulting net action values to determine

behaviour. The weights for model-free and model-based values are independent – a higher model-based weight does not necessarily imply a lower model-free weight or vice versa.

Although the RL model included 10 parameters, only the overall influence of the model-free and model-based components will be presented here. The influence metric is constructed using the model-free and model-based weight parameters as well as the learning and forgetting rates in each of these systems. Influence is formally defined as the mean over trials of the absolute difference between the action value estimates for the high and low central pokes. The influence measure is calculated separately for the model-free and model-based systems. Larger values indicate a greater influence of a particular system on behaviour.

7.3 Results

7.3.1 Trial rate

It took approximately 5.5 weeks to fully train animals on the 2-step task, by the end of which they performed approximately 300 trials per session (Figure 4). Mice exhibited the fastest trial rate at the beginning of the session, completing 8-10 trials per minute initially, and then slowed their responding, which decreased to a rate of 2-3 trials per minute by 30min into the session (Figure 4a and b). The trial rate remained fairly constant for the remainder of the session.

GBR-12909 significantly increased the rate at which animals performed trials (permutation test, $p = 0.001$). This can be seen in both the trial rate per minute (Figure 4a) and the cumulative trials performed over the course of the session (Figure 4c). By contrast,

tolcapone had no effect on the rate at which trials were performed (permutation test, p = 0.347) (Figure 4b and d).

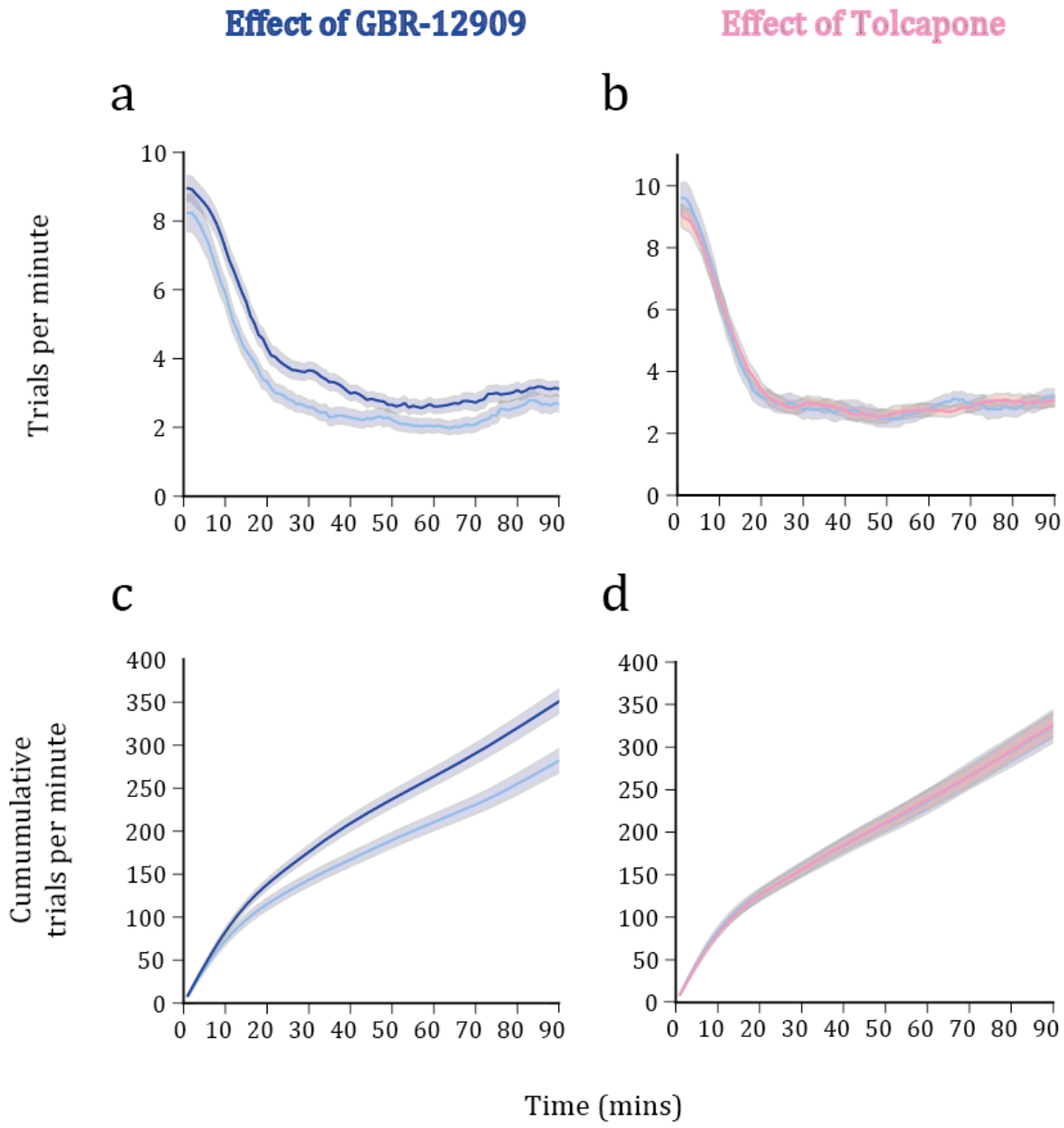

Figure 4: The rate at which animals performed trials over the first 90min of each session. a) The number of trials performed per minute following vehicle-15 (light blue) and GBR-12909 (dark blue) injections. b) As for (a) but following vehicle-90 (light blue) and tolcapone (pink) injections. c) The cumulative number of trials performed over the session following vehicle-15 and GBR-12909 injections. d) As for (c) but following vehicle-90 and tolcapone injections. Data are shown as the mean ± SEM across animals.

7.3.2 Reaction time

The reaction time that elapses between the animal nose poking in one of the central ports and then poking in the side port that is subsequently illuminated provides insight into how well the animal has learned the transition probabilities governing which side port activates following a nose poke in a given central port as well as into the animal's motivation to pursue reward. The reaction time was evaluated separately for common transitions (illumination of the side port that had 0.8 probability of activating following a nose poke at a given central port) and rare transitions (illumination of the side port that had 0.2 probability of activating following a nose poke at a given central port).

Reaction times on common transitions were consistently faster than on rare transitions, as shown in Figure 5. Permutation tests revealed that GBR-12909 significantly decreased reaction times compared to its vehicle control on both common ($p = 0.032$) and rare ($p = 0.001$) transitions. However, there was no difference in reaction time between tolcapone and its vehicle control on either type of transition (common transitions: $p = 0.795$; rare transitions: $p = 0.999$).

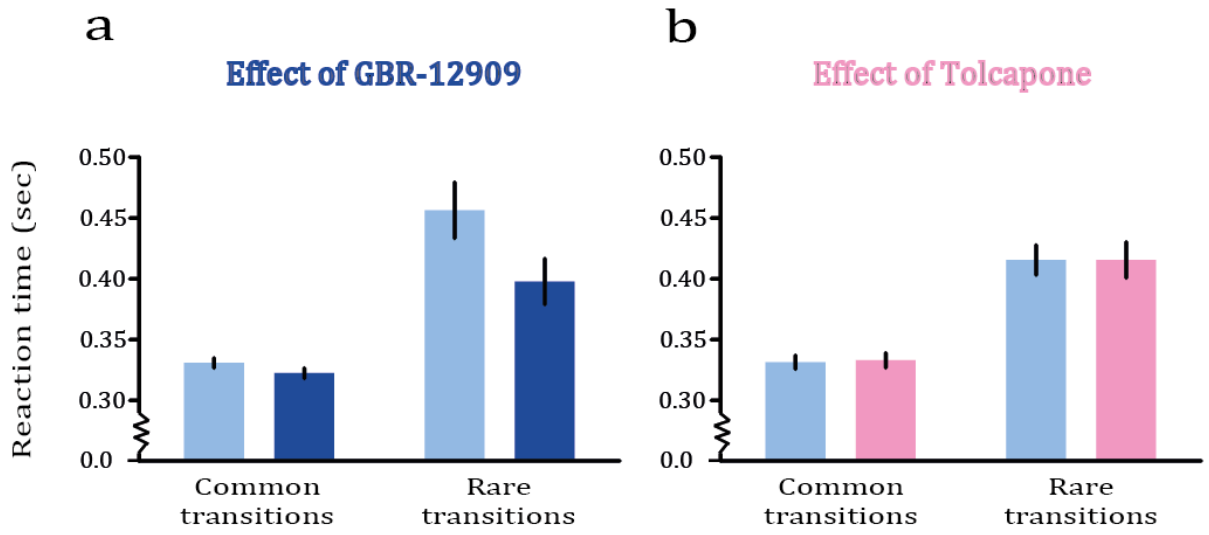

Figure 5: Reaction times (mean ± SEM across animals) for common and rare transitions during the 2nd step of the task. a) Reaction times following GBR-12909 injections (dark blue) compared to vehicle-15 injections (light blue). b) Reaction times following tolcapone injections (pink) compared to vehicle-90 injections (light blue).

7.3.3 Fraction correct choices

Once fully trained, animals made the correct choice in the 1st step of the task – selecting the central nose poke most likely to lead to the side port with the highest reward probability – slightly more than half of the time: the fraction of correct choices out of all trials was between 0.5 and 0.6. (Note that because this measure is calculated across the entire session, it covers both the portions of the session immediately following changes in reward or transition probability, when the animals perform less well while adapting to the new task structure, and the portions of the session immediately before changes in reward or transition probability, when the animals perform at their best. As a result, the fraction of correct choices is slightly lower than the block end choice probability calculated in the reversal analysis, which is presented in section 7.3.4 below.)

Neither GBR-12909 nor tolcapone substantially altered the fraction of correct choices. Administration of GBR-12909 appeared to decrease the fraction of correct choices (Figure 6a), and a paired-samples t-test showed that this effect approached but did not reach significance ($t(7) = 2.0$, $p = 0.081$). Administration of tolcapone had no consistent influence on the fraction of correct choices (paired-samples t-test: $t(7) = -0.9$, $p = 0.401$) (Figure 6b).

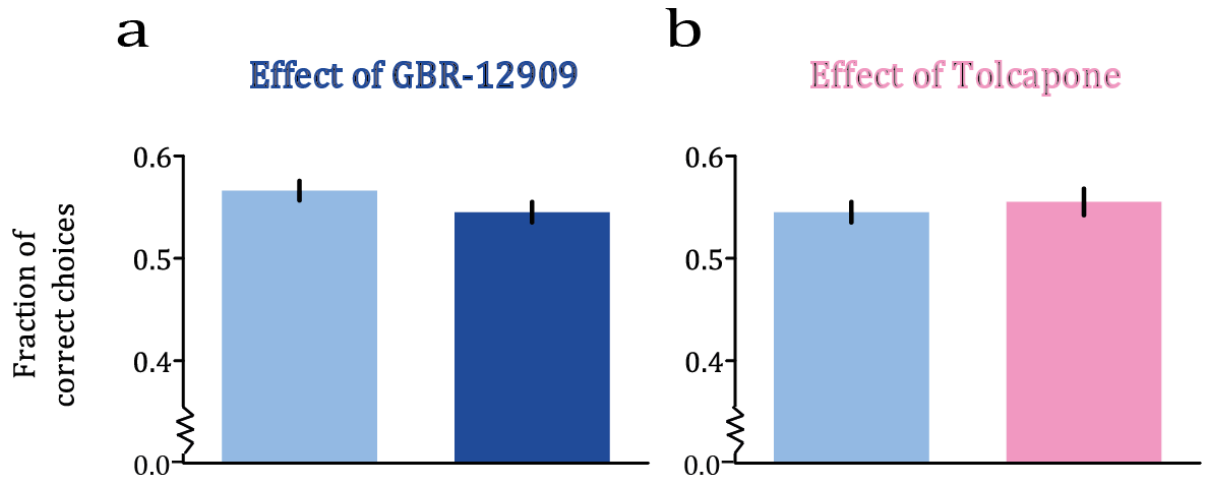

Figure 6: The fraction of correct choices out of total trials (mean ± SEM across animals). a) Fraction of correct choices following GBR-12909 injections (dark blue) compared to vehicle-15 injections (light blue). b) Fraction of correct choices following tolcapone injections (pink) compared to vehicle-90 injections (light blue).

7.3.4 Reversal analysis

The analysis of behaviour around block changes (reversals) assesses drug effects on both the block end choice probability – a measure of how well animals performed just before the reversal – and tau – a measure of how quickly animals adapted to the new task structure after the reversal. A higher block end choice probability value indicates that the

animal made the correct choice more frequently during the 15 trials leading up to the reversal. A lower tau value indicates that the animal adapted its behaviour more quickly over the 40 trials following reversal. Block end choice probability and tau were assessed separately on reversals that involved a change in reward probability and on those that involved a change in transition probability as well as on all reversals combined.

When fully trained, animals performed with a block end choice probability of between 0.6 and 0.7 correct choices and typically adapted their behaviour with a tau of less than 20 trials. Administration of GBR-12909 had no effect on the block end choice probability in comparison to its vehicle control (permutation test, $p = 0.552$) (Figure 7a). Permutation tests found that the effect of GBR-12909 on tau for reversals in reward probability approached significance ($p = 0.057$), indicating that mice on GBR-12909 were numerically slower to adapt their performance following a reward probability reversal. GBR-12909 had no effect on tau for reversals in transition probability ($p = 0.685$) or on tau for combined reversals ($p = 0.119$).

Like GBR-12909, tolcapone had no effect on the block end choice probability in comparison to its vehicle control (permutation test, $p = 0.618$) (Figure 7b). The tau for reversals in reward probability following tolcapone again approached significance ($p = 0.060$), indicating that tolcapone caused mice to adapt their performance more quickly following a reward probability reversal. By contrast, the taus for reversals in transition probability and for combined reversals were unaffected by tolcapone administration (transition probability reversals: $p = 0.495$; combined reversals: $p = 0.200$).

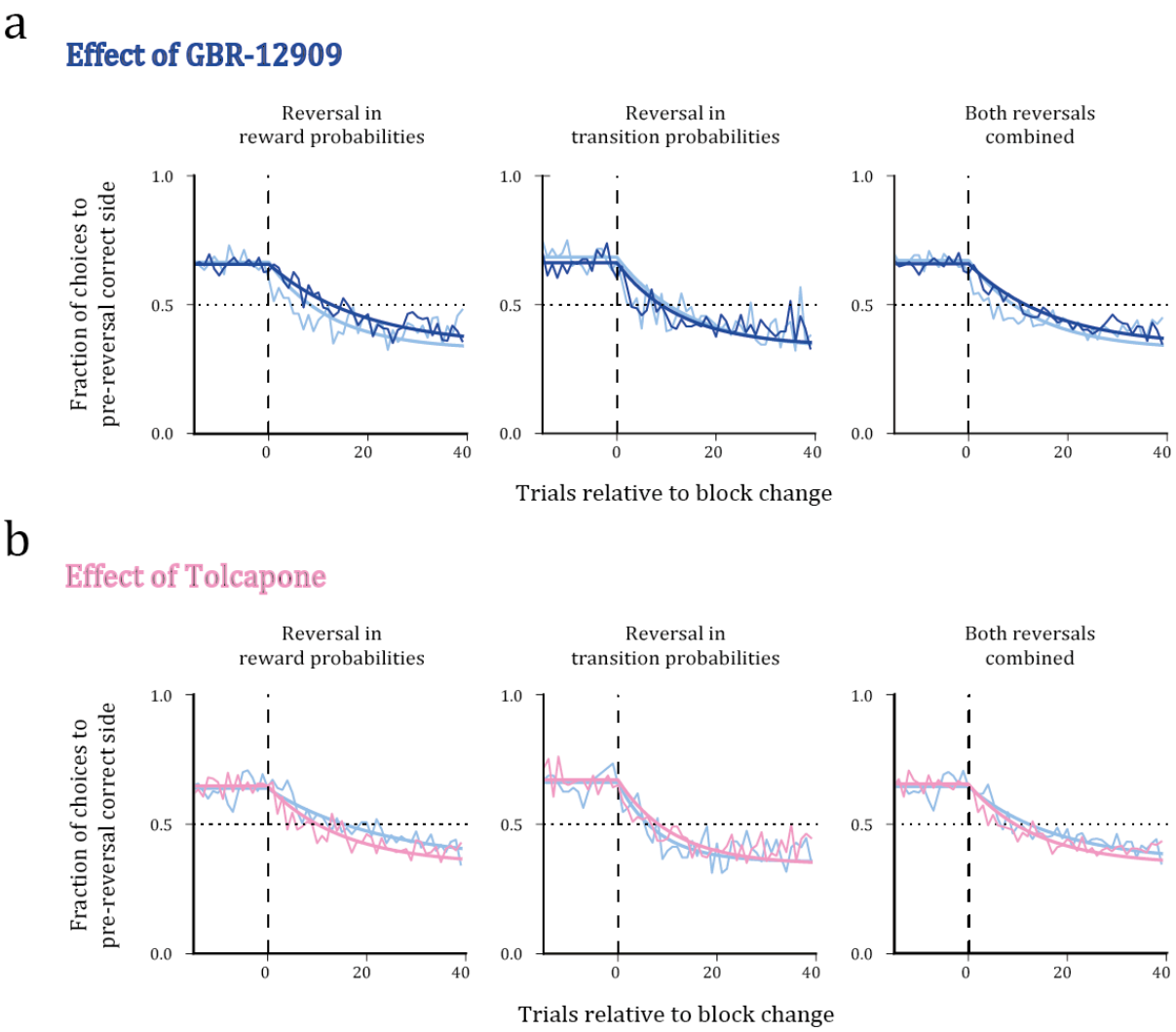

Figure 7: Reversal plots showing a) the effect of administration of GBR-12909 (dark blue) compared to vehicle-15 (light blue) and b) the effect of administration of tolcapone (pink) compared to vehicle-90 (light blue). Block changes occur at 0 on the x axis (vertical dotted line). The noisy trace depicts actual data, the smooth line to the left of the vertical dotted line represents the average probability of a correct choice before the reversal, and the smooth line to the right of the vertical dotted line represents the exponential fit to the 40 trials following the reversal. Thus, the value at which the smooth line meets the y axis is the block end choice probability, while the number of trials from the block change to the point at which the smooth line crosses the horizontal dotted line represents tau. Data are displayed for reversals in reward probability (left), reversals in transition probability (middle) and both reversals combined (right).

7.3.5 RL modeling

The results of fitting the RL model to the drug study data are shown in Figure 8.

Both model-free and model-based RL influence decision making on the 2-step task.

Following vehicle injections (whether administered 15 or 90min before testing), the influence of the model-free component was greater than the influence of the model-based component. GBR-12909 treatment significantly decreased the influence of the model-free component (permutation test, $p = 0.012$) and also caused a near-significant increase in the influence of the model-based component ($p = 0.057$) in comparison to its vehicle control. By contrast, tolcapone did not significantly alter the influence of either RL model component compared to its vehicle control (model-free component: $p = 0.231$; model-based component: $p = 0.750$).

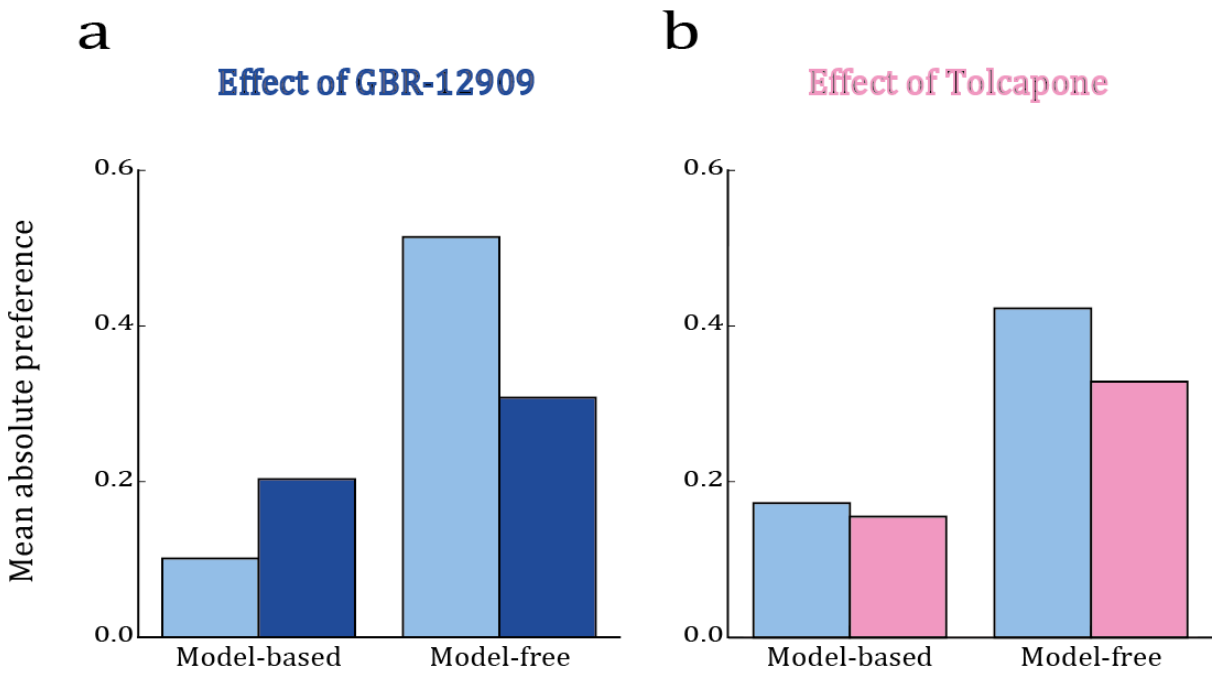

Figure 8: The influence of the model-free and model-based components of the RL model on behaviour, depicted as the mean absolute preference of each component. a) Results following GBR-12909 administration (dark blue) compared to vehicle-15 (light blue). b) Results following tolcapone administration (pink) compared to vehicle-90 (light blue).

7.4 Discussion

We took advantage of the specificity of DAT and COMT's effects in regulating striatal and cortical dopamine transmission, respectively, to assess the hypothesis that model-free RL is predominantly influenced by striatal dopamine and model-based RL by cortical dopamine. Unexpectedly, we found that administration of the DAT blocker GBR-12909 caused a significant *decrease* in the influence of the model-free strategy and a near-significant *increase* in the influence of the model-based strategy on decision making in the 2-step task (Figure 8a). Following GBR-12909, model-free RL still exerted more influence than model-based RL, but the difference in influence was less pronounced than it was under vehicle treatment. By contrast, the COMT inhibitor tolcapone had little to no impact on performance of the 2-step task and did not alter the influence of either model-free or model-based RL on decision making (Figure 8b).

7.4.1 Effects of DAT blockade on 2-step performance

As shown by our voltammetry recordings (see Chapter 4) as well as by previous literature (Budygin et al., 1999; Huotari et al., 1999, 2002a; Nakachi et al., 1995; Nomikos et al., 1990; Owesson-White et al., 2012; Raevskii et al., 2002), the DAT blocker GBR-12909 exerts its most pronounced effects in the striatum. In the striatum, GBR-12909 enhances both tonic dopamine transmission (slow, continuous dopamine release that establishes baseline levels of dopamine) and phasic dopamine transmission (short-lived bursts of dopamine release that are associated with behaviourally relevant events). Although a few studies have shown increased levels of cortical dopamine on a tonic timescale following GBR-12909 (Carboni et al., 2006; Tanda et al., 1997; Valentini et al., 2004), others have not replicated this finding (Mazei et al., 2002; Pozzi et al., 1994; Weikop et al., 2007), and we

found that this drug had no effect on dopamine transmission on a phasic timescale in our cortical voltammetry recordings (see Chapter 5). We therefore conclude that GBR-12909's effects on the 2-step task are exerted via changes in striatal dopamine transmission.

In light of this, our finding that manipulation of striatal dopamine transmission by GBR-12909 caused a near-significant increase in the influence of model-based RL on behaviour is both surprising and intriguing. Enhanced dopamine transmission has previously been linked to model-based RL. For example, systemic administration of dopamine enhancing drugs to both healthy subjects and Parkinson's patients has been shown to increase the influence of model-based RL on the human version of the 2-step task (Sharp et al., 2016; Wunderlich et al., 2012). However, these effects have usually been assumed to be mediated by cortical dopamine based on extensive evidence that working memory and other cognitive functions are modulated by cortical dopamine levels (Brozoski et al., 1979; Phillips et al., 2004; Robbins, 2005; Sawaguchi and Goldman-Rakic, 1991) and studies demonstrating that disruptions to cortical function can impair model-based RL and that higher working memory function supports more robust model-based processing (Eppinger et al., 2013; Otto et al., 2013a, 2013b; Smittenaar et al., 2013).

Our data, however, indicate that striatal dopamine can also influence model-based function. Several studies in the human literature have hinted at this. Imaging studies have found that the BOLD signal in both ventral striatum and prefrontal cortex reflects model-based influences during decision making (Daw et al., 2011; Deserno et al., 2015), indicating that both regions – and, potentially, dopamine transmission within them – are involved in model-based learning. In addition, RPE signals in the ventral striatum are correlated with increased fluid intelligence – a psychological measure of functions such as flexible problem

solving that are also important for model-based learning (Schlagenhauf et al., 2013). Finally, PET imaging has revealed that higher presynpatic dopamine in ventral striatum correlates with increased model-based behavioural control (Deserno et al., 2015). Our results not only support these findings but also point to a causative, and not merely correlative, role for striatal dopamine in model-based RL, which the human literature has not yet been able to demonstrate.

It remains unclear whether increased striatal dopamine enhances cortical dopamine transmission and the cognitive functions it mediates, thus bolstering model-based RL, or whether striatal dopamine affects model-based RL directly and separately to any effects cortical dopamine might have on model-based function. Intriguingly, increased striatal dopamine levels have been linked to higher working memory capacity (Cools, 2008) and to enhanced model-based signalling in the prefrontal cortex (Deserno et al., 2015). However, the imaging techniques used to acquire these data are insufficiently sensitive to detect dopamine levels in cortex and therefore do not indicate how dopamine in striatum might impact cognitive function. We have demonstrated a causal link between striatal dopamine and model-based RL, but further work is needed to determine by what means enhanced dopamine transmission in striatum affects model-based learning.

Our findings on GBR-12909's effects on model-free RL add a new twist to the link between model-free learning and striatal dopamine. One might have expected enhancements to striatal dopamine transmission induced by GBR-12909 to increase the influence of model-free RL on behaviour by facilitating model-free leaning, but GBR-12909 in fact reduced the influence of model-free RL. Several studies from the human literature align with this finding. PET imaging has revealed a correlation between higher presynaptic

dopamine in ventral striatum and reduced model-free signals in this region (Deserno et al., 2015). Furthermore, a study in which dopamine precursors were depleted found that this did not perturb habit learning and in fact enhanced model-free RL at the expense of model-based RL (de Wit et al., 2012). In addition, several studies of Parkinson's disease patients (who have diminished dopamine transmission, particularly in the dorsal striatum, but also in ventral striatum and cortex) have reported that patients retain the capacity for habit learning (de Wit et al., 2011) and have found that taking patients off their dopamine replacement medication did not affect model-free RL (Sharp et al., 2016). Together with these findings, our data indicate that the relationship between dopamine and model-free RL may be more complex than originally supposed.

In addition to its effects on RL, GBR-12909 both increased the trial rate (Figure 4a and c) and decreased the reaction time during the 2nd step of the task (Figure 5a). Together, these findings support the conclusions of Chapter 6 that animals are more motivated to engage in the task when DAT recycling is blocked. An alternative explanation is that GBR-12909 simply increased locomotor activity levels and caused animals to move faster throughout the session, resulting in a non-specific increase in trial rate and decrease in reaction time. The 2-step task lacks a measure to control for locomotor activity analogous to the inactive lever press parameter in the progressive ratio (PR) task presented in Chapter 6, and this alternative explanation cannot therefore be definitively ruled out. However, given that the same dose of GBR-12909 that was used during the 2-step task was found to selectively affect motivation to work for reward on the PR task but not to alter activity levels per se, we judge the motivational explanation of GBR-12909's effects on the 2-step task to be most convincing.

In contrast to its clear effects on motivation, GBR-12909 had only subtle effects on how well animals performed the task. The quality of performance can be assessed using both the fraction of correct choices and the reversal analysis. The analysis of the fraction of correct choices across the entire session indicated that GBR-12909 slightly diminished the animal's accuracy in making the correct choice during the 1st step of the task (Figure 6a) (although this did not reach statistical significance). The analysis of tau values (Figure 7) found that GBR-12909 had a near-significant effect on how quickly animals adapted their behaviour following a reversal in reward probabilities such that animals adapted more slowly following GBR-12909 injections. Although this result should be interpreted with caution, both this finding and the fraction of correct choices analysis suggest that DAT blockade slightly diminished the animals' sensitivity to changes in reward contingencies. These effects of GBR-12909 could potentially also be explained as increased perseveration in responding, which has previously been linked to striatal dopamine transmission (den Ouden et al., 2013; Rutledge et al., 2009). Interestingly, the effect on reversals in reward probabilities did not extend to the ability of animals to adapt to reversals in transition probabilities, which was unchanged by GBR-12909 administration.

7.4.2 Effects of COMT inhibition on 2-step performance

The lack of effect of tolcapone on the 2-step task was unexpected. COMT activity, as assessed by the Val^{158}Met polymorphism in humans, is linked not only with cortical dopamine levels (Slifstein et al., 2008) but also with working memory (Diaz-Asper et al., 2008; Egan et al., 2001; Farrell et al., 2012; Goldberg et al., 2003). Given the importance of working memory and other cognitive functions to model-based RL (see above), one would expect COMT to influence model-based learning. Indeed, a recent study found that

individuals homozygous for the Met allele of the Val^{158}Met polymorphism (who have less active COMT and, presumably, higher cortical dopamine levels) show enhanced model-based RL compared to individuals homozygous for the Val allele (who have more active COMT and are thus presumed to have lower cortical dopamine) on the human version of the 2-step task (Doll et al., 2016).

We failed to replicate these findings using pharmacological inhibition of COMT on the mouse version of the 2-step task. There are several potential explanations for this lack of effect. First, although tolcapone has been shown to alter dopamine on a tonic timescale (Lapish et al., 2009; Tammimaki et al., 2016; Tunbridge et al., 2004), we failed to find an effect of tolcapone on dopamine transmission on a phasic timescale in our voltammetry recordings (see Chapter 5). As the moment-to-moment decision making required by the 2-step task most likely relies on fast, phasic changes in dopamine transmission, COMT's longer-term effects on tonic transmission may not influence performance on this task. In addition, it is important to note that there are subtle differences in task design and structure between the human and rodent versions of the 2-step task and that therefore humans and mice solve the task differently. In particular, mice may not use working memory, in the sense in which we refer to it in humans, in the rodent 2-step paradigm to learn and perform the task on a trial-by-trial basis, and any effects of tolapone on working memory function may therefore not impact behaviour on the 2-step task. Finally, our results may be due to a difference between acute pharmacological manipulations and chronic genetic effects – the influence of COMT may only emerge in a complex interplay of genetic and environmental influences over time. In agreement with this view, mice genetically engineered to express COMT alleles analogous to the human polymorphism

show a difference in RL on the 2-step task only when stressed (Kristian Jensen and Anna Huber, personal communication).

In addition to its lack of effect on RL, tolcapone also failed to impact other measures of 2-step performance, including the rate at which trials were performed (Figure 4b and d), the 2nd step reaction time (Figure 5b), the fraction of correct choices (Figure 6b), and the block end choice probability and tau for combined reversals in the reversal analysis (Figure 7b). The one effect that approached significance was a decrease in the tau for reversals in reward probability, indicating that tolcapone slightly increased the speed with which animals adapted their behaviour following such reversals and suggesting that COMT inhibition subtly increased sensitivity to the change in reward contingencies. As in the GBR-12909 analysis, this effect of tolcapone was unique to reversals in reward probability and did not extend to reversals in transition probability.

7.4.3 Comparison across drug treatments

Because GBR-12909 and tolcapone act over different spans of time (Acquas et al., 1992; Männistö and Kaakkola, 1999; Nakachi et al., 1995; Raevskii et al., 2002), the two drugs had to be administered different amounts of time before the start of testing, and each drug therefore had to have its own vehicle control. Inspection of the data reveals an interesting pattern in that performance often appears to differ between the two types of vehicle controls – those that received an injection 15min before the start of testing and those that received the injection 90min before testing. This may be due to an injection stress effect similar to that observed on the PR task (see Chapter 6), which one would expect to be more pronounced in the vehicle-15 treatment condition than in the vehicle-90 condition. Such an effect can be assessed by comparing performance in each type of vehicle

control condition to performance on days when no injections were given. The impact of injection stress on 2-step performance during this study is presented in the MPhil thesis of a colleague with whom I conducted this experiment, and this topic will therefore not be discussed in detail here.

Nevertheless, it is possible that injection stress interacted with our experimental drugs to influence behaviour on the 2-step task. For example, part of GBR-12909's effects may be attributable to its ability to counteract the impact of injection stress – although we believe that the effects of DAT blockade on 2-step performance go beyond a simple reversal of stress effects. More interestingly, given the repeated indications in our lab's work that COMT's effects on dopamine transmission interact with arousal levels to influence behaviour, it may be that an effect of COMT inhibition on the 2-step task would emerge if performance under tolcapone were compared to performance under acute stress.

Chapter 8:
General Discussion

In this study, we investigated the potential interaction of reuptake by the dopamine transporter (DAT) and degradation by the enzyme catechol-*O*-methyltransferase (COMT) in the regulation of dopamine transmission and dopamine-mediated behaviours. DAT and COMT clearance mechanisms have traditionally been thought to operate separately in the striatum and cortex, respectively. However, human imaging studies indicate that polymorphisms in the DAT and COMT genes can produce interactive or additive effects on fMRI BOLD signal in both striatum and cortex (Bertolino et al., 2006, 2008; Caldú et al., 2007; Dreher et al., 2009; Prata et al., 2009; Yacubian et al., 2007), and several pharmacological studies in rodents have also found that DAT and COMT can interactively affect both dopamine transmission and behaviour (Budygin et al., 1999; Huotari et al., 1999; Raevskii et al., 2002). These findings suggest that DAT and COMT may interact to regulate dopamine transmission to a greater extent than previously thought and that this interaction may have functional consequences.

We probed this potential interaction by using pharmacological inhibitors to interfere with DAT recycling and COMT degradation in mice. We assessed the effects of these drugs on phasic dopamine transmission in both the nucleus accumbens (NAc) and medial frontal cortex (MFC) using fast scan cyclic voltammetry (FCV) and evaluated effects on motivation and reinforcement learning using a progressive ratio (PR) task and a 2-step decision making task.

Together, our results largely support the standard model that DAT and COMT influence dopamine transmission and behaviour separately, as we found no evidence of interactive effects. We confirmed that DAT exerts pronounced effects on striatal phasic dopamine transmission but has little influence on cortical phasic dopamine. Unexpectedly, we saw no effects of COMT inhibition on either cortical or striatal phasic dopamine transmission. This lack of effect on transmission was mirrored by an absence of clear behavioural effects of the COMT inhibitor. By contrast, the DAT blocker induced pronounced behavioural effects, both on motivation to work for reward and, intriguingly, on the reinforcement learning (RL) strategy used in reward-guided decision making.

8.1 Role of DAT recycling

We found that administration of the DAT blocker GBR-12909 reliably increased the size of evoked dopamine transients in the NAc, as expected based on previous findings (Budygin et al., 1999; Huotari et al., 1999, 2002a; Nakachi et al., 1995; Nomikos et al., 1990; Owesson-White et al., 2012; Raevskii et al., 2002). Our data add to the evidence that DAT blockade not only increases tonic dopamine levels, as indicated by microdialysis studies, but also enhances phasic dopamine transmission, which we measured here using FCV. At first glance, this appears to contradict the theory that tonic and phasic dopamine transmission in the striatum are inversely related (Grace, 1991). Tonic dopamine transmission is understood to influence phasic release in striatum via D2 autoreceptors, such that higher tonic levels of dopamine decrease phasic release probability (Ford, 2014; Grace, 1991; Sulzer et al., 2016). One would normally not expect DAT blockade to disrupt this feedback mechanism; however, drugs like GBR-12909 may increase striatal dopamine

levels to such an extent that both tonic and phasic release are affected concurrently. In any case, our recordings do not provide a definitive demonstration of how GBR-12909 affects the relationship between tonic and phasic dopamine, as we did not simultaneously assess tonic and phasic transmission. In addition, because we conducted our recordings in anaesthetised animals, our findings may not extend to awake animals – although a previous study conducted in awake rats found, like us, that systemic GBR-12909 increased phasic dopamine transients in the NAc (Owesson-White et al., 2012).

It would be useful to clarify whether tonic and phasic dopamine transmission in the striatum operate in tandem or in opposition (or interact in a more complex fashion), as this has important implications for understanding not just local dynamics within the striatum but also how striatal dopamine interfaces with the wider mesocorticolimic circuitry. Simultaneous microdialysis and FCV recordings could be used to investigate the relationship between tonic and phasic dopamine transmission. Alternatively, FCV alone may soon be able to address this issue, as modifications have recently been made to this technique that allow it to be used to monitor both tonic and phasic transmission at a single carbon fibre electrode (Atcherley et al., 2015).

We found no indication that DAT blockade affected evoked phasic dopamine release in the MFC, in agreement with previous reports of low DAT expression levels in cortex (Sesack et al., 1998) and of reduced efficacy of GBR-12909 in blocking reuptake in cortex compared to striatum (Cass and Gerhardt, 1995; Izenwasser et al., 1990; Mundorf et al., 2001). Although several previous microdialysis studies have, like us, failed to find an effect of GBR-12909 on cortical dopamine levels (Mazei et al., 2002; Pozzi et al., 1994; Weikop et al., 2007), others have observed an effect (Carboni et al., 2006; Tanda et al., 1997; Valentini

et al., 2004). The variability in these microdialysis results is not readily explainable by differences in experimental approach and so may simply be attributable to DAT's limited role in regulating cortical dopamine, which may make it difficult to consistently detect an effect of GBR-12909 on dopamine transmission in cortex. These studies suggest that whatever influence DAT reuptake has over cortical dopamine transmission occurs at longer timescales than we assessed with our FCV recordings. It is not known whether dopamine transmission operates in 'tonic' and 'phasic' modes in the cortex, as it does in the striatum. If cortical dopamine does exhibit multiple transmission modes, however, it is possible that DAT may influence one mode more prominently than another. Overall, however, our data concur with the literature that DAT's role in cortex is minimal.

Interestingly, the noradrenaline transporter (NET) appears to account for more reuptake of dopamine in the cortex than DAT does (Morón et al., 2002; Sulzer et al., 2016; Williams and Steketee, 2004), and numerous microdialysis studies have demonstrated that NET blockers increase cortical dopamine levels (Carboni et al., 2006; Mazei et al., 2002; Valentini et al., 2004; Yamamoto and Novotney, 1998). It would be interesting to assess the effects of a NET blocker on cortical dopamine in our preparation to see if these findings extend to the sub-second timescale at which FCV operates.

Our finding that GBR-12909 increases motivation to lever press for reward on the PR task is in agreement with the literature, which shows that elevated striatal dopamine transmission – induced by blockade of DAT reuptake or genetic knockdown of DAT – enhances animals' willingness to exert effort in the pursuit of reward (Cagniard et al., 2006a, 2006b; Young and Geyer, 2010; Zhuang et al., 2001). Our data also concur with reports that interfering with dopamine signalling in the striatum causes animals to choose

freely or immediately available rewards over rewards that can only be obtained through effort or after a delay, even at the expense of reward size or palatability (Denk et al., 2005; Phillips et al., 2007; Salamone et al., 2007). As such, our data add to the body of evidence indicating that one of mesolimbic dopamine's key roles in reward processing is to modulate behavioural activation, motivational drive, and cost/benefit decisions about how to allocate effort in pursuit of reward.

Previous work has most prominently implicated tonic striatal dopamine transmission in motivational and effort-related aspects of reward-guided behaviour (Cagniard et al., 2006b; Salamone and Correa, 2012), whereas phasic dopamine transients are more closely linked with reward learning (Schultz et al., 1997) as well as with action initiation (Hamid et al., 2016; Syed et al., 2016). Given that we observed effects of GBR-12909 on both phasic dopamine transients in the NAc (in our FCV recordings) and on motivation to work for reward (in the PR task), our study suggests that phasic dopamine transmission may contribute to motivational drive and the ability to exert effort for reward. Phasic transients can sum to influence tonic dopamine (Hamid et al., 2016), and such an effect could therefore be due to elevated tonic dopamine levels caused by the summation of increased phasic dopamine release. It is important to keep in mind, however, that the simplicity of the PR task makes it difficult to determine exactly why GBR-12909 causes an increase in active lever pressing. For example, it is possible that, by elevating striatal dopamine levels, GBR-12909 in fact minimises the impact of negative prediction errors that occur in response to the multiple unrewarded lever presses in each subsequent trial of the PR task. DAT blockade could thus impair learning and so allow animals to persist in lever pressing despite the increasing number of lever presses required

to obtain each reward. The PR task served our purpose of assessing potential interactive effects of DAT and COMT on reward-guided behaviour, but more sophisticated behavioural paradigms will be useful for determining exactly how DAT blockade affects reward processing.

The 2-step task offers one such approach. Indeed, this task yielded one of the most intriguing findings in our study by revealing that DAT blockade can shift the balance between the influence of model-free and model-based RL strategies on decision making. We found that GBR-12909 decreased the influence of model-free RL and increased the influence of model-based RL on this task. Because our voltammetry data as well as previous work show that DAT predominantly affects striatal dopamine transmission and plays little to no role in the regulation of cortical dopamine, the 2-step finding strongly indicates that striatal dopamine plays a role in both model-free and model-based learning. Critically, our data demonstrate a causal link between striatal dopamine transmission and RL that the correlational findings of human studies have only hinted at.

Our 2-step findings challenge the traditional view that model-free RL is mediated by striatal dopamine and model-based RL by cortical dopamine. We found instead that increased dopamine transmission in striatum is negatively correlated with model-free RL but positively correlated with model-based RL, in agreement with the results of several recent human studies (Deserno et al., 2015; Sharp et al., 2016; de Wit et al., 2012; Wunderlich et al., 2012). Our findings are also in line with data from mice that overexpress D2 dopamine receptors in the striatum, which have decreased dopamine transmission in the cortex and exhibit deficits in both motivation and cognitive function (Simpson and Kellendonk, 2016). Evidence of a causal influence of striatal dopamine transmission on

model-based learning is particularly intriguing because it shows that striatal dopamine can affect complex cognitive functions, which are traditionally thought of as the domain of the cortex. Understanding the extent of striatal dopamine's influence on cognitive processing is of great interest in the context of neuropsychiatric disorders, such as Parkinson's disease and schizophrenia, that involve abnormalities in both striatal dopamine and cognitive function. The cognitive symptoms associated with these illnesses are generally ascribed to changes in cortical function. However, our findings suggest that they may result, at least in part, from the alterations in striatal dopamine that occur in these disorders.

8.2 Role of COMT degradation

In contrast to the clear effects of DAT blockade on both striatal dopamine transmission and behaviour, the COMT inhibitor tolcapone had little effect in any of our experiments. Tolcapone had no effect on evoked dopamine transmission in the NAc in our FCV recordings, in agreement with previous studies showing that, although tolcapone can alter dopamine metabolite levels in the striatum, it does not on its own modulate either tonic or phasic dopamine transmission in this region (Acquas et al., 1992; Budygin et al., 1999; Garris and Wightman, 1995; Huotari et al., 1999; Li et al., 1998; Raevskii et al., 2002). We originally predicted that tolcapone might potentiate GBR-12909's effects on NAc dopamine transients, as several studies have reported that COMT inhibition can potentiate the effects of DAT blockers or other dopamine-enhancing drugs on dopamine transmission in this region (Budygin et al., 1999; Huotari et al., 1999; Raevskii et al., 2002). However, while there was a numeric increase in signal size under the combined influence of tolcapone and GBR-12909, we found no statistically significant evidence of such

potentiation. Our data thus support the traditional view that COMT degradation has little influence over striatal dopamine transmission.

Unexpectedly, tolcapone also had no effect on evoked dopamine transients in the MFC. This was surprising given that COMT is believed to play an important role in the regulation of cortical dopamine transmission. Indeed, evidence from both transgenic mice and humans genotyped for the Val^{158}Met COMT polymorphism indicates that lower levels of COMT activity result in higher levels of dopamine in the cortex (Gogos et al., 1998; Huotari et al., 2002b; Käenmäki et al., 2010; Slifstein et al., 2008), and previous microdialysis studies have shown that tolcapone can increase stimulated cortical dopamine release (Lapish et al., 2009; Tammimaki et al., 2016; Tunbridge et al., 2004). Based on these microdialysis results, we expected to see an effect of COMT inhibition on dopamine transmission in the MFC at least when tolcapone was administered together with GBR-12909, but we found no evidence of additive or interactive effects of the two drugs. Our findings thus indicate that COMT does not regulate dopamine transients in the cortex at the sub-second timescale assessed by FCV.

The lack of knowledge about what physiological functions cortical dopamine transmission serves at tonic versus phasic timescales makes it difficult to draw a firm conclusion on why we saw no effect of COMT inhibition in our recordings. It may be that degradation by COMT only influences cortical dopamine at the longer timescales measured by microdialysis and that other clearance mechanisms, such as reuptake by NET, regulate quicker and shorter-lived dopamine transients in the cortex. Furthermore, the lack of autoreceptors in cortically-projecting dopamine neurons (Gainetdinov and Caron, 2003; Lammel et al., 2008) may prevent tonic cortical dopamine transmission from regulating

phasic release in the cortex in the way that tonic levels of striatal dopamine can influence striatal phasic release via D2 autoreceptors (Grace, 1991; Sulzer et al., 2016). It is also important to keep in mind that we recorded electrically evoked dopamine release in anaesthetised animals and that COMT inhibition may produce effects on behaviourally evoked dopamine transmission in awake animals that are not reproducible in an anaesthetised preparation.

We found little indication that tolcapone boosted reward-guided responding in the PR task, either on its own or in combination with GBR-12909. As the motivational drive assessed by this task is primarily mediated by striatal dopamine, and as our FCV recordings showed no effect of COMT inhibition (with or without concurrent DAT blockade) on dopamine transmission in the striatum, this lack of effect of tolcapone on PR responding is not unexpected and is in line with the literature. We did observe one interesting effect of tolcapone on the PR task: tolcapone appeared to rescue a dampening of responding induced by vehicle injections. We interpret this as evidence that inhibition of COMT can modulate the effects of injection stress – a phenomenon we have observed repeatedly in studies conducted by our lab (Laatikainen et al., 2012).

As in the PR task, tolcapone had no direct effect on learning strategy in the 2-step decision making task, including on the relative influence of either model-free or model-based RL on behaviour. This was unexpected, as cortical dopamine is generally believed to influence model-based RL. Model-based learning clearly relies on cognitive functions that are mediated by the cortex (Eppinger et al., 2013; Otto et al., 2013a, 2013b; Smittenaar et al., 2013), including functions like working memory that are modulated by cortical dopamine levels (Brozoski et al., 1979; Phillips et al., 2004; Robbins, 2005; Sawaguchi and

Goldman-Rakic, 1991). Furthermore, in contrast to our findings, a recent study showed that differential model-based RL on the human version of the 2-step task correlated with Val^{158}Met genotype (Doll et al., 2015).

It may be that acute manipulation of COMT activity by tolcapone does not exert the same effects on RL as congenital, genetically-mediated differences in COMT activity levels. Alternatively, the effects COMT has on cortical dopamine and cognitive function may not come into play in the rodent version of the 2-step task. Working memory is an important mediator of performance on the human version of the 2-step task, but mice do not appear to solve the rodent version using the same approach. Indeed, 'working memory' in mice denotes retention of spatial or directional information in short-term memory and is not equivalent to humans' ability to remember and manipulate abstract information (Floresco and Jentsch, 2011; Sanderson and Bannerman, 2011). As a result, any effects of tolcapone on cortical dopamine and cognitive function in the mice may not translate to altered model-based RL in the 2-step task. A final consideration is that, as indicated by the PR experiment as well as by our lab's findings on injection stress mentioned above, COMT's influence on behaviour appears to be linked to arousal level. It is therefore possible that tolcapone might influence RL under conditions of higher arousal, such as following acute stress or even in combination with GBR-12909. It was not practically feasible for us to assess the combined effects of tolcapone and GBR-12909 on the 2-step task in our study, but future work may be more successful in clarifying COMT's role in RL by pairing manipulations of COMT activity with additional manipulations of dopamine transmission or arousal level.

8.3 Conclusions

One of the insights we hoped to gain from this study was an increased understanding of how the mesolimbic and mesocortical dopamine systems interface with one another. Although this insight did not come in the form of evidence of DAT-COMT interactions, as we initially anticipated, our 2-step task data do shed light on this issue by demonstrating the influence of striatal dopamine over both model-free and model-based RL. Both of these types of learning, particularly model-based RL, operate across corticostriatal networks. Our findings emphasise the importance of striatal dopamine for cognitive processes. The detailed mechanisms underlying this striatal influence remain to be determined and will require both a better understanding of the functional connectivity between striatum and cortex and of the dynamics of dopamine transmission within each of these regions. Our voltammetry data contribute insights to the latter question, particularly our finding that neither COMT nor DAT regulate cortical dopamine transmission at sub-second timescales. This indicates that dopamine in cortex may operate, as it does in striatum, via multiple transmission modes that produce different physiological and functional effects. It will be interesting to see whether this proves to be the case and what behavioural consequences different types of cortical dopamine transmission might have.

Abbreviations

ACC	Anterior cingulate cortex
ACSF	Artificial cerebrospinal fluid
ADH	Aldehyde dehydrogenase
Amy	Amygdala
ANOVA	Analysis of variance
AUC	Area under the curve
BOLD	Blood oxygen level dependent
Cgl	Cingulated cortex
COMT	Catechol-*O*-methyltransferase
DAB	Diaminobenzidine
DAT	Dopamine transporter
DHMA	3,4-dihydroxymandelic acid
DHPA	3,4-dihydroxyphenylacetaldehyde
DHPG	Dihydroxyphenylglycol
DOPA	Dihydroxyphenylalanine
DOPAC	3,4-dihydroxyphenylacetic acid
D2R-OE	D2 receptor overexpressor
fMRI	Functional magnetic resonance imaging
FR	Fixed ratio
GABA	Gamma-aminobutyric acid
GBR	GBR-12909
Hipp	Hippocampus
HVA	Homovanillic acid
ITI	Inter-trial interval
L-DOPA	L-3,4-dihydroxyphenylalanine
LDTg	Lateral dorsal tegmentum
LED	Light-emitting diode
LH	Lateral hypothalamus

LHb	Lateral habenula
LMA	Locomotor activity
MAO	Monoamine oxidase
MB-COMT	Membrane-bound catechol-*O*-methyltransferase
Met	Methionine
MFC	Medial frontal cortex
MHPA	3-methoxy-4-hydroxyphenylacetaldehyde
MO	Medial orbital cortex
MSN	Medium spiny neuron
3-MT	3-methoxytyramine
NAc	Nucleus accumbens
NET	Noradrenaline transporter
ns	Not significant
6-OHDA	6-hydroxydopamine
PBP	Paragrachial pigmented nucleus
PBS	Phosphate buffered saline
PC	Principal component
PCA	Principal component analysis
PCR	Principal component regression
PET	Positron emission tomography
PFC	Prefrontal cortex
pH	Potential of hydrogen
PN	Paranigral nucleus
PR	Progressive ratio
PrL	Prelimbic cortex
RL	Reinforcement learning
RMTg	Rostromedial tegmentum
RPE	Reward prediction error
S-COMT	Soluble catechol-*O*-methyltransferase
SEM	Standard error of the mean
SN	Substantia nigra

SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
TH	Tyrosine hydroxylase
Tolc	Tolcapone
Val	Valine
Veh	Vehicle
VMA	Vanillylmandelic acid
VNTR	Variable number of tandem repeats
VTA	Ventral tegmental area

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