Effects of Catechol-O-Methyltransferase on Reinforcement Learning and Mesolimbic Dopamine Transmission



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Abstract

The enzyme catechol-O-methyltransferase (COMT) plays an important role in the breakdown of dopamine in prefrontal cortex (PFC), but relatively little is known about its effects on nucleus accumbens (NAc) dopamine function. In this study, a transgenic mouse model of the Val158Met polymorphism found in the human COMT gene was used (a) to investigate the effects of COMT on associative learning, a process dependent on NAc dopamine, and (b) to relate this to potential changes in phasic dopamine release in the NAc core in awake behaving mice. In the first set of experiments (Chapter 3), animals were trained on a Pavlovian auditory discrimination task with white noise (WN) and a constant tone (Tone) as the rewarded (CS+) or unrewarded (CS-) conditioned stimulus (assignment of stimulus type counterbalanced between subjects). Whilst both COMT-Met mice and wild-type (WT) littermates acquired the cue-reward associations, learning rates of COMT-Met mice were significantly higher than those of WTs when WN was the CS+ and significantly lower when Tone was the CS+. Further experiments revealed that this effect was mediated by an interaction between COMT genotype and the unconditioned salience of the CS+, an effect that persisted even in the absence of a CS- (Chapter4). Cue-dependency of learning could not be explained by NAc dopamine release in response to auditory stimuli, as both genotypes showed similar response patterns as measured by fast-scan cyclic voltammetry at the start of training (Chapter 6). In a separate experiment, COMT-Met mice showed greater phasic dopamine levels in NAc core evoked by unsignalled reward than WT mice, especially when rewards were smaller than expected. Taken together, behavioural studies of associative learning in COMT-Met and WT mice revealed that COMT genotype affected the impact unconditioned cue salience had on associative learning. Direct measurements of phasic release of dopamine in the NAc core further showed a task-dependent effect of COMT genotype on dopamine release.

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Chapter 1: General Introduction

The enzyme catechol-*O*-methyltransferase (COMT) metabolises catechol-containing compounds, including dopamine and noradrenaline, and much research has focussed on its role in regulating prefrontal cortical dopamine as well as cognitive function. This thesis investigates whether COMT might also have an effect on reward learning and subcortical dopamine. I will first give an overview of the brain dopamine system and of what is currently known about the role of COMT in different brain regions, focussing on the prefrontal cortex (PFC) and the ventral striatum. I will then highlight the use of associative learning paradigms as a way of studying reward learning processes and dopamine signalling in the ventral striatum. Finally, I will introduce the transgenic mouse model that has been used to study the role of COMT in associative learning and dopamine release in the experiments presented in thesis.

1.1. The dopamine system

1.1.1. Projections

Dopamine-synthesising cells are located in certain nuclei of the midbrain, as well as the hypothalamus, from which they project to their target structures in the brain. The projections are frequently divided into separate, distinct pathways based on their projection targets: nigrostriatal, mesolimbic, and mesocortical pathways (and also the little mentioned tuberoinfundibular pathway, from the hypothalamus to the pituitary gland, where it controls the secretion of prolactin and somatotrophin). While there is good evidence for dopamine projections targeting non-overlapping regions, it should also be noted that there is increasing evidence for a diversity of output targets from the different midbrain nuclei (Beier et al., 2015; Menegas et al., 2015) as well as diversity of

properties of midbrain dopamine neurons (Lammel et al., 2008; Margolis, Mitchell, Ishikawa, Hjelmstad, & Fields, 2008; Schiemann et al., 2012). For the sake of simplicity, these will only be discussed if of direct relevance to the hypotheses being advanced by the thesis.

The nigrostriatal pathway originates in the substantia nigra pars compacta (SNc) and projects most strongly to the dorsal striatum. It is important in motor control and has been extensively studied in the context of motor disorders – the primary loss in Parkinson's disease is of SNc dopamine neurons – although more recently there has been research into its role in action selection and decision-making (Balleine, Delgado, & Hikosaka, 2007; Cui et al., 2013; DeLong, 1990; Wise, 2009). The mesolimbic pathway projects from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) core and shell regions and the olfactory tubercle, which collectively form the ventral striatum. The NAc, and especially dopamine release in this structure, has been extensively studied in the context of reward, motivation and reinforcement learning and its function shall be discussed in more detail in the sections below. Finally, the mesocortical pathway describes projections from the VTA to cortical and limbic structures, most notably the PFC, amygdala, and hippocampus (Moore & Bloom, 1978; Ungerstedt, 1971). What role dopamine release in cortex and temporal lobe nuclei plays remains a matter of much debate (Floresco, 2013). Most relevant for the studies presented in this thesis are the pathways projecting to the NAc and the PFC, and their respective roles and interactions.

1.1.2. Biosynthesis, release and inactivation of dopamine

Dopamine is part of the family of catecholamines and is found both in the central nervous system and in peripheral tissues, although this thesis will focus on its role in the brain. Like all catecholamines, dopamine is synthesised from the amino acid tyrosine by

the action of tyrosine hydroxylase (TH; Figure 1-1), the rate-limiting step in catecholamine synthesis, and is loaded into vesicles by the vesicular monoamine transporter for release via exocytosis.

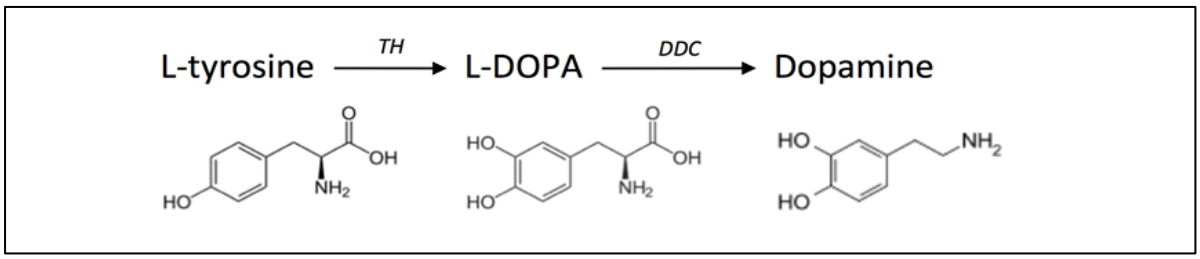


Figure 1-1. Dopamine synthesis*. Dopamine is synthesised from the amino acid precursor L-tyrosine. The rate-limiting step in their formation is under the control of the enzyme tyrosine hydroxylase (TH). L-DOPA, 3,4-dihydroxyphenylalanine; DDC, DOPA-decarboxylase*

Once released, dopamine can act on five types of receptors (D1-D5) – all are G-protein-coupled metabotropic receptors – which can be grouped into two functional classes: the D1-like receptors, comprised of dopamine receptors D1 and D5; and the D2-like receptors, comprised of D2, D3, and D4 (Beaulieu & Gainetdinov, 2011). D1-like receptors are positively coupled to the intracellular adenylate cyclase pathway, whereas D2-like receptors are negatively coupled, leading to excitatory and inhibitory effects downstream, respectively (Sealfon & Olanow, 2000; West & Grace, 2002). High affinity D2 receptors (autoreceptors) exist on the presynaptic membranes and provide feedback inhibition, regulate cell firing and the release and uptake of dopamine (reviewed by Ford, 2014). There are regional differences in the expression of the different dopamine receptor subtypes. D2 autoreceptors, for example, are found at high densities in the NAc and the dorsal striatum, but at lower densities in the hippocampus, the amygdala and cortical regions (De Mei, Ramos, Iitaka, & Borrelli, 2009; Schmitz, Benoit-Marand, Gonon, & Sulzer, 2003).

Electrophysiological recordings have revealed three major patterns of activity in midbrain dopamine neurons: an inactive, hyperpolarised state; a slow firing pattern, which consists of individual action potentials (1-10Hz) and shall be referred to as "tonic" from here on (Grace & Bunney, 1984); and a fast burst (>20Hz), or phasic, firing mode (Hyland, Reynolds, Hay, Perk, & Miller, 2002; Robinson, Smith, Mizumori, & Palmiter, 2004). The distinction between tonic and phasic firing of dopamine neurons is important as these two firing modes have been proposed to lead to non-linear effects on dopamine release and differential activation of D1 versus D2 receptors at the terminal sites. Increases in phasic firing leads to a greater release of dopamine and greater occupancy of dopamine D1 receptors, whereas tonic firing leads to less dopamine release and comparably greater occupancy of D2 receptors; pauses in dopamine cell activity cause a decrease in occupancy in both D1 and D2 receptors (Dreyer, Herrik, Berg, & Hounsgaard, 2010; Floresco, West, Ash, Moore, & Grace, 2003; Gonon, 1988; Grace, 1991). It should be noted, however, that the distinction between tonic and phasic firing modes is not necessarily as clear-cut as often described in the literature (see, for example, (McNamara, Tejero-Cantero, Trouche, Campo-Urriza, & Dupret, 2014).

Phasic dopamine release can bidirectionally modulate plasticity on the post-synaptic site through interactions with glutamate (Kombian & Malenka, 1994; Shen, Flajolet, Greengard, & Surmeier, 2008; Wickens, Reynolds, & Hyland, 2003). The magnitude of the phasic dopamine response is proposed to be modulated by tonic dopamine, which acts on highly sensitive autoreceptors (Grace, 1991), as well as by interactions with other neurotransmitters such as acetylcholine (Threlfell et al., 2010). Interestingly, the ratio of phasic-to-tonic dopamine differs across different dopaminergic projection targets (Garris & Wightman, 1995; Zhang, Doyon, Clark, Phillips, & Dani, 2009).

Once dopamine has been released, there are two major ways of terminating its action, in addition to diffusion: reuptake and catabolism. Extracellular dopamine can be transported out of the extracellular space by the dopamine transporter (DAT), the noradrenaline transporter, or the extra-neuronal monoamine transporter (Jonker & Schinkel, 2004; Moron, Brockington, Wise, Rocha, & Hope, 2002). After reuptake into the cell, dopamine can either be degraded or repackaged into vesicles for release. The second way of terminating the action of dopamine is via its enzymatic degradation. This process can be initiated by two enzymes, COMT, and monoamine oxidase (MAO; Figure 1-2). The prevailing mechanism of dopamine signal termination depends on a number of factors, including the relative expression levels of reuptake transporters and their distance from the site of dopamine release.

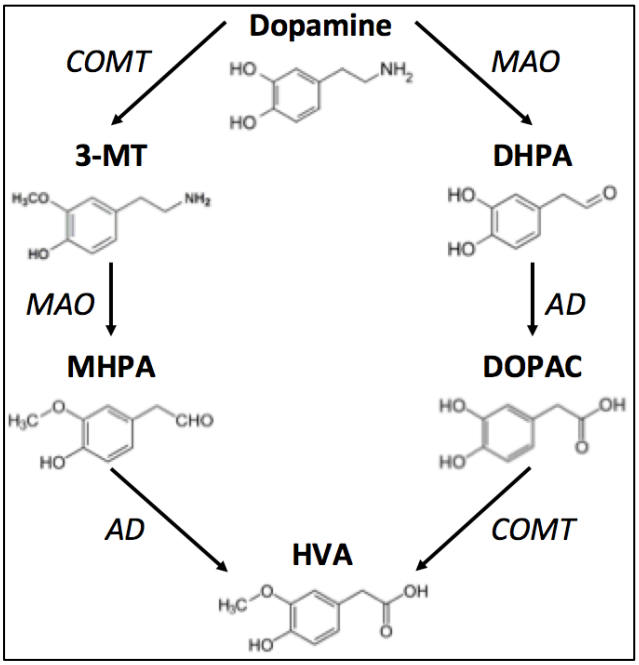


Figure 1-2. Dopamine catabolism. *Dopamine is degraded by the enzymes catechol-o-methyltransferase (COMT), monoamine oxidase (MAO), and aldehyde dehydrogenase (AD). The intermediate products are 3-methoxytyramine (3-MT), 3,4-di-hydroxyphenylacetaldehyde (DHPA), 3-methoxy-4-hydroxyphenylacet-aldehyde (MHPA), and 3,4-dihydroxy-phenylacetic acid (DOPAC). The end product is homovanillic acid (HVA).*

1.2. Dopamine function

1.2.1. Dopamine and cognitive function

A landmark study by Broski and colleagues, demonstrating that catecholamine depletion of the PFC impaired spatial memory in rhesus monkeys (Brozoski, Brown, Rosvold, & Goldman, 1979), triggered a surge of research into the role of dopamine in cognitive function. Indeed, several lines of research over the past 40 years have confirmed a crucial influence of PFC dopamine levels on cognitive function. Microdialysis studies in the PFC of primates and rodents have revealed an increase in extracellular dopamine levels during performance of working memory tasks (Phillips, Ahn, & Floresco, 2004; Watanabe, Kodama, & Hikosaka, 1997), whilst dopamine depletions, as well as administration of D1 receptor antagonists, reliably impair cognitive function (Goldman-Rakic, 2000; Sawaguchi & Goldman-Rakic, 1991; Williams & Goldman-Rakic, 1995). Interestingly, there seems to be an inverted-U relationship between dopamine D1 receptor activation and working memory task performance, whereby performance is best at intermediate levels of D1 activation and deteriorates as levels either decrease or increase (Floresco & Phillips, 2001; Goldman-Rakic, 2000) (Figure 1-3).

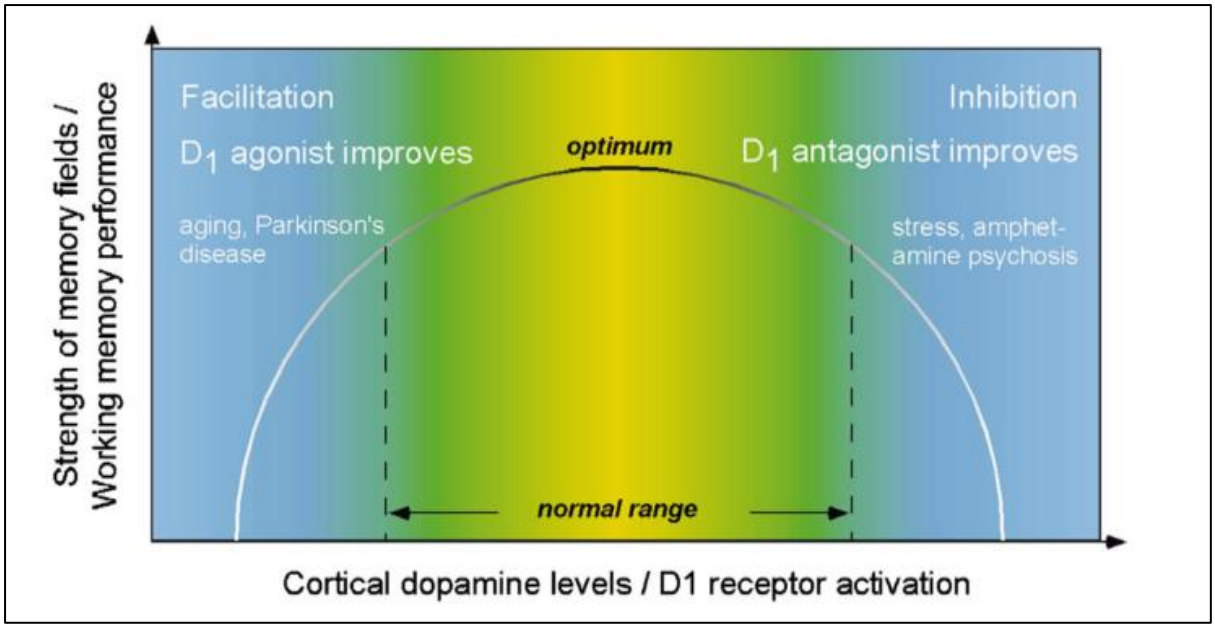


Figure 1-3. Inverted-U relationship between cortical D1 receptor activation and working memory performance. *(Taken from* Goldman-Rakic, 2000*).*

Durstewitz and Seamans (2008) adapted and extended the idea of this inverted-U relationship between D1 receptor occupancy and working memory performance by including the action of cortical dopamine D2 receptors into their model (Figure 1-4). According to their "dual-state theory of PFC dopamine function", microcircuits in the PFC can exist in states of high and low activity, which depend on the ratio between local D1 and D2 receptor activation. D1 receptor activation increases local NMDA and other voltage-dependent currents (Yang & Seamans, 1996; Zheng, Zhang, Bunney, & Shi, 1999), boosting recurrent excitation within neighbouring cell assemblies, while increasing $GABA_A$ currents and therefore competition between different active assemblies of neurons (Seamans, Gorelova, Durstewitz, & Yang, 2001). In electrophysiological terms, D1 receptor activation increases the signal-to-noise ratio by boosting the firing rates of neurons involved in encoding the currently active state and decreasing those of neurons associated with non-active states, as well as suppressing spontaneous activity. In the

theoretical framework, this means an increase in the energy barrier that needs to be overcome in order for a switch between different active states to occur (Durstewitz, Seamans, & Sejnowski, 2000). In more behaviourally relevant terms, this means that D1 receptor activation leads to a more stable neural representation of task-relevant items, whilst reducing interference from irrelevant items.

D2 receptor activation, on the other hand, has opposite effects on local NMDA and $GABA_A$ currents and on the excitability of pyramidal cells in the PFC (Gulledge & Jaffe, 1998; Trantham-Davidson, Neely, Lavin, & Seamans, 2004). Within the proposed framework, increased D2 receptor activation therefore reduces the energy barrier between activity states, allowing activity arising in surrounding cell assemblies to push the system from one state into another. Whilst this might be detrimental to behavioural performance in situations that require stable maintenance of items in working memory, it might be beneficial in situations that require switching between different task requirements (Fallon, Williams-Gray, Barker, Owen, & Hampshire, 2013; Floresco, Magyar, Ghods-Sharifi, Vexelman, & Tse, 2006; O'Reilly, 2002). D2-dominant states are thought to prevail both at very low and very high concentrations of dopamine, possibly mediated by high and low affinity receptor subtypes respectively (Richfield, Penney, & Young, 1989; Seeman et al., 2005; Sibley, De Lean, & Creese, 1982). Importantly, however, at intermediate levels of dopamine, more D1 than D2 receptors are activated, bringing the cortical network into a D1-dominant state (Durstewitz & Seamans, 2008).

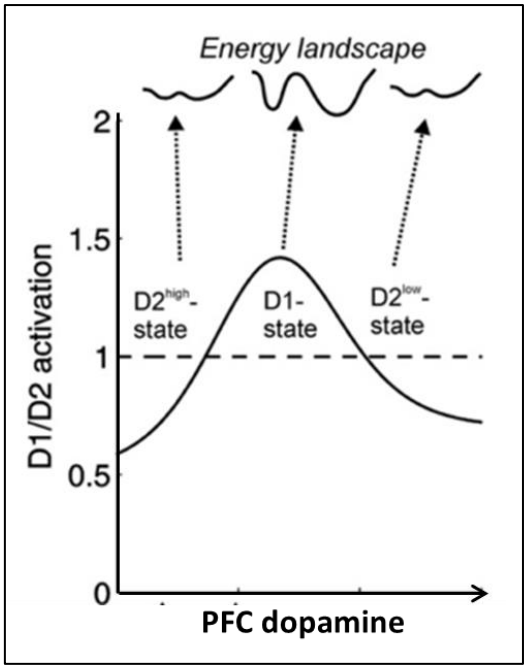


Figure 1-4. Hypothesized dopamine dependency of PFC dynamical regimes proposed by the dual-state theory of PFC dopamine *by Durstewitz and Seamans (2008). The D1/D2 activation ratio, and the resulting dynamical states and associated attractor landscapes are depicted in relationship to PFC dopamine concentration. Adapted from Durstewitz and Seamans (2008).*

Studies investigating the differential roles of D1 and D2 agonists and antagonists in tasks that require either stable working memory or flexibility have provided experimental support for this hypothesis (reviewed in Durstewitz & Seamans, 2008). The implications of the foregoing evidence on understanding the effect of differences in COMT activity on PFC dopamine levels shall be discussed further in section 1.3.5.

1.2.2. Dopamine and reward processing

The term "reward" is often used interchangeably to describe distinct psychological components of reinforcement, incentive motivation, information, homeostasis and hedonic pleasure, all of which have at some points in the literature been argued to be mediated by mesolimbic dopamine (Berridge & Robinson, 1998; Robbins & Everitt, 2007; Salamone & Correa, 2012; Schultz, 2007; Wise & Rompre, 1989). While it

is now generally agreed that dopamine is not needed for hedonic appreciation - or "liking" - of reward or the signalling of all reward delivery (Berridge, 1996; Salamone & Correa, 2012; Schultz, 1997), its influence over the other aspects of reward function – and aversion – remains a matter of much debate. One potential reason for the lack of consensus is that there are discrepancies between ideas that have emerged from electrophysiological recordings of putative midbrain dopamine neurons and those that have come from measuring or interfering with dopamine release in terminal regions. For instance, unsignalled delivery of appetitive reward has been shown to cause a brief increase in activity in a large proportion of midbrain dopamine neurons in both SNc and VTA (Cohen, Haesler, Vong, Lowell, & Uchida, 2012; Mirenowicz & Schultz, 1994). However, studies using voltammetry to record dopamine release have found that unsignalled reward only causes consistent phasic increases in dopamine levels in the NAc core region (Brown, McCutcheon, Cone, Ragozzino, & Roitman, 2011).

For the purposes of the current thesis, these debates can be largely put to one side as (a) the precise functional role of dopamine release does not directly impact on the basic question of how COMT might influence striatal dopamine release, and (b) all electrochemical recordings have been made in the NAc, where patterns of release most closely resemble observed properties of midbrain dopamine cells during learning and behaviour (i.e., increases in dopamine are observed to unsignalled rewards, novel cues, and cues that predict reward). Of particular relevance to the work presented here, dopamine release in the NAc has been shown to scale with reward size (Gan, Walton, & Phillips, 2010; Hollon, Arnold, Gan, Walton, & Phillips, 2014; Howe, Tierney, Sandberg, Phillips, & Graybiel, 2013) and to adapt during Pavlovian conditioning as cue-reward associations are acquired (Day, Roitman, Wightman, & Carelli, 2007; Parker et al., 2010).

The importance of dopamine release in the NAc for aspects of associative learning will be discussed in more detail in Section 1.4.2.

1.3. Catechol-*O*-methyltransferase

COMT initiates the breakdown of catechol-containing molecules by catalysing the transfer of a methyl group to one of the hydroxyl groups of the catechol ring of its substrates (Figure 1-2). This transfer takes place in the active site of the enzyme, which has been shown to be conserved across species, suggesting a similar enzymatic process in rodents and humans (Tenhunen et al., 1994).

1.3.1. Structure, expression, and genetic variants

The human COMT gene is located on chromosome 22, band q11.2 (Grossman, Emanuel, & Budarf, 1992), and consists of two non-coding and four coding exons (Figure 1-5). It contains two promoter regions, P2 and P1, which drive the transcription of a long transcript, containing two start codons (AUG), and a short transcript, containing only one start codon, respectively (Lotta et al., 1995; Tenhunen et al., 1994). Translation initiated from the first start codon produces the longer, membrane bound (MB)-COMT protein, whilst translation initiated from the second start codon produces the shorter, soluble (S)-COMT protein. The longer isoform includes additional hydrophobic amino acids which form a transmembrane domain (Lotta et al., 1995). MB-COMT is the predominant form found in the brain (60-70%) while S-COMT prevails in peripheral tissues (~70%, Tenhunen et al., 1994). In addition to its greater prevalence in the brain, MB-COMT also shows 10 times greater affinity for dopamine and noradrenaline, and is therefore thought to play the predominant role in the inactivation of catecholamine neurotransmitters, whilst S-COMT is predominantly responsible for eliminating toxic, exogenous catechols

in the periphery (Kaakkola, Gordin, & Männistö, 1994; Männistö & Kaakkola, 1999; Männistö et al., 1992).

It is also noteworthy that the promoter regions contain hormone response elements and binding sites for estrogens and glucocorticoids (Salama et al., 2006; Wentz, Jamaluddin, Garfield, & Al-Hendy, 2006; Xie, Ho, & Ramsden, 1999) which may contribute to interactive effects of sex and stress with COMT (Torda & Kvetnanský, 1983).

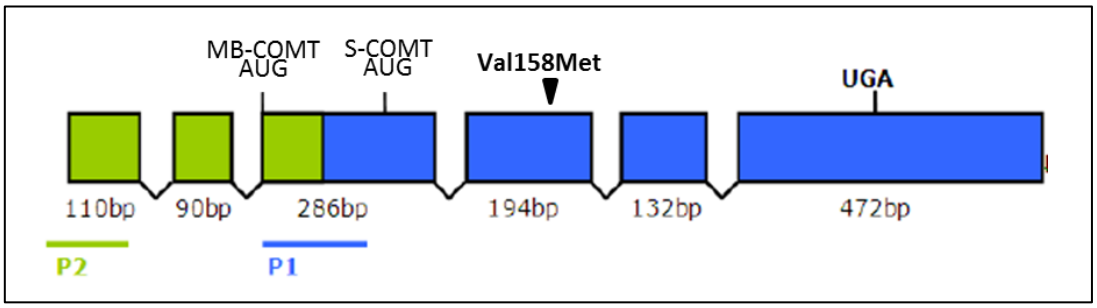


Figure 1-5. The structure of the human COMT gene. *Exons are shown as boxes and introns as connecting lines. Exon length is indicated by numbers of base pairs below the boxes. The green portion indicates the exon sequence unique to the mRNA transcript encoding MB-COMT. Labels highlight the promoters P1 and P2, the AUG translation start sites for MB-COMT and S-COMT, the UGA translation stop codon, as well as the location of the Val158Met polymorphism (adapted from Tenhunen et al 1994).*

1.3.2. Cellular and subcellular localisation

There has long been controversy about the cellular and subcellular localisation of COMT. Depending on which particular study to look at, COMT has been found only in glia (Kaplan, Hartman, & Creveling, 1979; Reenilä, Tuomainen, Soinila, & Männistö, 1997), only in neurons (Matsumoto, Weickert, Akil, et al., 2003), or in both glia and neurons (Broch & Fonnum, 1972; Garbarg, Baudry, Benda, & Schwartz, 1975; Karhunen, Tilgmann, Ulmanen, Julkunen, & Panula, 1994; Karhunen, Tilgmann, Ulmanen, & Panula, 1995; Myöhänen & Männistö, 2010). One way to reconcile these findings would be to selectively target S- and MB-COMT, which had been suggested to be differentially

localised to glia and neurons, respectively (Rivett, Francis, & Roth, 1983). However, such studies are difficult to carry out due to the lack of isoform-specific antibodies.

Determining the subcellular localisation of COMT has proven even more difficult. Initially, MB-COMT has been suggested to be bound to the plasma membrane, protruding into the extracellular space, and potentially even into the synapse (Aprille & Malamud, 1975; Karhunen et al., 1995; Kastner et al., 1994; Raxworthy, Gulliver, & Hughes, 1982; Traiger & Calvert, 1969). However, this view was challenged as increased concentrations of calcium ions in the extracellular space are expected to inhibit COMT function (Jeffery & Roth, 1984). Instead, COMT has been suggested to be located on intracellular membranes (Broch & Fonnum, 1972; Grossman et al., 1985; Ulmanen et al., 1997).

Whether COMT acts intra- or extra-cellularly is potentially of great importance: in the former scenario, the rate of dopamine inactivation by COMT would depend on transport or endocytosis of dopamine into the cell, whilst in the latter scenario, COMT would be able to act on extracellular dopamine directly. A study by Chen and colleagues has recently shed some more light on this issue (Chen et al., 2011). By conducting a series of elegant experiments, Chen and colleagues not only showed that at least some of the MB-COMT enzyme protrudes into the extracellular space, but also demonstrated that it is able to fulfil its catalytic function there, thereby suggesting that COMT might indeed directly inactivate extracellular dopamine, independent of reuptake processes.

1.3.3. Expression and functional importance across the brain

COMT is expressed across the entire brain, with slight regional variations. COMT expression was observed in the spinal cord, cerebellum, VTA and the cerebral cortex. Comparatively lower levels of COMT were found in the hippocampus, amygdala, and thalamus (Kastner et al., 1994; Mitsuyuki Matsumoto, Weickert, Akil, et al., 2003;

Tenhunen et al., 1994; reviewed in Myöhänen & Männistö, 2010). Studies in human and rodent post-mortem brain tissue have found higher levels of COMT mRNA in the PFC, compared to the striatum (Hong, Shu-Leong, Tao, & Lap-Ping, 1998; Matsumoto, Weickert, Akil, et al., 2003), although in mice there were no differences between these two brain regions when levels of COMT protein were measured (Myöhänen & Männistö, 2010).

Despite only minor differences in COMT expression levels, region-specific differences in other enzymes involved in the termination of dopamine signaling, such as MAO and DAT, might determine to what extent COMT contributes to the regulation of local dopamine signaling. For instance, reuptake of dopamine by DAT is thought to be the main factor in terminating dopamine signaling in the striatum (Budygin et al., 1999), where DAT is highly expressed and localized close to the synapse, in an ideal position to limit spillover from the synaptic cleft (Hersch, Yi, Heilman, Edwards, & Levey, 1997).

On the other hand, DAT is expressed relatively sparsely in the PFC and is located at a distance from sites of synaptic release (Lewis, Melchitzky, Sesack, Whitehead, & Sampson, 2001; Sesack, Hawrylak, Matus, Guido, & Levey, 1998), thereby allowing dopamine to diffuse further and increasing the importance of enzymatic degradation in the termination of its action. Indeed, there are several lines of evidence demonstrating an important role for COMT in the regulation of dopamine levels in the PFC. For instance, by measuring tissue levels of 3-methoxytyramine, the product formed when dopamine is o-methylated by COMT, Karoum and colleagues determined that COMT contributed to over 60% of total dopamine turnover in the PFC but only 15% in the striatum (Karoum, Chrapusta, & Egan, 1994). In line with this, Yavich and colleagues found that elimination of electrically-stimulated phasic dopamine from the PFC was two-fold slower in COMT knockout mice, compared to wild type controls, without any effect in the dorsal striatum

(Yavich, Forsberg, Karayiorgou, Gogos, & Männistö, 2007). Similarly, COMT knockout mice showed a two-fold increase in dopamine tissue content and a 50% increase in extracellular dopamine levels, as measured by no net flux microdialysis, in the PFC, without any effects observed in NAc or dorsal striatum (Gogos et al., 1998; Käenmäki et al., 2010).

At this point it should be noted that tissue levels of noradrenaline were not altered in PFC or striatum in COMT knockout mice (Gogos et al., 1998).

1.3.4. COMT Val158Met polymorphism

Given the important role of COMT in regulating PFC dopamine levels, it is not surprising that there has been substantial interest in studying the effects of COMT on PFC-mediated behaviour. One way of doing so in the human population is by comparing brain activity and task performance between individuals with high and low COMT enzyme activity genotypes, conferred by functional polymorphisms in the COMT gene.

A single nucleotide polymorphism (SNP) consisting of a guanine-to-adenosine substitution in the coding sequence of the human COMT gene results in the substitution of valine with methionine at position 158 in the human COMT enzyme (Val158Met, rs4680 polymorphism; Bertocci et al., 1991; Lundström, Salminen, Jalanko, Savolainen, & Ulmanen, 1991; Figure 1-5). The valine with methionine substitution leads to reduced thermostability of the enzyme. Consequently, the Val-form of the enzyme is more active at body temperature, with Val homozygotes showing 40% higher enzyme activity in the central nervous system than Met homozygotes (Chen et al., 2004; Lotta et al., 1995). Because the two alleles are codominant, there is a trimodal distribution of high (Val/Val), intermediate (Val/Met), and low (Met/Met) COMT activity in the human population (Floderus, Sääf, Ross, & Wetterberg, 1981; Lachman et al., 1996).

The Val158Met polymorphism is not present in rodents, and the high activity Val form of the enzyme is thought to be the ancestral form (Palmatier, Kang, & Kidd, 1999). Instead of a valine, rodents have a leucine at the corresponding position. Thermostability and therefore activity of the rodent COMT-Leu form is similar to, or slightly greater than, the human COMT-Val form (Chen et al., 2004; Lotta et al., 1995).

Although several other polymorphisms have been identified in the COMT gene, none have been as intensively studied as the Val158Met polymorphism, nor does any other single nucleotide polymorphism within COMT have a comparable effect on enzyme activity (Chen et al., 2004; Gothelf et al., 2014). Indeed, the human Val158Met polymorphism has proven to be an interesting tool for studying the role of individual differences in PFC dopamine in cognition and other behaviours. Some of these studies will be summarised below.

1.3.5. COMT and cognition

Given the importance of dopamine in PFC function, and the role of COMT in regulating PFC dopamine levels, it is not surprising that the COMT Val158Met polymorphism has been implicated in PFC-dependent behaviours.

In a landmark study, Egan and colleagues demonstrated that performance on the Wisconsin Card Sorting Test (WCST), a test of executive control, was significantly correlated with the number of Met alleles that participants carried: Met homozygotes performed better than Val homozygotes, with heterozygotes showing intermediate levels of performance. This was accompanied by lower PFC activity in Met-allele carriers, as measured by the blood-oxygen-level dependent (BOLD) signal in fMRI, compared to Val homozygotes, which the authors interpreted as representing more efficient PFC functioning associated with the Met allele (Egan et al., 2001). In another critical study,

Slifstein and colleagues demonstrated higher D1 receptor availability in the PFC of Val homozygotes relative to Met allele carriers by using positron emission tomography (Slifstein et al., 2008). This was interpreted as a measure for lower PFC dopamine levels in Val homozygotes than Met allele carriers and therefore a confirmation that the human Val158Met polymorphism indeed modulated PFC dopamine levels.

Findings from an ever-growing body of studies examining the relationship between the Val158Met polymorphism, PFC activity and cognition have been mixed. Many support the initial finding that Met-allele carriers have an advantage over Val homozygotes in a variety of cognitive tasks, both on the level of behavioural performance, and, more robustly, on the so-called efficiency of PFC activation (Barnett, Jones, Robbins, & Müller, 2007; Bertolino et al., 2006; Blasi et al., 2005; Farrell, Tunbridge, Braeutigam, & Harrison, 2012; Gallinat et al., 2003; Goldman, Weinberger, Malhotra, & Goldberg, 2009). However, there are also studies reporting no effects of the Val158Met polymorphism on cognitive task performance. Results of meta-analyses have been similarly mixed (Barnett, Scoriels, & Munafò, 2008; Mier, Kirsch, & Meyer-Lindenberg, 2010; Nickl-Jockschat, Janouschek, Eickhoff, & Eickhoff, 2015; Stefanis et al., 2004). Age-related changes in dopamine might interact with COMT and have been suggested to contribute to some of these divergent findings (Ho, Wassink, O'Leary, Sheffield, & Andreasen, 2005; Wahlstrom, Collins, White, & Luciana, 2010). Moreover, "cognitive function" is a broad term that can be studied using a variety of experimental paradigms, not all of which might be sensitive to the neural effects of the Val158Met polymorphism (Tunbridge, Harrison, & Weinberger, 2006). In some cases, the Met allele has even been found to be detrimental to behavioural performance, particularly in tasks that involve

switching and cognitive flexibility (Colzato, Waszak, Nieuwenhuis, Posthuma, & Hommel, 2010; Nolan, Bilder, Lachman, & Volavka, 2004).

More consistent results have been obtained with studies that examine the effect of the Val158Met polymorphism on performance after pharmacological manipulations of the dopamine system (Apud et al., 2007; Farrell et al., 2012; Kelm & Boettiger, 2013; Mattay et al., 2003). For instance, the COMT inhibitor tolcapone improved working memory performance, as measured by the *n*-back task, in Val homozygotes while impairing performance in Met-allele carriers (Farrell et al., 2012). A similar effect on behaviour was obtained after administration of amphetamine, and this was accompanied by more efficient PFC activity, as measured by the BOLD signal in fMRI, in Val homozygotes compared to Met homozygotes (Mattay et al., 2003). These results are consistent with the idea that Val- and Met-allele carriers might occupy different positions on the inverted-U curve describing the relationship between PFC dopamine and working memory due to genotype differences in D1 receptor activation in PFC (Tunbridge et al., 2006; Figure 1-6).

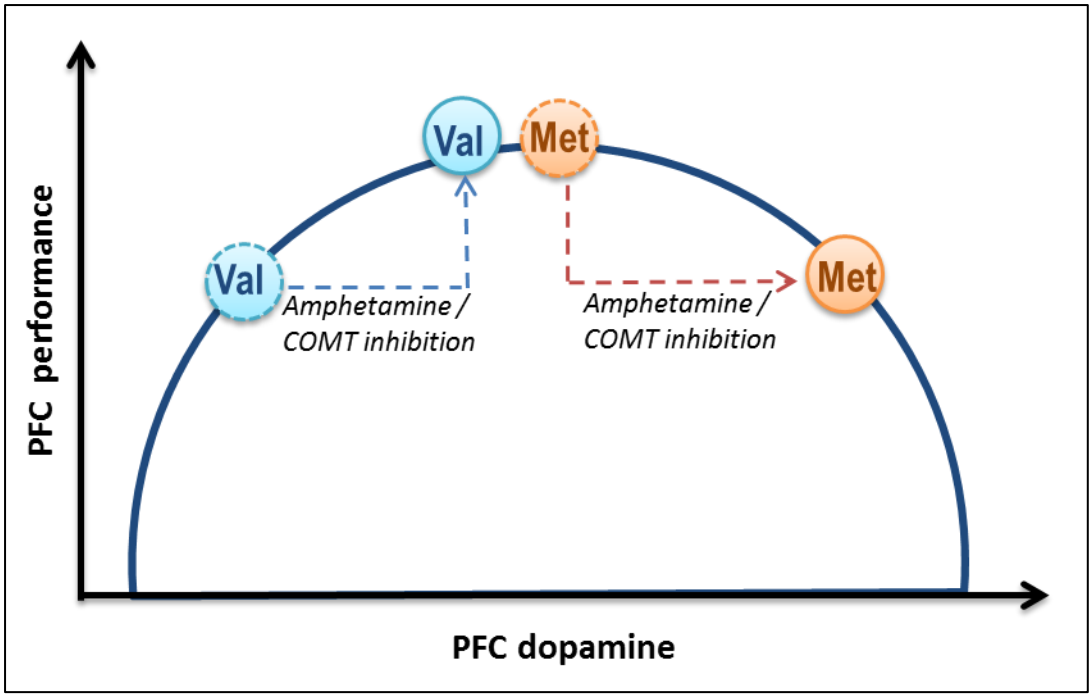


Figure 1-6. COMT and the inverted-U relationship between PFC dopamine and performance. *Subjects homozygous for the Val allele have higher COMT enzyme activity and lower PFC dopamine levels compared to Met homozygotes. They therefore occupy a position left of Met homozygotes on the inverted-U curve. Pharmacologically induced increases in dopamine levels move all subjects along the curve, to the right. Adapted from Tunbridge et al., 2006.*

1.3.6. Does COMT affect reward processing and subcortical dopamine?

Although most studies to date have focused on the role of COMT in PFC dopamine and cognition, some evidence for a role of COMT in reward processing is starting to emerge from human neuroimaging studies investigating the role of the Val158Met polymorphism in monetary reward tasks (reviewed by Tunbridge et al., 2012). Whilst a number of these studies failed to show any effect of COMT on striatal activity during anticipation or receipt of reward (Forbes et al., 2009; Nikolova, Ferrell, Manuck, & Hariri, 2011; Stice, Yokum, Burger, Epstein, & Smolen, 2012), studies that *did* find an effect have been astonishingly consistent with respect to the direction of the effects, despite the use of a range of different behavioural paradigms. For instance, several studies report a positive correlation between the number of Met alleles and ventral striatal and prefrontal

activation (measured by the BOLD signal in fMRI) during the anticipation of upcoming reward (Dreher, Kohn, Kolachana, Weinberger, & Berman, 2009; Yacubian et al., 2007), although in some studies, this effect was only observed during the anticipation of losses, compared with gains (Schmack et al., 2008). Second, Val-allele carriers have been repeatedly found to have greater differences in ventral striatal activation between losses and gains at the reward outcome phase, suggesting that COMT may have an impact on the neural correlates of reward prediction errors (Camara et al., 2010; Krugel, Biele, Mohr, Li, & Heekeren, 2009; Marco-Pallarés et al., 2009). In line with this, when given a reversal learning task which requires the updating of expected reward contingencies, Val homozygotes demonstrated faster learning rates and better performance, which might be due to a more adaptive striatal response to reward prediction errors conferred by the Val as compared to the Met allele (Krugel et al., 2009).

Despite these emerging data suggesting the presence of a link between reward processing and COMT, there is to date no direct evidence about which particular elements of reward-driven behaviour are affected by COMT and how such effects may relate to dopamine transmission in the NAc.

How could COMT affect NAc dopamine?

Given the evidence from neuroimaging studies demonstrating an effect of the COMT Val158Met polymorphism on striatal activation in response to rewards, it is tempting to speculate that this effect might be mediated by effects on NAc dopamine. There is a range of evidence to suggest that changes in ventral striatal BOLD can be directly driven by dopamine release (Knutson & Gibbs, 2007; Schott et al., 2008). As described previously, COMT is not thought to play a major role in the turnover of striatal dopamine due to a high density of DAT in the striatum. However, there are at least two

possible ways in which COMT could directly or indirectly affect dopamine release in the NAc.

One possibility is that the effect of COMT on PFC dopamine might affect dopamine release downstream in the NAc. Indeed, there are several lines of evidence suggesting that dopamine levels in PFC and NAc are reciprocally related, whereby increases in PFC dopamine lead to decreases in NAc dopamine release (briefly referred to as "the inverse relationship" between PFC and NAc dopamine in this thesis). For instance, electrical stimulation of PFC neurons or pharmacologically mediated increases in PFC dopamine levels lead to a decrease in striatal dopamine levels (Jackson, Frost, & Moghaddam, 2001; Kolachana, Saunders, & Weinberger, 1995), possibly via inhibition of dopaminergic mesolimbic neurons in the VTA (Carr, O'Donnell, Card, & Sesack, 1999). Conversely, increases in striatal dopamine release have been observed after dopamine depletion of PFC (Pycock, Carter, & Kerwin, 1980; Pycock, Kerwin, & Carter, 1980). Moreover, a study by Kellendonk and colleagues demonstrated that overexpression of D2 receptors in the striatum impacts dopamine levels, rates of dopamine turnover, and activation of D1 receptors in the prefrontal cortex (Kellendonk et al., 2006), providing some evidence for bi-directionality of the "inverse relationship" between PFC and NAc dopamine. Taken together, these results would predict that increases in PFC dopamine conferred by the Met-allele might lead to a *decrease* in dopamine release in the NAc.

Another potential mechanism by which COMT might affect dopamine signalling in the striatum has been proposed by Bilder and colleagues (Bilder, Volavka, Lachman, & Grace, 2004) – it shall be referred to as the "tonic-phasic hypothesis of COMT" in this thesis.

At the basis of this hypothesis is the notion that COMT might increase *tonic* levels of dopamine in the striatum. Tonic levels of dopamine in the striatum have been shown to be regulated by glutamate released locally by corticostriatal afferents (Borland & Michael, 2004; Floresco et al., 2003). Increased PFC dopamine levels conferred by the Met-allele would be predicted to lead to an increase in the activation of corticostriatal fibres, which in turn would raise tonic dopamine release in the striatum. Moreover, Bilder and colleagues argue that tonically-released dopamine in the striatum might also be subject to *direct* catabolism by COMT as it might diffuse further than phasically-released dopamine, thereby becoming less likely to be cleared by DAT (Bilder et al., 2004; Floresco et al., 2003). Importantly, tonic dopamine levels are thought to regulate phasic dopamine response via presynaptic D2 autoreceptors, whereby higher tonic levels lead to greater inhibition of phasic dopamine release (Grace, 1991). Reduced COMT activity conferred by the Met-allele would therefore be predicted to *reduce phasic* dopamine release by *increasing tonic* dopamine levels in the striatum.

1.4. Associative learning

The previous section has summarised existing evidence for a role of COMT in reward processing and reward-guided learning. However, which exact factors of reward learning are affected by COMT has not been systematically studied. One possible way of doing so is by studying associative learning – the processes by which an animal learns to associate the occurrence of one event or stimulus with the occurrence of another. The following section will introduce the concept of associative learning and what is currently known about the neural mechanisms it engages.

1.4.1. Theories of associative learning

Whilst exploring the world, an animal is likely to encounter many different stimuli and events, and interacting with these might have widely varying consequences for the animal's survival. For instance, the colour of a fruit eaten might have positive metabolic consequences (e.g. an energy boost), or might cause an aversive physiological response (e.g. illness). The ability of an animal to represent and act upon the relationships between one event (seeing/eating a fruit) and another (the physiological consequence) is therefore of vital importance. One way in which this process has been described involves the formation of an association between stimulus and outcome, based on the extent to which the former is a valid and effective predictor of the latter. Given its importance for the survival of an animal, it is not surprising that this mechanism of associative learning has been the subject of intense investigation for over a century.

There are two main classes of paradigms used to understand associative learning. In operant conditioning, the presentation of a reinforcing stimulus (such as food) is contingent upon an animal's response (such as a lever press). In classical conditioning, presentation of the reinforcing stimulus is contingent upon the presentation of another stimulus (such as an auditory cue). The important distinction to note here is that classical conditioning does not require the performance of a response by the animal. It should be noted that the main theories of associative learning attempt to describe the mechanisms of learning in both operant and classical conditioning tasks. The focus of the present thesis is classical, or Pavlovian, conditioning. During Pavlovian conditioning, a neutral stimulus (conditioned stimulus, CS) such as an auditory cue, is paired with a biologically significant stimulus (unconditioned stimulus, US), such as a food reward. As animals learn that the auditory cue predicts reward, they start approaching either the cue itself ("sign

tracking") or the location of reward delivery (i.e. the food magazine; "goal tracking") during cue presentation (Boakers, 1977; Hearst & Jenkins, 1974). In mice, these processes often occur in combination (Tomie, Lincks, Nadarajah, Pohorecky, & Yu, 2012). This change in behaviour (conditioned response, CR) to the previously neutral stimulus is used as a readout of the strength of the Pavlovian association, i.e. the strength of the connection formed between internal representations of the CS and US. (e.g. Peterson 1972).

A number of theoretical frameworks have been developed to describe the psychological processes underlying associative learning (Esber & Haselgrove, 2011; Le Pelley, Haselgrove, & Esber, 2012; Mackintosh, 1975; Pearce & Hall, 1980; Rescorla & Wagner, 1972; Sutton & Barto, 1990). All associative models of conditioning are based around the concept of prediction errors – a mathematical term that results from the discrepancy between the observed and expected outcomes of an event. There are two key factors that influence the speed and extent of conditioning that is possible between a CS and a US. First, the intensity (or magnitude) of the US limits the amount of conditioning that can be sustained by an associated CS. Second, the attention paid to a CS governs the speed with which it will enter into association with a US, but will not limit the amount of learning that can be achieved.

Models of associative learning can be split into US-processing and CS-processing categories. US-processing models are based on the premise that learning occurs as a result of animals attempting to discover the cause of a surprising outcome. One example that uses such a framework is the Rescorla-Wagner model (Rescorla & Wagner, 1972). The main assumption of this model is that learning only proceeds when a reinforcing

event is not fully predicted, and the prediction is updated on each trial according to the following updating rule:

$$V_{new} = V_{old} + \alpha\,\beta\,(outcome - prediction)$$
$$= V_{old} + \eta\,(R - V_{old})$$

whereby V_{old} is the prediction associated with the observed stimulus on the previous trial, R is the quality of the actual outcome experienced, and η is a learning rate parameter ($0 < \eta \leq 1$) which is comprised of the salience of the CS (α) and the salience of the US (β), and which determines how much the current experience affects the prediction for the next trial. It is important to highlight, for the purpose of this thesis, that according to Rescorla-Wagner, the learning rate parameter is proportional to the *unconditioned* salience (i.e., the physical intensity) of the cue: cues of high physical salience are thought to attract more attention and therefore enable more learning.

A related model of reinforcement learning, which originates from artificial intelligence and that has gained considerable popularity is the temporal difference (TD) learning model (Sutton & Barto, 1990; Sutton, 1988). Like the Rescorla-Wagner model, learning in the TD model is based on the discrepancy between a prediction and the outcome (prediction error). However, whilst in the Rescorla-Wagner model predictions and outcomes are evaluated at the end of each discrete *trial*, TD learning takes into account the fact that instead of the world being divided up into trials, it is a *continuous* flow of experience, and that within this flow, stimuli and outcomes can occur at different points in time. The goal of TD learning is therefore to predict, at each point in time, all future outcomes given current and past stimuli (Niv & Schoenbaum, 2008). One

important implication of this distinction is that in TD learning, prediction errors can occur not only at the time of the outcome, but also at the time of cue presentation.

Despite some differences, TD learning and the Rescorla-Wagner model have in common that prediction errors are *directly* driving learning. According to CS-processing models, on the other hand, instead of driving learning *per se*, prediction errors drive the *attention* a stimulus receives, which can then in turn determine the rate at which the stimulus is learned about. These models emphasise that animals experience a great number of stimuli, but that the amount of attention that can be attributed to stimuli within an environment at any one time is *limited*. Therefore animals must increase or decrease the attention paid to stimuli *selectively*, based upon the extent to which they predict the presence of an outcome. For instance, Mackintosh (1985) argues that as a stimulus becomes a more valid predictor of the presence or absence of reward, the attention it will receive increases. In contrast, Pearce and Hall (1982) argue that as a stimulus becomes more predictive of reward, the attention paid to it will decrease, and will be diverted to other stimuli about which little is known.

1.4.2. Neural mechanisms

Perhaps the most important piece of evidence advancing our understanding of the neurobiological mechanisms of associative learning has been the finding that midbrain dopamine neuron firing follows a pattern that mirrors the reward prediction error term in the TD learning model (Fiorillo, Tobler, & Schultz, 2003a; Schultz, Dayan, & Montague, 1997; Tobler, Fiorillo, & Schultz, 2005; Figure 1-7). Crucially, it was recently demonstrated that the firing patterns are not only *correlated* with the mathematical prediction error term, but are indeed *causally* linked to cue-reward learning (Steinberg et al., 2013).

As noted previously, midbrain dopamine neurons project to a variety of brain structures and it is difficult to conclude from the studies cited above into which of these structures the prediction error signal is carried. However, there is now a growing number of studies attempting to record dopamine release in terminal regions, especially in the striatum. These studies have demonstrated that dopamine release in the NAc core follows a pattern consistent with reward prediction error coding (Day et al., 2007; Flagel et al., 2011; Parker et al., 2010). Accordingly, disruption of dopamine transmission in the NAc core leads to impairments in the formation of cue-reward associations and in the performance of appetitive Pavlovian approach behaviour (Di Ciano, Cardinal, Cowell, Little, & Everitt, 2001; Flagel et al., 2011; Parkinson et al., 2002a). Consistent with the animal literature, ventral striatal activity, as measured by the BOLD signal in fMRI, has been shown to follow a reward prediction error-like pattern in response to rewards and reward-predictive cues in humans (O'Doherty et al., 2004) - in a way that could be differentially modulated by administration of dopamine agonists and antagonists (Pessiglione, Seymour, Flandin, Dolan, & Frith, 2006).

Associative learning relies on the acquisition and storage of sensory and motivational information to provide motor output. Dopaminergic inputs from the VTA and glutamatergic inputs from a number of cortical and limbic structures, including the ventromedial PFC, basolateral amygdala, and hippocampus, converge in the NAc (Brog, Salyapongse, Deutch, & Zahm, 1993; Groenewegen, Vermeulen-Van der Zee, te Kortschot, & Witter, 1987; McGeorge & Faull, 1989; Wright, Beijer, & Groenewegen, 1996) thereby making it a prime region to serve this function (Ikemoto & Panksepp, 1999; Pennartz, Groenewegen, & Lopes da Silva, 1994).

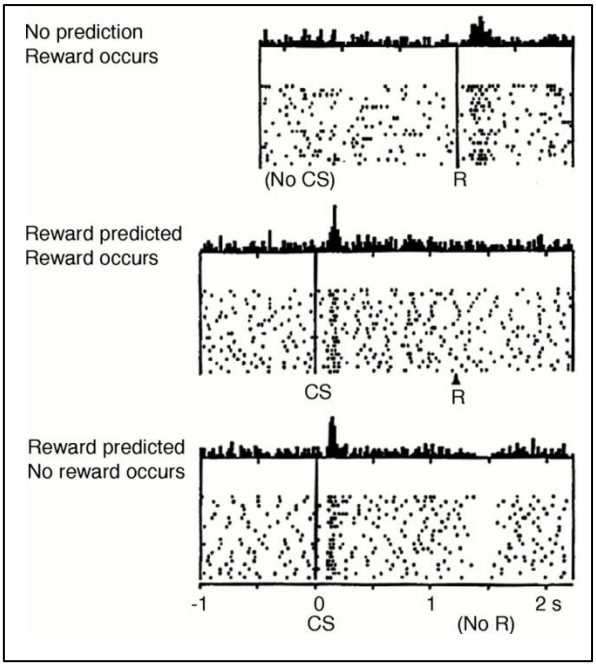


Figure 1-7: Changes in dopamine neuron signalling during Pavlovian conditioning, *where an animal was trained to associate a distinct visual cue with reward delivery (R). Initially, SNc dopamine neuron burst firing only occurs at the time of reward delivery (top panel). However, once an animal has learned associations, burst firing is instead only elicited by the predictive cue (CS) and not by the reward (middle panel). Omission of a predicted reward leads to a dip in dopamine neuron firing (bottom panel). Taken from Schultz et al., 1997.*

1.5. The COMT-Met mouse model

As described in previous sections, the Val158Met polymorphism in the human COMT gene has attracted significant interest due to its role in modulating PFC dopamine and PFC function. However, recent studies in the human population suggest that it might also affect reward-guided behaviours and that this might be mediated by subcortical structures. Whether and which elements of subcortical dopamine signalling are affected by COMT is difficult to study in humans due to the lack of non-invasive methods for the recording of fast changes in dopamine. Such measurements are, however, possible in rodents (Clark et al., 2010).

In this thesis, I have therefore used a novel mouse model of the human Val158Met polymorphism, where the Met allele was knocked into the native mouse COMT gene using a PCR- and site-directed mutagenesis-based strategy (Barkus, Korn, Stumpenhorst et al., *in preparation*). Importantly, this genetic modification proved highly specific: it does not affect the expression of neighbouring genes in frontal cortex or striatum (see Supplementary Figure S1-1 for details). In the present experiments, COMT-Met mice were compared to wild-type (WT) littermates, whose COMT enzyme shows similar, or slightly higher, activity levels, compared to the human Val-form (Chen et al., 2004; Lotta et al., 1995).

Enzyme activity in COMT-Met mice is reduced by approximately 50% (Supplementary Figure S1-2). Microdialysis studies performed in our laboratory have not shown any differences in extracellular dopamine levels in NAc shell or dorsal striatum between COMT-Met and WT mice at baseline (i.e., whilst *not* performing behavioural task), although levels of dopamine metabolites were affected in a way that was consistent with reduced COMT action (for comparison of tissue levels of the metabolites DOPAC and HVA, see Supplementary Figure S1-3). Gene expression, as surveyed by microarrays performed on tissue from NAc, dorsal striatum, and frontal cortex, is not affected in COMT-Met mice. Importantly, COMT-Met mice show normal performance on a range of basic behavioural measures, including locomotor activity, anxiety, feeding behaviour, and sensorimotor gating (Table 1-1).

In summary, the experiments in this thesis were performed in a highly specific mouse model of the Val158Met polymorphism, in which COMT enzyme activity was reduced by approximately 50% in COMT-Met compared to WT mice. Importantly, although they showed neurochemical changes consistent with reduced COMT activity,

COMT-Met mice did not differ in basic behavioural characteristics such as locomotion or sucrose preference, compared to WTs. These initial findings therefore provide a good basis for the exploration of more subtle behavioural effects in the behavioural paradigms used in this thesis.

Table 1-1: Overview of behavioural tests performed in COMT-Met and WT mice.

Behavioural Task	**Result: COMT-Met vs. Wt**
Locomotor activity	n.s.
Feeding behaviour	
Body weight	n.s.
Sucrose preference test	n.s.
Anxiety related tasks	
Elevated plus maze	n.s.
Light-dark box	n.s.
Novelty suppressed feeding task	n.s.
Anxiogenic open field	n.s.
Sensorimotor gating	
Habituation to acoustic startle	n.s.
Prepulse inhibition	n.s.
Learning and memory	
Spatial novelty preference	Subtle increase in COMT-Met
Discrete trial spontaneous alteration	n.s.
Novel object recognition	n.s.
Acquisition of appetitively-motivated reference Y memory task	n.s.
Morris water maze	n.s.

n.s. : no statistically significant genotype differences

1.6. Aims of this thesis

The dopamine-metabolising enzyme COMT gene has attracted significant interest due to its role in modulating PFC dopamine and PFC function. Recent studies in the human population suggest that it might also affect reward processing and that this might be mediated by the ventral striatum. However, which particular aspects of reward-guided behaviour are affected by COMT and how this relates to any potential effects on striatal dopamine transmission is not currently understood.

The two main aims of this thesis were therefore to use a mouse model of the Val158Met polymorphism to (1) investigate the role of COMT in associative learning, a process known to depend on NAc dopamine, and (2) directly measure behaviourally-evoked dopamine release in the NAc using fast-scan cyclic voltammetry in awake behaving mice. An overview of the methods used in this thesis is given in Chapter 2.

Chapters 3 and 4 investigate the role of COMT in associative learning by using a Pavlovian auditory discrimination (Chapter 3) as well as a single-cue Pavlovian conditioning paradigm (Chapter 4).

A prominent theory (the "tonic-phasic hypothesis of COMT) suggests that COMT might have differential effects on tonic and phasic dopamine subcortically (Bilder et al., 2004). Chapter 5 therefore addresses the question whether COMT affects phasic dopamine in the NAc by using fast-scan cyclic voltammetry to record dopamine release in response to rewards.

The last study of this thesis (Chapter 6) combines Pavlovian auditory discrimination conditioning with fast-scan cyclic voltammetry in order to examine whether changes in the effects of COMT on cue-reward learning might be explained by differences in dopamine release early in training.

Finally, Chapter 7 provides a general discussion of the results of this thesis and suggests further directions of research.

Chapter 2: General Methods

2.1. Animals

Breeding and maintenance of animals

All experiments described in this thesis were performed on COMT-Met mice and their Wild-type (WT) littermates as controls. The COMT-Met mice have a Met allele knocked into the position in the mouse COMT gene homologous to the human Val158Met locus (Leu148). The animals used in this thesis had been backcrossed onto a C57BL/6J background for 10 generations. They were bred in individually ventilated cages in the University of Oxford's Biomedical Building and were transferred into open cages at the age of 7 weeks. Male mice were held in single-family groups of 2-4, unless otherwise indicated, and female mice were held in mixed-litter groups of 3-6 animals per cage in a temperature-controlled holding room (19-22°C) under a controlled light-dark cycle (lights on from 7:00 - 19:00). All experiments were performed during the light phase. Mice were food restricted and their body weights were kept at approximately 85-90% of their free feeding weights for the duration of all experiments described in this thesis. During food restriction, mice were fed once daily, shortly after completion of their experimental session. Water was available *ad libitum*. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and associated Home Office guidelines. Experiments described in Chapters 3 and 4 were performed on both male and female mice, although only data from male mice is presented in this thesis.

Genotyping

Mice were genotyped using a restriction fragment length polymorphism assay. The primers Met-Mouse-Geno-F (5' GCC AAA TCA TGG ATG CAG TGA TTC 3') and Met-

Mouse-Geno-R (5' CTT TGG GGC TTT GGA GAG GAA 3') were used to amplify a fragment of COMT, producing an amplicon of 471bp. The amplicon was digested by StuI and the products resolved and visualized on 3% w/v agarose gels. The presence of the Met allele abolishes a StuI site, which cleaves the amplicon into 244bp and 227bp fragments in WT mice; thus, Met homozygotes show only a 471bp band, WT homozygotes show only 244bp and 227bp bands, and heterozygotes show bands at all three sizes.

2.2. Pavlovian conditioning experiments

Apparatus

The behavioural experiments described in Chapters 3 and 4 were conducted in Med Associates modular test chambers (ENV-307W) with inner dimensions of 21.6 cm x 17.8 cm x 12.7 cm. Each chamber was equipped with an audio speaker (ANL-926) on the rear wall. Liquid reward was delivered by a single speed syringe pump (PHM-100A-UK) into a liquid reward receptacle (ENV-303HD) on the opposite wall (Figure 2-1). The reward receptacle was equipped with an infrared beam, and beam breaks were used to detect the animal's head entries into the receptacle. ENV-313W nose poke detectors with illuminated apertures were used for the conditioned reinforcement test. Each chamber was housed within a sound attenuating cabinet ventilated with a fan which provided background noise of 68-70dB.

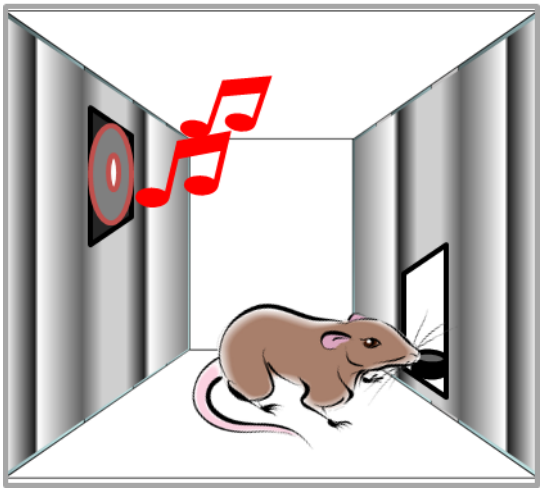


Figure 2-1. Set up of operant boxes for auditory Pavlovian conditioning. *Auditory cues were played from a speaker on the rear wall. Sucrose solution was delivered inside a magazine on the opposite wall. In the behavioural studies described in Chapters 3 and 4, the magazine was recessed (shown) whilst in the recording chambers, the magazine protruded into the chamber. Entries into the magazine were counted and referred to as "magazine responding".*

The recording experiments described in Chapters 5 and 6 were conducted in Med Associates modular test chambers identical to those used in the behavioural experiments described above, with the following modifications to enable voltammetry recordings: the metal wall components were replaced by plastic components, the roof was removed to allow for tethering and the walls were extended to prevent the mice from climbing out of the chamber. Instead of using a recessed reward magazine, a magazine extending into the chamber was used in order to allow for more space for movement with cerebral implants. In order to reduce electrical noise that could interfere with recordings, the syringe pumps for reward delivery were replaced with a custom-made gravity-based system for the delivery of liquid rewards. The background noise provided by a ventilating fan was at ~61dB. During recordings, signals were transmitted from chronically implanted electrodes to the data acquisition system via a head-mounted voltammetric amplifier and an electrical commutator (Crist Instrument Co.) mounted above the recording chamber.

Behavioural protocols

Habituation and magazine training: In all experiments described in this thesis, mice were habituated to the experimental setup and the reward by being placed into the chambers and being allowed to consume 20% sucrose solution from the magazine for 15 minutes. Mice that did not start consuming sucrose solution within 10 minutes were removed from the chamber and the session was repeated after a break of several hours or on the next day. Mice then underwent magazine training sessions during which they obtained 20 rewards (40 µL of 20% sucrose solution). Rewards were delivered with a mean inter-trial-interval (ITI) of 60s (ITI60, range: 35-83s) in session 1, and 120s (ITI120, range 90-155s) on session 2. Onset of reward delivery coincided with a 0.1s click (30Hz, approx. 75dB). In the behavioural studies in Chapters 3 and 4, reward delivery was not contingent upon magazine entry and sucrose solution could therefore accumulate in the food magazine in the course of a session if the mouse did not visit the magazine. Sucrose solution leftovers were therefore measured, and any mice that had consumed < 80% of the sucrose solution delivered on magazine training session 2 were given additional magazine training sessions until they achieved > 80% consumption.

Pavlovian conditioning: Auditory stimuli (white noise (WN) and a 3kHz constant tone (Tone) were used as conditioned stimuli (CS) in all of the Pavlovian conditioning experiments described in this thesis. Stimuli were always presented for 8s. In cases where conditioned stimuli were paired with reward (CS+), cue termination coincided with reward delivery (40 µL of 20% sucrose solution) and a 0.1s click sound (30Hz, approx. 75dB). Trials were separated by an ITI with a mean of 120s (range: 90-155s). Each animal was tested in the same test chamber each day. Experimenters were blind to genotype for all tasks. For details of trial numbers and cue assignments to experimental conditions, see the methods sections of the individual results chapters.

Conditioned reinforcement test: Conditioned reinforcement tests were conducted in some of the behavioural experiments (see respective methods sections). Two nose poke detectors were inserted left and right of the food receptacle. Nose poking into one of the two apertures produced a 2s presentation of the CS+ cue while poking into the other aperture elicited a 2s CS- cue presentation (Chapter 3) or no cue (Chapter 4). The assignment of the cues to the left and right sides of the food receptacle were counter-balanced across subjects. During cue presentation, the nose poke devices were switched to an "inactive state", such that nose pokes made while a cue was played were registered but did not prolong the presentation of the ongoing cue. Active and inactive states were signalled by the inbuilt aperture lights being either on or off. The entire test was conducted under extinction conditions, i.e. cue presentations were not rewarded.

Data analysis

In all of the Pavlovian conditioning experiments, learning was indexed by the rate of magazine responding during cue presentation. Both the number of head entries into the food magazine and the duration of these magazine entries was recorded throughout training (commonly summarised as "magazine responses" below). Magazine responses were first averaged across trials within each session, and then, where appropriate, grouped into blocks of multiple sessions. To determine whether there were overall differences in response rates between COMT-Met and WT mice, magazine responding in an 8s period prior to cue onset ('baseline') were assessed. For the key analysis of cue-reward learning across sessions, response rates during cue presentation were normalised on an animal-by-animal basis by subtracting each mouse's responses in this pre-cue baseline period on a trial-by-trial basis. Normality of data was tested using the Shapiro-Wilks test. To correct for skewness of the data, logarithmic data transformations were applied where required (see individual chapters' methods sections). Data were

analysed using repeated measures ANOVA, with genotype as a between-subject factor and session block and, where appropriate, cue type as within-subject factors.

Sphericity was tested using Mauchly's Test of Sphericity and, where violated, Greenhouse-Geisser corrections were applied. Significant effects and interactions ($p < 0.05$) were followed up using pairwise comparisons, corrected for multiple comparisons using Bonferroni's correction, unless indicated otherwise. All analyses were conducted in SPSS Statistics version 20 (IBM, Portsmouth, UK).

2.3. Overview of principles of fast-scan cyclic voltammetry

The details of fast-scan cyclic voltammetry (FSCV) using acute and chronic electrodes *in vitro* and *in vivo* have been extensively described elsewhere (Clark et al., 2010; Heien, Phillips, Stuber, Seipel, & Wightman, 2003; Phillips, Robinson, Stuber, Carelli, & Wightman, 2003; Phillips & Wightman, 2003; Robinson, Venton, Heien, & Wightman, 2003). The following paragraphs will summarise the basic mechanism underlying FSCV, and briefly address some of the methodological considerations that are relevant to the questions posed in this thesis.

FSCV is an electrochemical technique that was employed in this thesis to measure changes in extracellular dopamine concentration at sub-second time resolution (Clark et al., 2010; Robinson et al., 2003). In contrast to constant potential amperometry, in FSCV, the potential of the working electrode is linearly scanned. In the course of this, a large background current is produced, which is proportional to the scan rate and capacitance of the electrode. In addition, electrochemically active molecules present at the surface of the electrode are oxidised or reduced when the electrode reaches the oxidation or reduction potential of the given molecule, thereby adding to the background current at the respective potential (Figure 2-2). Since the background current is rather stable across

several seconds, it is possible to subtract it in order to reveal changes in current after an event of interest, such as electrical stimulation or the delivery of a behaviourally salient stimulus. For instance, an increase in the presence of dopamine around the electrode produces a small change in the background current, which is greatest at dopamine's oxidation potential. The magnitude of the current detected at peak oxidation potential is directly proportional to the concentration of the given analyte. To assess changes in analyte levels over time, current at the peak oxidation potential can be plotted across successive voltammetric scans.

The location of the oxidation and reduction peaks in the background-subtracted cyclic voltammogram (current during a scan plotted against the applied potential) can be used for chemical identification of the analyte contributing to those current changes. A range of electroactive molecules in the brain can be detected using FSCV, including catecholamines, indolamines, ascorbic acid, and pH. Most of these molecules can be resolved based on the location of their reduction and oxidation peaks as well as kinetics. The main interferent for the dopamine signal is a shift in pH, which is often detected as a result of protonation and deprotonation of groups at the electrode surface. Changes in current attributable to shifts in pH can be subtracted out from the FSCV signal, leaving behind the current attributable to dopamine.

The only molecule that cannot be distinguished from dopamine electrochemically is norepinephrine, as both molecules have nearly identical cyclic voltammograms at the FSCV settings used in the present study. However, the electrode is considerably more sensitive to dopamine than norepinephrine. Moreover, in the present study, recordings were taken from a brain region (the nucleus accumbens core) with a relatively low norepinephrine content. I can therefore be confident that signal changes recorded using

FSCV in this study are indeed attributable primarily to changes in dopamine concentration.

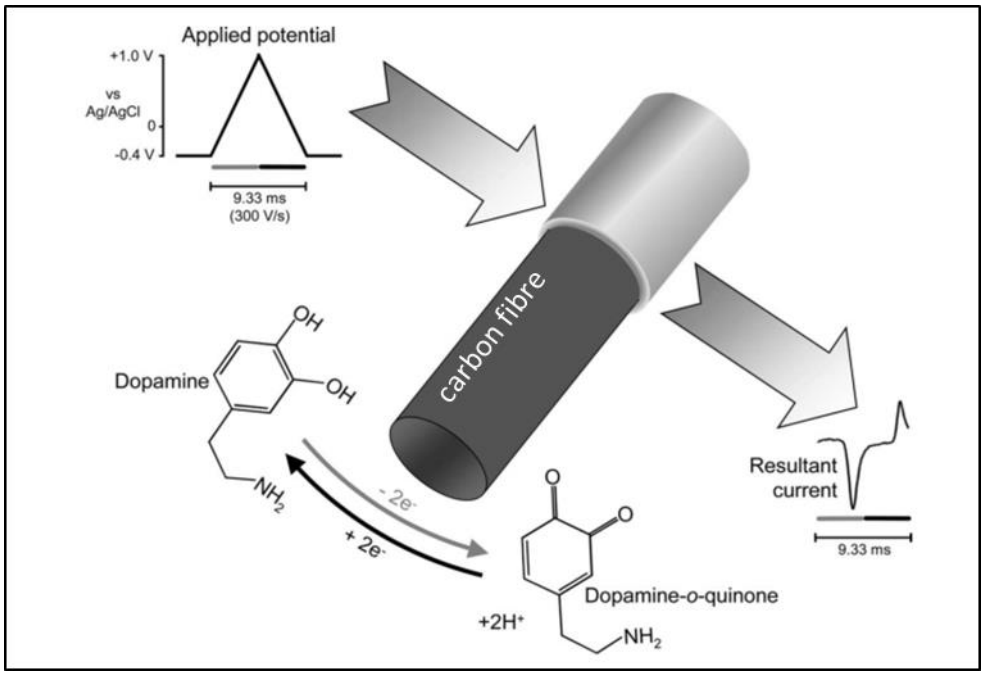


Figure 2-2. Voltammetric detection of dopamine. *When sufficient potential is applied to the electrode, dopamine is oxidized to dopamine-o-quinone, donating two electrons that are detected as current. When the potential is returned, any dopamine-o-quinone remaining at the electrode surface is reduced back to dopamine by accepting electrons, producing current in the opposite direction. The current resulting from FSCV comprises time-resolved peaks that enable analyte identification. Adapted from Phillips & Wightman, 2003.*

Importantly, only *relative* changes in analyte concentrations can be measured with FSCV; information about absolute dopamine concentrations can therefore not be obtained in the experiments described in this thesis. Moreover, whilst stable in the range of seconds, the background current might be subject to a slow drift across minutes. As a consequence, long-term changes in analyte concentration, such as changes in tonic dopamine levels, cannot be reliably measured. Fast, transient changes, such as those evoked by phasic release of dopamine can be, however, easily measured using FSCV.

In theory, changes in current can be converted into changes in dopamine concentration by use of an *in vitro* calibration after completion of recordings. Such post-recording calibrations were, however, not possible in the experiments described in this thesis, as the electrode was burned in the process of making an electrical lesion in the brain tissue to allow for subsequent confirmation of the location of the recording site, which would have not been visible otherwise. Differences in dopamine current recorded between subjects could therefore result from a) real differences in dopamine concentration present around the electrode, and b) differences in electrode sensitivity. The impact of b) on the overall result can be reduced by increasing the sample size and averaging across electrodes of different sensitivities and/or by first computing within-subject measures (e.g. relative current change to stimulus A versus stimulus B) and then comparing those between subjects.

2.4. Fast-scan cyclic voltammetry – experimental methods

Electrodes

Construction of carbon fibre electrodes. Carbon fibre microelectrodes for voltammetric recordings were constructed by threading carbon fibres (Goodfellow; Grade 34-700, C 005722, 7µm) through polyamide-coated fused silica capillaries (outer diameter: 90µm; inner diameter 20µm; polyimide coating 12µm) cut to a length of 0.8cm. The capillary opening at the end that would be inserted into the brain was sealed using Devcon two-component epoxy, leaving some carbon fibre exposed which was later cut to a length of 150-200µm. A gold pin (Mill-Max, USA) was attached to the carbon fibre protruding from the other end of the silica capillary using conductive silver epoxy and insulated with non-conductive epoxy glue (Figure 2-3).

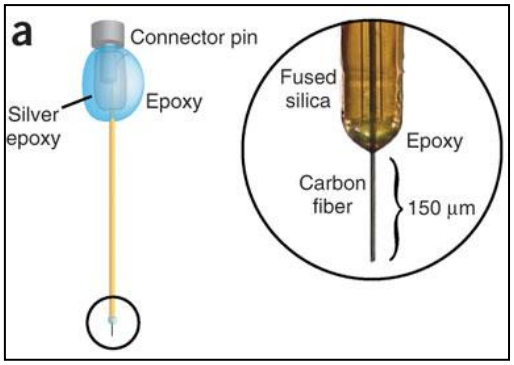


Figure 2-3: Schematic of constructed carbon fibre electrode *(left) and photograph of the tip of the microelectrode. Taken from Clark et al., 2010.*

Construction of reference electrodes. For the construction of Ag/AgCl reference electrodes, coated silver wire was cut to 1cm, the protective coating was scraped off and the wire placed into sodium hypochloride solution overnight. The chloride layer was then scraped off at approximately one third of the length of the wire. This part of the wire was inserted and glued into a silver pin using conductive silver epoxy with the remaining, chlorinated part of the wire protruding out of the pin.

Different lengths and thicknesses of silver wire were tested in a small cohort (n = 7) of C57BL/6J mice in order to determine the optimum configuration for chronic implantation in mice (data not shown).

Electrode calibration (proof of principle). Note that electrodes were not routinely calibrated prior to implantation into the brain. However, in the initial stages of establishing the protocols and equipment for FSCV recordings in mice, the sensitivity of a small number of electrodes to dopamine was assessed *in vitro* (Figure 2-4). Electrodes were calibrated in a tissue bath apparatus routinely used for FSCV recordings in brain slices. The bath was perfused with a constant flow of oxygenated and carbonated artificial cerebrospinal fluid (aCSF) and kept at 37 °C.

The following waveform was applied during the acquisition of FCV recordings: At the start of the waveform, there was a voltage step from 0V to -0.4V and the voltage was kept at -0.4V for 5ms, followed by a sweep to +1.3V and back down to -0.4V at a scan rate of 340V/s. The voltage was then held at -0.4V for another 5ms, followed by a voltage step back to 0V. This waveform was applied at 16Hz for approximately 15min after initial insertion of the electrode into aCSF. Once the background current had stabilised, scan frequency was switched to 8Hz and voltage changes at the electrode [mV] were recorded in response to different concentrations of dopamine flushed through the bath. Peak voltage changes caused by oxidation of dopamine were converted into current [nA] by using the given Faradaic current gain (20) and the full current gain (2.9 mV/nA).

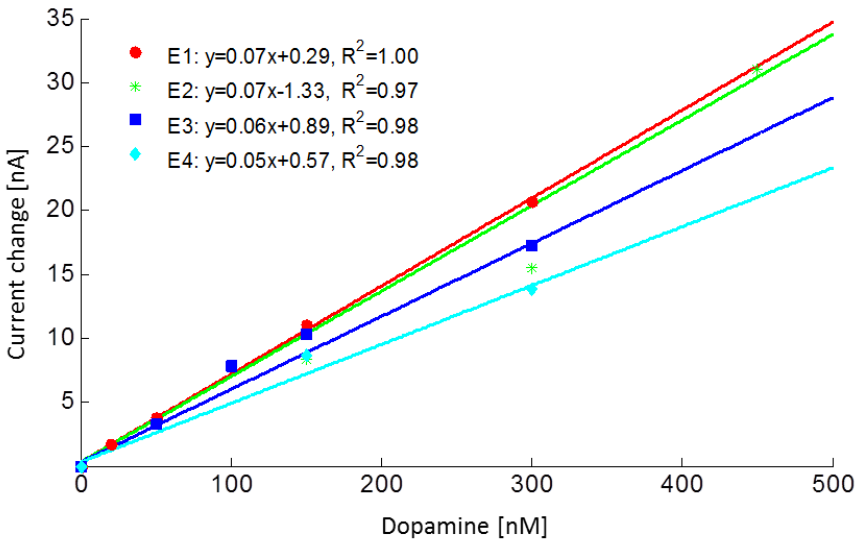


Figure 2-4: Background-subtracted peak faradaic current measured at four different electrodes (E1-E4) *in response to different dopamine concentrations in vitro. Approximation of the data by linear regression yielded $R^2 \geq 0.97$ for all electrodes.*

Implantation of electrodes

COMT-Met and WT mice were anesthetised in an induction chamber (Vet-tech Solutions, Cheshire, UK) filled with 3-4% isoflurane at an oxygen flow rate of 2-3 L/min. Once unconscious, meloxicam (Metacam, Boehringer Ingelheim; 5mg/kg) and

buprenorphine (0.08mg/kg, Vertergesic, Alstoe Animal Health, UK) were given subcutaneously. The scalp of the mouse was shaved, cleaned with Hibiscrub (4% w/v chlorhexidine gluconate, Regent Medical Ltd), and secured in a stereotaxic frame (Kopf Instruments, Model Clark Electromedical, Reading, UK). Corneal drying was prevented by the application of sterile eye drops (Viscotears®, Novartis Pharma AG, Basel, Switzerland). A surgical level of anaesthesia was maintained using isoflurane at concentrations of 1-3% and an oxygen flow rate of 1.5L/min. The skull was exposed by an incision along the midline and local anaesthesia was provided by topical application of Bupivacain (2mg/kg, Marcain). Bregma and lambda were used as landmarks, helping to adjust the position of the head in the horizontal plane. Using a dental drill, holes were drilled into the skull at the sites of electrode implantation and for the insertion of three jewellers' screws as depicted in Figure 2-5.

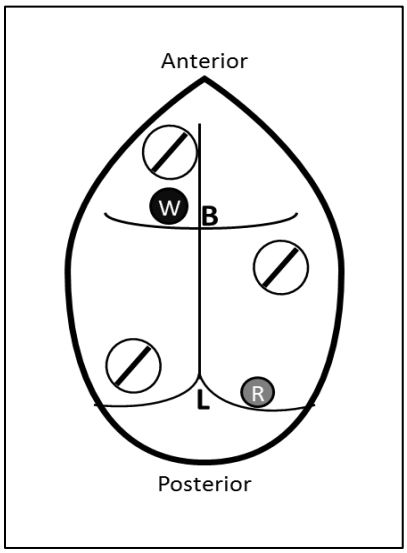


Figure 2-5: *Schematic of the exposed skull, depicting insertion sites for the working electrode (W), reference electrode (R) and bone screws, relative to bregma (B) and lambda (L). In a subset of mice, a second working electrode was inserted in the right hemisphere (not shown).*

A carbon fibre electrode was implanted into the core of the left nucleus accumbens (NAc) according to the stereotaxic coordinates: anterior-posterior 1.5 mm and medio-lateral 1.0 mm, relative to bregma, and dorso-ventral: -3.6 mm, from the brain surface. In some mice, a second carbon fibre electrode was inserted into the NAc core of the right

hemisphere. A reference electrode was inserted into the right hemisphere near lambda. The electrodes were held in place using Kemdent Simplex Rapid dental cement (Associated Dental Products Ltd), which was applied to the whole area of the exposed skull. The inserted head screws provided additional contact between the skull and the headcap, helping to hold the headcap in place. The metal contacts at the ends of the electrodes were connected to a 2-pin or 3-pin (if 2 working electrodes were implanted) plastic socket (Plastics One, Roanoke, VA, USA) which was then securely cemented to the skull. This "headcap" covered the entire skull surface, leaving no tissue from the incision exposed.

After surgery, mice received a subcutaneous injection of 1mL of 0.9% saline. Lasting analgesia was provided by subcutaneous injections of buprenorphine and meloxicam a few hours after surgery and the following morning. The behavioural and health status of the mice was monitored daily using distress scoring sheets for at least two weeks after surgery. Daily Metacam and glucose-saline injections were continued for up to 7 days, as required, to aid recovery. Mice were housed either singly or in pairs, and allowed to recover for approximately 4 weeks before starting voltammetry recordings.

Recording sessions

Animals were tethered to a head-mounted voltammetric amplifier, and a triangular waveform (0.4V holding voltage and +1.3V peak voltage) with a sweep rate of 400 V/sec was applied. The voltage was kept at -0.4V between scans (Clark et al., 2010). At the start of each recording session, the electrodes were conditioned by applying the waveform described above at 60Hz for approximately 45min, in order to improve the sensitivity and stability of the recordings. During this time, the doors of the sound attenuating box remained open, the fan was off, and the reward magazine was obscured by a piece of brown cardboard. Waveform application frequency was then switched to

10Hz, and the background current was allowed to adjust for at least 15min. It should be noted that the potential of the Ag/AgCl reference electrode may shift with long-term implantation, resulting in an offset of approximately -0.2V (Heien et al., 2005; Moussy & Harrison, 1994). This change can be diagnosed by assessing the positions of Faradaic peaks within the background current and was observed in all of the subjects tested here. To account for this offset, the potential limits of the voltammetric waveform were adjusted - as previously done by others (Clark et al., 2010) - and electrodes allowed to condition for another 30min at 60Hz, followed by 15min at 10Hz using the adjusted waveform.

Just prior to the testing session, the doors of the sound attenuating box were closed and the cardboard removed, before mice were presented with up to four sucrose rewards (40µL of 20% sucrose solution) to assess the presence of reward-evoked dopamine. The fan was then turned on and the session commenced (for details of the behavioural sessions, see methods sections of the respective results chapters). During the session, delivery of rewards, and where applicable, of auditory stimuli, were controlled by Med-PC software (Med Associates, St. Albans, VT) and head entries into the magazine were automatically detected by infra-red beam breaks. Reward delivery and head entries were communicated to the voltammetry recording software by TTL (Transistor-Transistor Logic) pulses delivered by the Med-PC programme, allowing alignment of electrochemical data with behavioural events.

Dopamine data extraction

Criteria for inclusion of data. The chemical sensitivity of the recording electrode to dopamine was assessed at the start of each recording session according to previously established criteria (Gan et al., 2010). To that end, the background-subtracted cyclic

voltammogram (current-vs-voltage profile) of the signal measured in response to unexpected reward delivery was extracted and compared to the voltammogram obtained from electrically-evoked dopamine, recorded in the NAc during medial forebrain bundle stimulation. Behaviourally evoked signals met electrochemical criterion for dopamine if the cyclic voltammogram was significantly correlated with that of the template, with $r^2 \geq 0.75$ set as the criterion based on the discriminability of dopamine from other common neurochemicals in a flow cell (Gan et al., 2010). Recording sessions and electrodes that met this criterion were included in data analysis.

Dopamine data extraction and processing. Voltammetric analysis was initially carried out using software ('CV_Analysis') written in LabVIEW (National Instruments). Data were low pass filtered at 2kHz and aligned to events of interest (e.g., cue onset / reward delivery). The average signal in the 0.5s prior to the event of interest was used for background subtraction. To isolate changes in dopamine concentration from other electrochemical signals, a principal component analysis was performed on the background-subtracted signal using a standard training set of stimulated dopamine release detected by chronically implanted electrodes, with dopamine treated as the first principal component among other unrelated electrochemical fluctuations such as changes in pH (Heien, Johnson, & Wightman, 2004; Heien et al., 2005). The data were smoothed using a 0.5s moving window. Once dopamine-related current changes were extracted, all further analyses were undertaken using Matlab® 2010 (Mathworks, MA, USA; for details see methods sections of respective results chapters).

Validation of electrode placements

At the end of the experiment, mice were deeply anaesthetised with sodium pentobarbitone (200mg/kg) and electrolytic microlesions were made at the electrode

locations before they were transcardially perfused with saline followed by a 10% formalin-saline solution. Brains were extracted and placed into a formalin-saline solution. Subsequently, the brains were placed in a sucrose/formalin solution for 24 hours, before being frozen and sectioned in 40 µm coronal slices using a cryostat. Sections were stained with cresyl violet and electrode locations were identified (Figure 2-6).

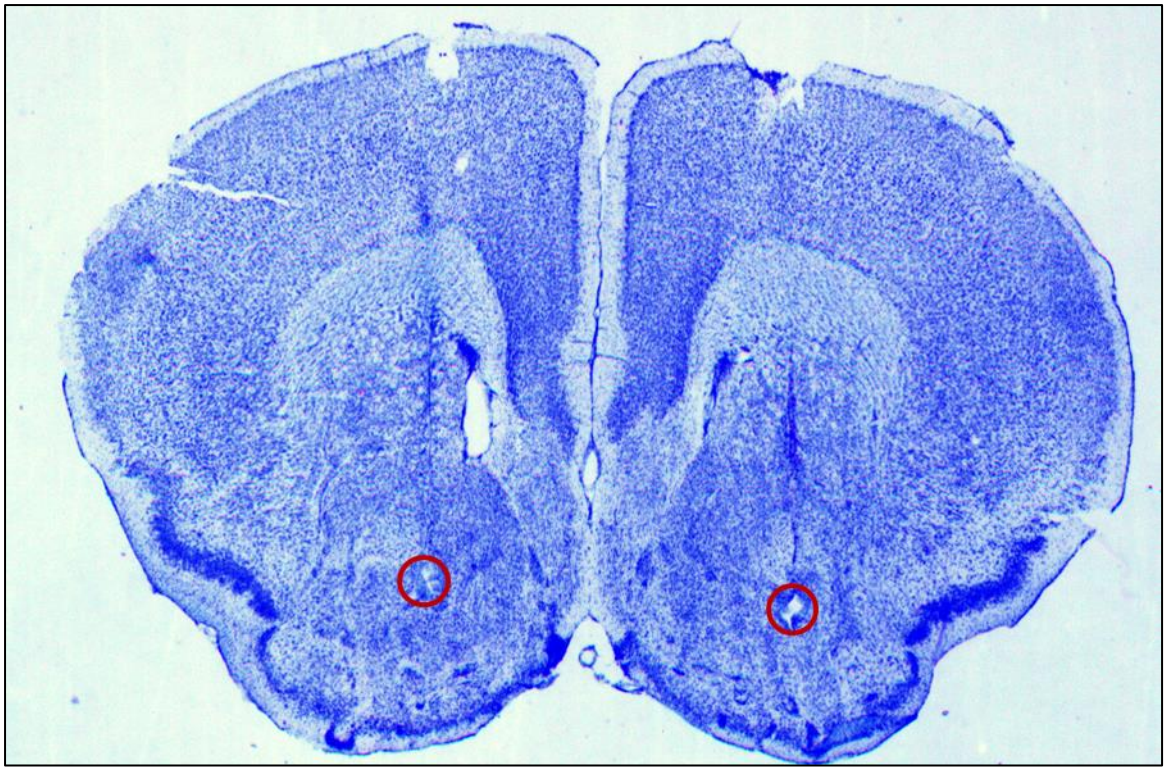


Figure 2-6. Example of a cresyl violet stained coronal brain section showing electrolytic lesions at electrode location (red circles).

Chapter 3: COMT modulates the effect of cue salience on appetitive conditioning

3.1. Introduction

As detailed in Chapter 1, most of the research into the behavioural effects of COMT has focussed on PFC function and PFC-dependent tasks. More recently, however, there has been an increase in interest in the role of COMT in reward learning, and several functional neuroimaging studies have linked the COMT Val158Met polymorphism with ventral striatal activity during reward processing. However, which particular components of reward learning are affected by COMT has not been systematically studied. Therefore, the aim of the present chapter was to dissect some of these components in COMT-Met and WT control mice using Pavlovian discrimination learning.

Pavlovian discrimination learning

Animals encounter a myriad of stimuli in their environment and their survival depends on successful discrimination between those that predict positive, negative or unimportant events. In classical or Pavlovian conditioning, discrimination therefore occurs when an animal shows one conditioned response to one stimulus and another (or none) to a different stimulus. Generalisation, on the other hand, occurs when an animal makes the same response to different stimuli that might not be associated with the same outcome. Typically, the greater the

resemblance between stimuli, the greater the generalisation effect (Moore, 1972; Thomas, 1981).

The first experiment presented in this chapter focused on auditory Pavlovian discrimination learning to investigate the role of COMT genotype in associative learning processes typically believed to be mediated by striatal dopamine (Day et al., 2007). As cue-reward associations are learned, animals increase approach behaviour to the location of reward delivery during presentation of the rewarded cue (conditioned response). Increase in magazine responding rates during cue presentation, compared to pre-cue baseline, was therefore used as a readout of the strength of the Pavlovian association, i.e. the strength of the connection formed between internal representations of the CS and US (e.g. Peterson 1972).

Based on the predictions made by the tonic-phasic hypothesis of COMT (Bilder et al., 2004), as well as the reciprocal relationship between prefrontal and striatal dopamine levels (Carr & Sesack, 2000; Kolachana et al., 1995; Sesack, Carr, Omelchenko, & Pinto, 2003), it would be predicted that Met-COMT mice should have *decreased* phasic dopamine in the NAc. Given that NAc core dopamine is causally implicated in appetitive Pavlovian learning and performance (Di Ciano et al., 2001; Flagel et al., 2011; Parkinson et al., 2002b) and phasic dopamine cell activity and dopamine release in the NAc is proposed to be central to the updating of values associated with stimuli through encoding reward prediction errors (Bayer & Glimcher, 2005; Day et al., 2007; Flagel et al., 2011; Trevor W. Robbins & Everitt, 1996; Schultz et al., 1997), we therefore predicted *attenuated* Pavlovian learning in COMT-Met compared to WT mice.

Even for something as seemingly simple as Pavlovian learning, there are potentially several factors that can influence the speed of acquisition of the association. One factor that is known to play a role is the salience of the events that are encountered (Mackintosh, 1965; Pearce & Hall, 1980; Rescorla & Wagner, 1972). Whilst there is consensus on the importance of cue salience in associative learning, it must be noted that the term "salience" in itself might refer to subtly distinct concepts, depending on the formal framework it is used in. It can, for example reflect the intrinsic attention-grabbing properties of a cue, the novelty of a cue within a given context, or the cue's ability to predict reward. In this chapter, "salience" is used in the sense of *unconditioned salience*, i.e. the extent to which the perceptual properties of an environmental cue inherently capture the attention of the animal.

One way of assessing how attention-grabbing a cue might be is to infer salience from the rate at which an animal is able to learn about it (Hull, 1949). Conditioning to a more salient cue should proceed more readily than to a less salient cue (Kamin & Schaub, 1963) – an assumption tested in experiment 2 presented in this chapter, where cue salience was controlled by manipulating the volume at which the cues were presented.

Another rapid way to measure how attention-grabbing a cue might be without relying on associations with reward is to directly assess the way in which an animal orients to that stimulus. Rodents will typically exhibit a motor response oriented towards the perceived direction of a novel stimulus when it is presented, and assessing the duration and extent of this 'orienting response' provides one method of estimating the attention a cue receives in its current context. A more perceptually salient cue will elicit a greater orienting response from a rodent than a

less salient cue. Such responses can either be scored visually or measured indirectly by assessing the degree to which an animal interrupts ongoing behaviour such as lever pressing (Walasek, Wesierska, & Zieliński, 1994) or head entries into the food magazine (Sanderson, Sprengel, Seeburg, & Bannerman, 2011), when a stimulus is presented. In experiment 3 described in this chapter, the change in magazine responding caused by unexpected cue presentation in naïve COMT-Met and WT mice was used as an index for the unconditioned salience of the auditory cues.

Therefore, the aim of the present study was to investigate the effect of COMT on Pavlovian discrimination learning and to explore what factors might predict any change in learning rate between the groups. The working hypothesis was that learning in COMT-Met mice would proceed more slowly than in WT mice. Interestingly, the effect of COMT on learning in Experiment 1 interacted with the identity of the stimulus (white noise – WN – or Tone) used as the rewarded cue. We hypothesised that this effect might be mediated by salience differences between the stimuli used and that associative learning in COMT-Met mice might be more sensitive to such differences. Experiments 2 and 3 were set up to test these hypotheses.

In Experiment 2, the sensory salience of the cues was manipulated by altering their volumes. Given the results of Experiment 1, it was predicted that learning rates would increase with a louder / more salient Tone cue, and that COMT-Met mice would be more strongly affected by this. Finally in Experiment 3, I examined the unconditioned orienting responses in naïve COMT-Met and WT mice to the stimuli used in Experiment 1 to determine whether the WN cue, which caused faster

learning, provoked stronger orienting responses than the Tone, and whether this was modulated by genotype in the absence of learning.

3.2. Methods

Experiments 1 & 2: Pavlovian auditory discrimination

Subjects. A total of 37 male mice (20 COMT-Met and 17 WT) were used in this study (47 female mice were tested in parallel, but the data from these is not reported in this thesis). The mice were 12-18 weeks of age at the start of the experiment. Mice were food restricted throughout the study and their body weights were kept at approximately 85-90% of their free feeding weights. Water was available ad libitum.

Apparatus. See Chapter 2 for a description of the apparatus used in the experiments detailed in this chapter.

Auditory stimuli. White noise (WN) and a 3kHz pure tone (Tone, T) auditory stimuli were used as conditioned cues in both Pavlovian conditioning experiments. In Experiment 1, the volume of each was set to 75dB (6-7dB above background noise levels). The assignment of the two auditory cue types to the CS types (CS+ and CS-) was randomised and counterbalanced between subjects (n = 27, see Table 3-1).

In Experiment 2, mice (n = 10, see Table 3-1) were again trained with a Tone CS+ and a WN CS- (stimuli <u>not</u> counterbalanced across mice). However in this experiment, the Tone was played at 88dB and WN was played at 72dB.

An overview of the conditions and the numbers of subjects used in each condition is given in Table 3-1.

Table 3-1. Overview of Pavlovian discrimination experiments

	Experiment 1: Standard Pavlovian Discrimination Learning		Experiment 2: Salience Manipulation
Name of condition	**WN+/T-**	**T+/WN-**	**'boosted' T+/WN-**
CS+ stimulus identity	WN (75dB)	T (75dB)	T (88dB)
CS- stimulus identity	T (75dB)	WN (75dB)	WN (72dB)
Number of subjects	Met n = 7 WT n = 6	Met n = 8 WT n = 6	Met n = 5 WT n = 5

WN: white noise
T: 3kHz pure tone

Magazine training: Mice were habituated to the test chambers and trained to retrieve rewards from the food magazine as described in Chapter 2. One mouse (Met-COMT, Experiment 2) required 3 additional magazine training sessions until it consistently retrieved rewards from the magazine.

Pavlovian conditioning: Following magazine training, the mice underwent 24 appetitive Pavlovian conditioning sessions. Each session consisted of 15 rewarded (CS+) and 15 unrewarded (CS-) cue presentations lasting 8s each. The average inter-stimulus interval was 120s. Two "probe trials" on which the CS+ cue was presented but not followed by reward were additionally included in sessions 5, 6, 11, 12, 16, 17, 21 and 22 (data not shown). In Experiment 1, both stimuli were presented at the same volume (~75dB) whereas in Experiment 2, the volume of the CS+ was increased to 88 dB and the CS– decreased to 72 dB.

Note that at the end of Experiment 2, cue-reward associations were then extinguished for 4 sessions, reinstated for 14 sessions, and then reversed. A Conditioned Reinforcement Test session (see Chapter 2) was also run after each of these stages (i.e., after initial acquisition, extinction, reinstatement, reversal). However, for reasons of focus, these post-acquisition data will not be discussed in

the main part of this thesis but for an overview of the results, see Supplementary Figure S3-1 in the Appendix.

Data analysis. Data were averaged across trials within each session and then grouped into four-session blocks for data presentation as well as statistical analysis. For details of statistical analysis, see Chapter 2.

Experiment 3: Unconditioned responding to auditory cues

Subjects. This experiment was initially run on a cohort of 19 male mice (Met COMT n = 9, WT n = 10) and was replicated in a second cohort of 17 mice (COMT-Met n = 9, WT n =8). Data analysis and presentation was collapsed across both cohorts. The mice were experimentally naïve and were at the age of 8-12 weeks at the start of the experiment. They were food restricted and their body weights were kept at approximately 85-90% of their free feeding weights. Water was available ad libitum.

Apparatus. The apparatus was identical to that described in the Pavlovian conditioning experiment (3.2.1.) above.

Procedure. The perceptual salience of WN (75dB) and Tone (75dB) were assessed in experimentally naïve animals by making use of the phenomenon that unexpected presentation of salient stimuli is able to interrupt ongoing behaviour (Sanderson et al., 2011; Walasek et al., 1994). In order to promote head entries into the magazine to act as a baseline level of responding against which orienting could be measured, mice were first given four sessions during which 20 rewards (60μL of 10% sucrose solution; 4.2s total time required to deliver full amount) were delivered on a pseudorandom interval schedule (mean 120s, range 12-484s, calculated according to the Fleshler and Hoffman scale (Fleshler & Hoffman, 1962)). This delivery

schedule maximised the temporal uncertainty of reward delivery and therefore promoted magazine checking behaviour. Reward delivery was unsignalled, except for the sounds made by the sucrose pump operating, and was not contingent upon magazine head entries. Stimulus-driven orienting was assessed in a fifth and sixth session. In each of these sessions, 8s WN (75dB) and Tone (75dB) were each presented four times on a pseudorandom interval schedule with a mean of 300s (range 70-450s). Stimuli lasted 8s and were not contingent on reward delivery or magazine responding (i.e., cue presentation and reward delivery times were independent). The order of stimulus presentation was counterbalanced between subjects. Magazine responding was recorded throughout all sessions.

Data analysis. Unconditioned responding to auditory cues was measured indirectly by measuring the extent to which magazine-directed behaviour was suppressed during stimulus presentation (Sanderson et al., 2011). Suppression of magazine responding was calculated by subtracting the time spent in the magazine during the 4s pre-stimulus baseline from the time spent in the magazine in the first 4s of stimulus presentation. Analysis was focussed on the first half of stimulus presentation as salience effects were expected to be strongest at stimulus onset and to decline in the course of stimulus presentation in a process of habituation. Data were analysed using repeated measures ANOVA with genotype as the between-subjects factor and stimulus type (2 levels: T, WN) and exposure session (2 levels: first, second) as within-subject factors. Significant main effects or interactions ($p < 0.05$) were followed up by pairwise comparisons. Moreover, one-sample t-test against 0 was used for each stimulus type and within each genotype group to test whether responding had significantly changed from baseline.

3.3. Results

3.3.1. Experiment 1: Pavlovian auditory discrimination

All 27 mice (COMT-Met n = 15, WT n = 12) completed the 24 Pavlovian conditioning sessions (presented and analysed in 4-session blocks below). The average rate of baseline responding, as assessed by time spent in the food magazine during the 8s period before cue onset, was similar in both genotype groups, with a decrease between the first two and the third block (main effect of session block: $F_{5,125} = 5.0$, $p < 0.01$) but no effect of genotype ($F_{1,25} = 0.4$, $p = 0.53$) or genotype × block interaction ($F_{5,125} = 0.6$, $p = 0.70$, Figure 3-1).

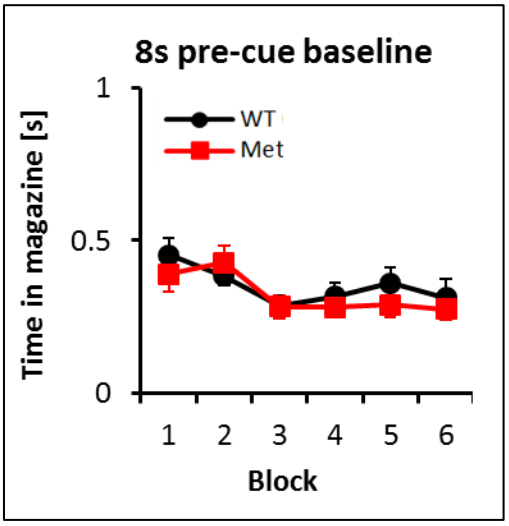


Figure 3-1. Baseline magazine responding. *Average time spent in magazine during the 8s preceding auditory cue onset (collapsed across all trials).*

Both genotypes learned the cue-reward associations, exhibiting increased time spent in the magazine during presentation of the CS+ over sessions (main effect of CS: $F_{1,23} = 256.5$, $p < 0.01$; and CS × block interaction: $F_{3.6,81.9} = 177.6$, $p < 0.01$; Figure 3-2 *left*). Notably, animals both acquired the cue discrimination faster and spent more time in the magazine during CS+ when the rewarded cue was white

noise (main effect of stimulus identity: $F_{1,23} = 22.6$, $p < 0.01$; stimulus identity × CS interaction: $F_{1,23} = 20.2$, $p < 0.01$; stimulus identity × CS × block interaction: $F_{1.6,81.9} = 7.8$, $p < 0.01$). These effects of CS+ stimulus identity were not present at the start of Pavlovian conditioning and became apparent from session block 2 onwards.

Intriguingly, whilst there was no main effect of COMT on conditioning (main effect of genotype: $F_{1,23} < 0.1$, $p = 0.87$; genotype × CS and genotype × CS × session interactions: both $F < 0.4$, $p > 0.52$), the extent to which stimulus identity affected conditioned responding *was* modulated by genotype (stimulus type × genotype interaction: $F_{1,23} = 9.5$, $p < 0.01$; Figure 3-2). Whilst numerically, both genotype groups spent more time in the magazine during CS+ when the WN was paired with reward compared to when the Tone was paired with reward, this difference was only significant in COMT-Met ($p < 0.001$) but not WT mice ($p = 0.27$). Moreover, COMT-Met mice spent significantly *more* time in the magazine than WT mice when the CS+ was the WN ($p = 0.03$), but significantly *less* than WT when the CS+ was the Tone ($p = 0.048$). These differences were not present right at the start of testing, but emerged for the CS+ after block 3 (CS × stimulus type × session × genotype: $F_{3.6,81.9} = 6.6$, $p < 0.01$).

Exploration of this interaction for CS- trials revealed a significant genotype difference only for session block 3 in mice trained in the T+/WN- condition: WT mice responded more strongly to WN- than did COMT-Met mice ($p < 0.01$). This was due to an increase in responding to WN- in WT mice from session block 1 to block 3, reaching its peak and being significantly above baseline on session block 3 (one-sample t-test, $t_5 = 2.9$, $p = 0.03$; Figure 3-2 *bottom*).

In order to test whether WT mice generally responded more to CS- cues than COMT-Met mice, CS- trials were also analysed separately from CS+ trials. Overall, all animals significantly increased responding during CS- cues across the first three session blocks (main effect of block: $F_{5,115} = 4.3$, $p < 0.01$), suggesting generalisation of magazine responding to the CS- from the CS+ in the first half of the course of training. There was also trend level evidence that generalisation was stronger in WT than in COMT-Met mice (main effect of genotype: $F_{1,23} = 3.9$, $p = 0.06$, but no interaction with stimulus type: $F_{1,23} = 2.4$, $p = 0.14$).

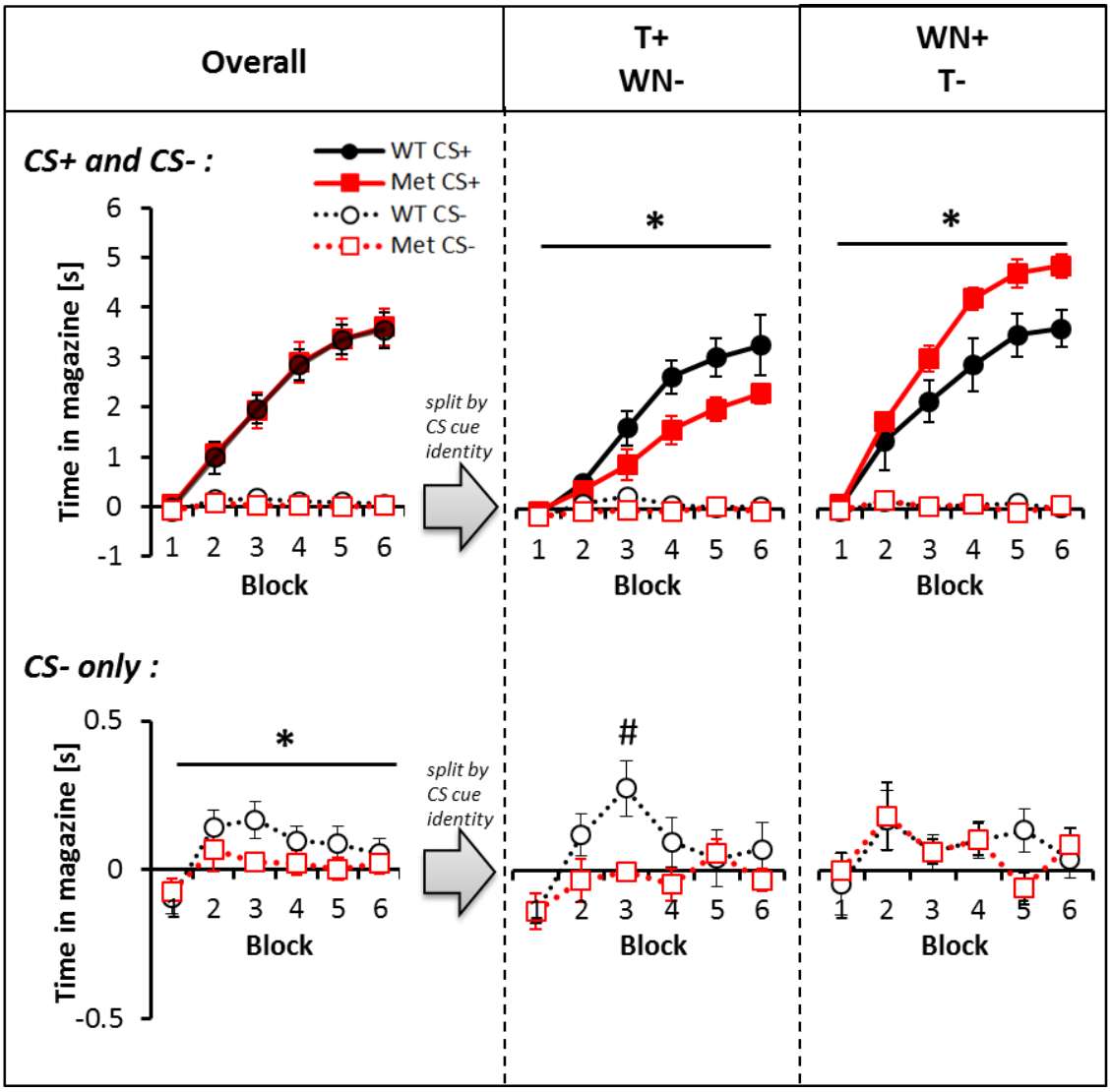


Figure 3-2. Overview of Pavlovian conditioning results. *Average time spent in the magazine, normalised to the 8s pre-cue period, during CS+ and CS- presentations, presented as an average of all COMT-Met and WT mice (left), as well as separately for mice where CS+ was signalled by Tone (middle; * WT > Met, $p < 0.05$) and mice where CS+ was signalled by White Noise (right;*, Met > WT, $p < 0.05$). Bottom row depicts CS- data at more refined scaling of the y-axis (*, main effect of genotype, $p < 0.05$; #, one-sample t-test against 0, $p < 0.05$). Data points represent the mean of blocks of 4 sessions ± SEM.*

3.3.2. Experiment 2: Pavlovian auditory discrimination with salience manipulation

Experiment 1 showed that COMT genotype interacted with auditory stimulus identity (WN, T) to influence the rate of acquisition and strength of the conditioned response. One possibility was that this effect was driven by differences in stimulus

salience between the cues. However, a second possibility was that the learning differences reflected some other intrinsic property of the WN and T. To disentangle these, in Experiment 2, the sensory salience of the Tone and WN was manipulated by changing the volumes at which the stimuli were presented (Tone volume increased to 88dB and WN decreased to 72dB). If the relatively lower stimulus salience of the Tone compared to the WN in Experiment 1 was driving the learning rate difference between the groups, then this manipulation should reduce or abolish the effect; if however the learning rate difference was driven by the intrinsic properties of the Tone, then increasing its volume should alter both groups' learning rates equally, leaving COMT-Met mice still impaired compared to the WTs.

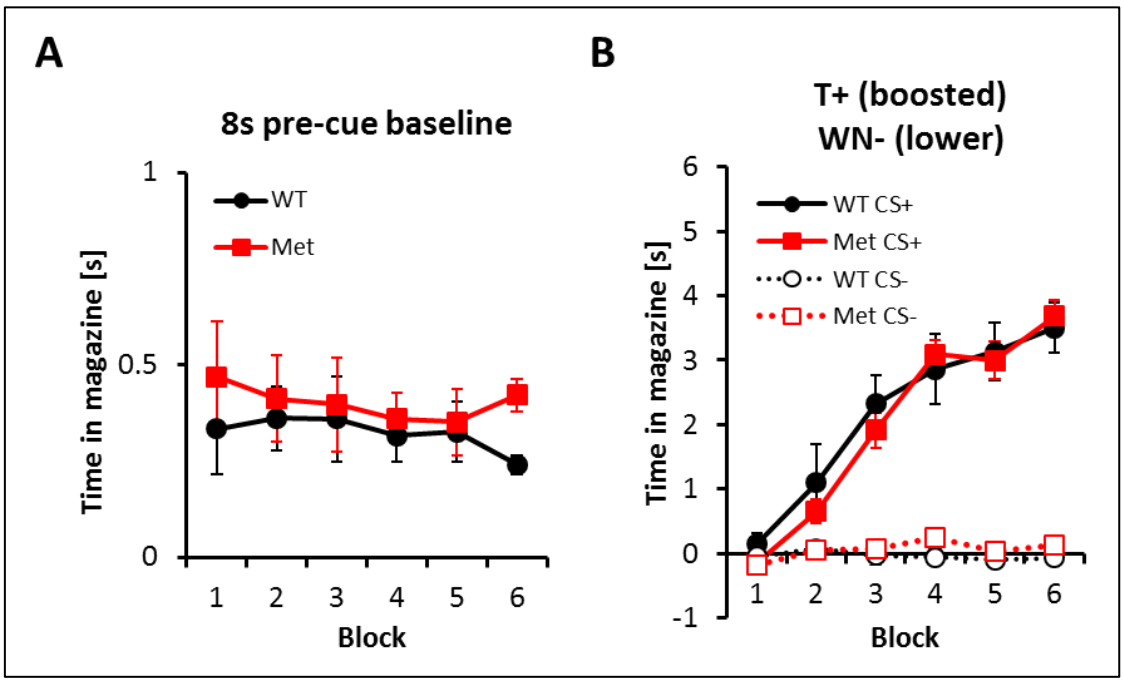


Figure 3-3. ***Pavlovian conditioning results for manipulated salience condition.*** ***A.*** *Average time spent in magazine during the 8s preceding auditory cue onset (collapsed across all trials).* ***B.*** *Average time spent in the magazine during CS+ and CS- presentations, normalised to the 8s pre-cue period. Data points represent the mean of blocks of 4 sessions ± SEM.*

Baseline and cue-elicited magazine responding can be observed in Figure 3-3. As in Experiment 1, genotype did not affect the average rate of baseline responding, as assessed by the time spent in the food magazine during the 8s period before cue onset (no main effect of genotype: $F_{1,8} = 0.7$, $p = 0.44$; no genotype × session interaction: $F_{5,40} = 0.4$, $p = 0.83$; Figure 3-3A). Both genotype groups acquired a conditioned response to the rewarded cue (main effect of CS: $F_{1,8} = 122.4$, $p < 0.01$; CS × session interaction: $F_{5,40} = 38.0$, $p < 0.01$), with significant discrimination of CS types emerging from session block 2 ($p < 0.01$; Figure 3-3B). Importantly, there were no effects of or interactions with genotype (main effect of genotype: $F_{1,8} < 0.1$, $p = 0.92$; all interactions with genotype: $F < 0.8$, $p > 0.60$).

These results show that performance of COMT-Met mice trained on a Tone CS+ and WN CS- discrimination can match that of WT mice if the volume of the Tone is increased to be more salient.

Finally, magazine responding during the CS+ was compared between the two genotype groups and across all three cue assignment conditions of Experiments 1 and 2 (Figure 3-4), revealing a significant stimulus type × genotype interaction ($F_{2,31} = 4.6$, $p = 0.02$). Notably, magazine responding differed significantly between each of the three cues in COMT-Met mice (pairwise comparisons: WN > Tone (boosted) > Tone, all $p < 0.05$), suggesting that learning in COMT-Met mice varied with cue salience in a systematic manner. No such effect was observed in WT mice (all $p > 0.25$).

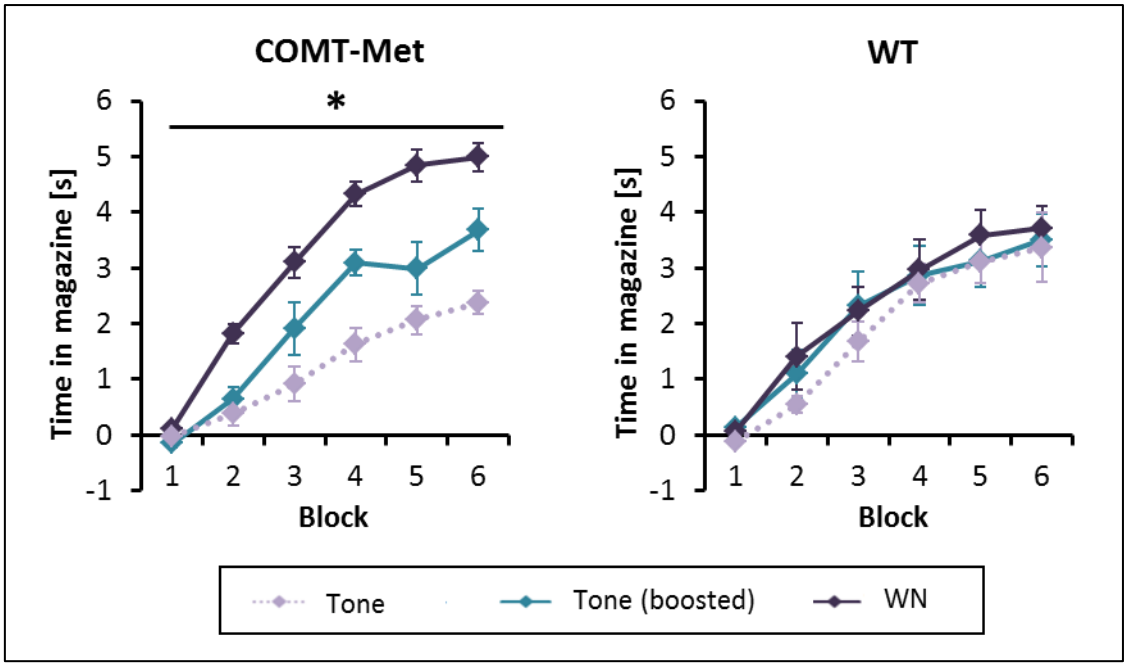


Figure 3-4. ***Direct comparison of CS+ responding in Experiments 1 and 2.*** *Average normalised time spent in the magazine during CS+ presentations, compared across all three cue assignment conditions: T+/WN- ('Tone'); WN+/T- ('WN'); and boosted T+ / lower WN- ('Tone boosted'). Magazine responding differed significantly between all three conditions in COMT-Met but not WT mice (*, RM ANOVA, genotype*stimulus type interaction, $p < 0.05$; WN > Tone (boosted) > T in COMT-Met mice, all $p < 0.05$). Data points represent the mean of blocks of 4 sessions ± SEM.*

3.3.3. Experiment 3: Unconditioned responding to auditory cues

Experiment 1 showed that the effects of COMT on learning were modulated by stimulus identity (WN vs Tone). Experiment 2 provided some evidence supporting the hypothesis that these effects might be driven by differences in stimulus salience. The aim of experiment 3 was to test whether genotype differences in the response to the two stimuli were already present *prior* to learning, by measuring the suppression of magazine responding elicited by the presentation of the stimuli.

Animals spent on average 16% (±13 SD) of the 4s pre-stimulus baseline in the magazine on test session 1 and 11% (± 12 SD) on test session 2, and there were

no effects of or interactions with stimulus type or genotype (all $F < 2.5$, $p > 0.12$). Magazine responding was more suppressed by WN than by Tone presentation (main effect of stimulus type: $F_{1,34} = 7.4$, $p = 0.01$; Figure 3-5). Furthermore, there was a significant stimulus type × session interaction ($F_{1,34} = 6.3$, $p = 0.02$): whilst responding to the two auditory stimuli was significantly different on exposure session 1 ($p < 0.01$) no differences were present when re-exposed to the stimuli on session 2 ($p = 0.72$). However, there was no main effect of or interactions with genotype (all $F < 0.6$, $p > 0.43$). In both genotype groups, magazine responding was significantly suppressed below 0 during the WN (one sample t-test against 0, both $t_{17} > 2.8$, $p < 0.02$) but did not significantly deviate from baseline during the Tone (both $t_{17} < 0.7$, $p > 0.55$) on the first exposure session. On exposure session 2, both stimuli failed to cause significant changes in magazine responding from baseline in either genotype group (all $t_{17} < 1.4$, $p > 0.21$).

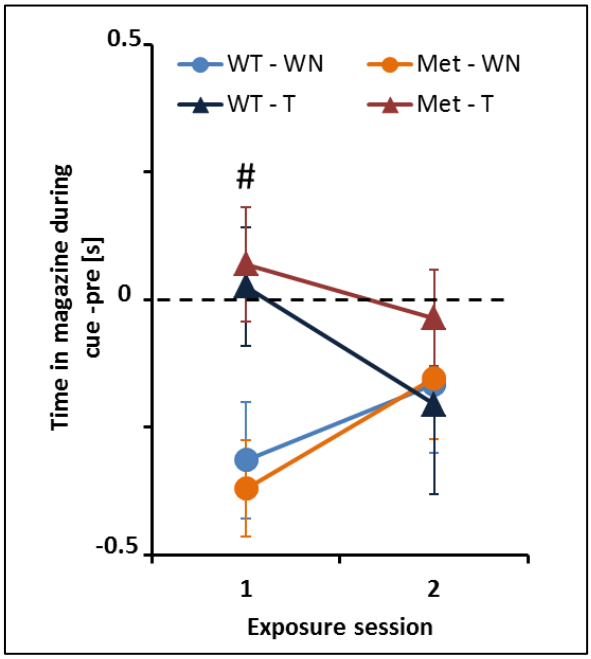


Figure 3-5. Change in magazine responding elicited by non-contingent presentation of WN and Tone (T) across 2 exposure sessions. *Data points are mean (± SEM) of all four stimulus presentations (#, one-sample t-test against 0, $p < 0.05$ for WN trials on exposure session 1)*

3.4. Discussion

The aim of this chapter was to dissect the elements of associative learning affected by COMT by using a Pavlovian discrimination learning paradigm. As detailed in Chapter 1, deterministic appetitive Pavlovian conditioning processes are typically thought to be largely independent of PFC and instead dependent on NAc function (Christakou, Robbins, & Everitt, 2001), with phasic dopamine release in the NAc playing a critical role (Day et al., 2007; Flagel et al., 2011; Robbins & Everitt, 1996). According to the predictions from the tonic-phasic hypothesis (Bilder et al., 2004) as well as from what is known about the inverse relationship between prefrontal and striatal dopamine, phasic dopamine signalling in the striatum is hypothesised to be reduced in COMT-Met mice. If so, it was anticipated that associative learning would be attenuated in these animals.

Taken together, the present data provide little evidence in support of this hypothesis, as there was no main effect of COMT genotype in the associative learning task used in the present study. However, there was instead a strong interaction between COMT genotype and the identity of the rewarded cue, suggesting an unexpected and more complex role for COMT in the processing of environmental stimuli and the way they are used to guide learning and behaviour. More specifically, COMT-Met mice appeared to be more sensitive to stimulus salience influences on learning rates. All animals learned faster about, and oriented more strongly to, the WN than the Tone even though it was presented at the same absolute volume, implying that the WN was of higher salience than the Tone. However, the change in learning rate, when these stimuli were used as conditioned stimuli in the present learning paradigm, was strikingly greater in COMT-Met compared to WT mice. Since

these results were not part of our initial hypothesis, the following discussion shall first provide an overview of what is known about the use of different cues in learning paradigms from a behavioural science perspective, before proceeding to a discussion of potential neural mechanisms underpinning the findings of the present study.

Is WN intrinsically different from Tone?

The first possibility to be considered as a reason for a cue being conditioned faster than another, is that of *selective association*, a term describing the phenomenon that some CS-US relationships are learned about more readily than others (Thorndike, 1898). For instance, pigeons learn to associate an appetitive US faster when the stimulus predicting it is a visual cue (light) than when it is an auditory cue (tone). Importantly, this cue-dependent difference in learning cannot be explained by differences in sensory salience alone, as conditioning proceeds faster with tone than light when the outcome is aversive (Shapiro, Jacobs, & LoLordo, 1980). One explanation for this phenomenon is that some stimulus dimensions have been more relevant to the occurrence of some biologically significant events in the past than others – either in the experience of the animal itself or in the course of evolution (Rozin & Kalat, 1971; Seligman, 1970).

However, it seems unlikely that selective association could explain the learning differences observed here. Such studies typically compare cues (and/or unconditioned stimuli) of different modalities, whilst the two cues used here were of the same modality. It is also not immediately obvious why WN should have acquired greater evolutionary significance in predicting food than a Tone. More

importantly, there is no reason why any such effect would interact with genotype here. It therefore seems unlikely that mice learning WN+ associations faster than T+ in Experiment 1 can be explained by the phenomenon of selective association.

Is WN more salient than Tone?

Instead of selective association, an alternative explanation for the WN's greater associability is that the WN is *perceptually* more salient than the Tone. This explanation is supported by the results of Experiment 3, where non-contingent presentations of WN were found to elicit a stronger unconditioned response than the Tone, which can be interpreted as a heightened ability to attract attention. Moreover, all mice were found to increase responding to the CS+ faster when it was the WN rather than the Tone, suggesting that the WN exhibits a greater degree of associability than the Tone, a term that has been attributed to greater cue salience in formal models of reinforcement learning (Rescorla & Wagner, 1972).

The perceptual salience of a cue typically increases with the intensity at which the cue is presented. In an attempt to match cue intensities, both the Tone and WN were presented at 75dB in Experiment 1. Nevertheless, it is possible that the excitation of the auditory system was more sustained during WN presentation than during the Tone: WN covered a wider range of frequencies with random on- and offsets, whilst the Tone was a standing wave of a constant frequency, which is known to lead to greater habituation in the auditory system (Pérez-González & Malmierca, 2014; Westerman & Smith, 1984).

It is also possible that the increased salience of the WN might relate to it being mildly aversive, at least at high volumes. Indeed, some studies of COMT in humans,

as well as in animals, show that the Met-allele increases anxiety as well as reactivity to stressful and negative affective stimuli (Drabant et al., 2006; Heinz & Smolka, 2006; Papaleo et al., 2008; Smolka et al., 2005), although these effects seem to be more robust in females than males (Domschke et al., 2012; Montag et al., 2008; Risbrough, Ji, Hauger, & Zhou, 2014), or were shown to have an opposite direction (Domschke et al., 2015)

It should be noted, that COMT-Met and WT mice showed no differences on behavioural tests of anxiety performed in our laboratory (Barkus et al., *in preparation*). Moreover, the learning rate effects observed here cannot be explained solely by the intrinsic properties of the WN as learning was also rapid to the Tone cue when its salience was increased by boosting its volume. Moreover, in Experiment 3, both genotypes oriented similarly to the WN cue.

Do cues of different salience interact with each other during discrimination learning?

It is well established that learning of a cue-reward association proceeds more readily with a perceptually more salient cue than that with a less salient cue (Hull, 1949; Kamin & Schaub, 1963). However, what is not immediately clear is whether and how stimuli with differences in perceptual salience as well as reward outcomes might interact when presented within the same session. One possible explanation for the results of the present study could be that COMT-Met mice pay more attention to the perceptually most salient stimulus presented in a session, at the expense of the less salient stimulus, thereby learning about the reward contingency of the most salient stimulus *first*. This will lead to enhancement of cue-reward learning if the more salient stimulus is assigned to be the CS+. However, if the more salient

stimulus is assigned to be the CS-, the first association the animal will learn is that between an auditory stimulus and *no* reward, which could generalise to the less salient, rewarded stimulus, thus attenuating the formation of its association with reward (see literature on latent inhibition; Lubow, Weiner, & Schnur, 1981; Rescorla, 1976).

Potential neural substrate of COMT's influence on learning

The results obtained in the present study point towards the possibility of a critical influence of attentional processes, and their modulation by COMT, even during the simple Pavlovian discrimination conditioning task used here. Given that the genotype effects found here were bi-directional and cue-dependent, they cannot be easily explained by a universally enhancing effect of the Met-allele on task performance, as suggested in studies of COMT effects on cognitive function (Babovic et al., 2008; Bruder et al., 2005; Dickinson & Elvevåg, 2009; Lapish et al., 2009; Mattay et al., 2003; Papaleo et al., 2008; Tunbridge, Bannerman, Sharp, & Harrison, 2004).

Simple appetitive Pavlovian conditioning processes are typically thought to be PFC-*independent* (Christakou et al., 2001). The question of whether COMT modulates striatal dopamine release is the subject of experiments in later chapters. However, it is known that COMT affects PFC dopamine levels. Therefore, it is worth considering whether the PFC, as well as other cortical regions modulated by dopamine, may play a subtle and underappreciated role in this type of learning.

One potential locus could be the anterior cingulate cortex (ACC). Several lesion studies in rats have shown that cue-elicited *discriminated* approach is

dependent on the ACC (Bussey, Everitt, & Robbins, 1997; Cardinal et al., 2003; Parkinson, Cardinal, & Everitt, 2000). For instance, using a visual cue discrimination task, Bussey and colleagues showed that rats with ACC lesions were able to learn the association between CS+ and reward and therefore approach the cue as expected, but failed to suppress responding to the CS- cue (Bussey et al., 1997). Interestingly, in the present study, in addition to the striking effect of COMT genotype on CS+ learning rates, there was also a subtle effect on CS- responding, with WT mice responding to the CS- cue more strongly than did COMT-Met mice. Indeed, the cingulate cortex receives a dense innervation from the dopaminergic cells of the VTA (Seamans & Yang, 2004), and the COMT Met-allele has been associated with more efficient/altered ACC function as well as increased grey matter in humans (Blasi et al., 2005; McIntosh et al., 2007; Tian et al., 2013). The present finding of COMT-Met mice being superior to WT mice at suppressing responding to the CS- is therefore consistent with the notion of improved ACC function conferred by the Met-allele.

Of further relevance to the present findings is the fact that the ACC is one node consistently linked with what has been termed "the salience network" in human functional imaging studies (Seeley et al., 2007; Sridharan, Levitin, & Menon, 2008). The proposed role of the salience network is to segregate the most relevant among external and internal stimuli, and drive behaviour accordingly (Menon & Uddin, 2010). In this model, the salience network identifies behaviourally relevant stimuli and drives attention and cognition towards them by switching between the default mode and task-related brain networks (Dosenbach et al., 2006; Menon & Uddin, 2010). The task-related brain networks then operate based on the identified behavioural relevance (Seeley et al., 2007). The salience network integrates bottom-

up attention switching with top-down biasing of sensory input, enabling the animal to adjust attentional gain for task-relevant stimuli (Yantis, 2008). Consistent with this proposal, activity within the salience network is disrupted in patients with psychosis, and its activity is modulated by dopaminergic drugs (Gradin et al., 2013; Palaniyappan & Liddle, 2012). It is therefore conceivable that effects within this network might also underlie direct or indirect modulation by COMT.

In light of the results of the present study, a key question to ask is that of which factors determine how the behavioural relevance of a stimulus is judged. In the present study, one could speculate that in COMT-Met mice "behavioural relevance" is more strongly influenced by the sensory salience of a cue, thus enhancing attention towards and learning about more perceptually salient cues, than in WT mice. There are several ways in which this could occur, none of which are mutually exclusive. First, this could potentially be the result of higher levels of PFC dopamine associated the COMT-Met genotype shaping overall network properties to be more likely to be in a state where only the most salient information can disrupt stable network properties (Durstewitz & Seamans, 2008). More perceptually salient cues might be classified as more behaviourally relevant by COMT-Met than WT mice, in which relevance might, in turn, be more strongly determined by a cue's association with reward (Hickey, Chelazzi, & Theeuwes, 2010). In this scenario, the neurobiology conferred by the COMT genotypes would shape the salience network's interpretations of incoming sensory input with respect to its behavioural relevance (Hickey et al., 2010).

An alternative explanation would be that the difference between genotypes arises at an earlier stage in stimulus processing, namely at the level of the inputs

into the salience network. Responses to specific stimuli can be filtered and amplified at multiple levels in the ascending neural pathways that bring external stimuli to the sensory cortices, as well as in the sensory cortices themselves (Barceló, Suwazono, & Knight, 2000; Fecteau, Bell, & Munoz, 2004). Of particular relevance in this context is the finding that dopamine D1 receptor stimulation in PFC influences the response to stimuli in visual cortex (Noudoost, Chang, Steinmetz, & Moore, 2010; Noudoost & Moore, 2011). It is therefore conceivable that, in the present study, an increase in PFC dopamine levels in COMT-Met mice could lead to increased top-down attentional influence on the detection of the most salient stimulus, i.e. WN, potentially at the expense of the less salient stimulus, i.e., the Tone. Indeed, it has been repeatedly demonstrated that increased attention on particular features of an auditory stimulus, such as its frequency or intensity, comes at the expense of stimuli outside the attended range (Luce & Green, 1978; Mondor & Bregman, 1994; Nosofsky, 1983; Scharf, Quigley, Aoki, Peachey, & Reeves, 1987). In line with this proposal, a recent study shows increased top-down but not bottom-up attentional control in healthy human Met-allele carriers compared to Val-allele carriers (Schneider, Schote, Meyer, & Frings, 2014)

In addition to indirect top-down attentional modulation of stimulus processing in sensory cortices via dopamine levels in the PFC, dopamine plays an important role in adaptive mechanisms in the sensory cortices themselves during learning: sensory stimuli have been shown to drive plasticity in the corresponding sensory cortices in a process that is both modulated by local dopamine and related to the behavioural relevance of the stimuli (Happel, Deliano, Handschuh, & Ohl, 2014; Stark & Scheich, 1997). The role of COMT in sensory cortices has not been

studied to date, although it is conceivable that an increase in dopamine levels conferred by the Met-allele might lead to enhanced plasticity, or 'tuning' of sensory areas to certain stimuli, potentially at the expense of others.

Finally, COMT could affect the flow of information between sensory cortices and PFC, as demonstrated by a study that found a reduction in some components of auditory gating in Met-allele carriers (Majic et al., 2011), although it is not clear whether this was primarily driven by COMT effects on dopamine in PFC, the auditory cortices, or subcortical structures known to be involved in sensory gating mechanisms (Adler, Rose, & Freedman, 1986; Bickford-Wimer et al., 1990; Freedman, 1996; Light et al., 1999; Mann et al., 2008).

Conclusions

In conclusion, while there was no main effect of COMT genotype in the Pavlovian auditory discrimination task used in the present study, more detailed analysis revealed that learning in COMT-Met mice was significantly affected by the salience of the stimuli used. This could either be mediated by genotype differences in bottom-up or top-down attentional processes, or the way both systems interact to guide behaviour. The results of the present chapter therefore uncover a complex role for COMT in associative learning.

Chapter 4: COMT and single cue conditioning

4.1. Introduction

The Pavlovian auditory discrimination study presented in Chapter 3 revealed a striking interaction between COMT genotype and the auditory stimuli used for discrimination. It was hypothesised that these findings were driven by the different ways in which the two genotype groups assign attention when discriminating between stimuli with differences in perceptual salience as well as reward outcomes. The aim of the present chapter was to further test this hypothesis and extend the findings using the multi-stage experiment described below.

The results of Chapter 3 showed that in a Pavlovian discrimination learning setting, COMT-Met mice increased conditioned responding significantly faster when the more salient stimulus was rewarded and significantly slower than WT mice when the more salient cue was unrewarded. One possible explanation put forward for this finding was therefore that COMT-Met mice might pay more attention selectively to the perceptually most salient stimulus presented in a session, leading either to enhancement or attenuation of cue-reward learning, depending on whether the more salient cue is assigned to be CS+ or CS-. Such an idea has parallels with the literature on latent inhibition, where animals' learning about a CS+ is retarded if the same cue has been previously paired with no consequences in an earlier stage (Mackintosh, 1975; Pearce & Hall, 1980; Weiner, 2003), and also with recent theories around the concept of *acquired salience*, relating to the sum of cue-outcome and cue-no outcome associative strengths (Esber & Haselgrove, 2011;

Ogawa et al., 2013). However, an alternative explanation could be that learning rates are determined by the absolute perceptual salience of the CS+ alone and do not depend on its interaction with the CS-.

The main aim of the present study was therefore to test whether the cue-dependent effects of COMT observed during Pavlovian discrimination learning would be attenuated in the absence of a CS-. In Stage 1 of the present experiment, separate groups of mice were trained to acquire either a Tone-reward or a WN-reward association, in the absence of any unrewarded cue. It was predicted that there would be no interaction between genotype and stimulus type if the previously observed effects were indeed driven by competition between two stimuli presented within the same session. On the other hand, if learning in COMT-Met mice was solely affected by the absolute, rather than relative, salience of the CS+, then the genotype by stimulus type interaction – faster learning about the WN and slower learning about the Tone in COMT-Met compared to WT mice – would be expected to persist even in the absence of a second stimulus.

The next question investigated was whether the impact of unconditioned cue salience on learning would be affected by prior learning experience. Therefore, in Stage 2 of the experiment, initial cue-reward associations were first extinguished and then, in Stage 3, mice were subjected to Pavlovian cue-reward training using the other stimulus type. It was hypothesised that if the influence of stimulus salience on learning rate in COMT-Met mice simply reflected the intrinsic alerting properties of the stimulus, then the speed of acquisition in Stage 3 should again scale with the salience of the stimulus used at that stage. If, however, stimulus salience had an impact on the acquisition of a rudimentary model of the environment (i.e. the notion

that auditory cues in general may predict reward), the speed of acquisition in Stage 3 should be influenced by the salience of the stimulus used in Stage 1.

Finally, one question that might arise from the finding that learning rate in COMT-Met mice is retarded in the Tone condition and improved when Tone volume is boosted (Chapter 3), is the possibility that COMT-Met mice might have a subtle hearing impairment. The aim of the final experiment in this chapter was therefore to explore the possibility that genetic modification of COMT might confer a hearing impairment specific to stimuli in the 3kHz range. To address this question, a "volume response curve" was obtained by systematically varying Tone volumes and recording magazine responding in mice that had previously been trained to respond to Tone at plateau levels. It was hypothesised that magazine responding would decrease with decreasing stimulus volumes and the volume of cue presentation where responding did not rise above baseline would indicate that the hearing threshold was surpassed. This test would provide an insight into the question of whether the absolute hearing threshold for the Tone was altered in COMT-Met mice compared to WT.

4.2. Methods

Subjects. A total of 25 male mice (14 COMT-Met and 11 WT) were used in this study (23 female mice were tested in parallel, but the data from these is not reported in this thesis). All subjects went through all experiments, as summarised in Figure 4-1. The mice were 10-13 weeks of age at the start of the experiment. Mice were food restricted throughout the study and their body weights were kept at approximately 85-90% of their free feeding weights. Water was available ad libitum.

Apparatus. See Chapter 2.2 for a description of the apparatus used in the experiments detailed in this chapter.

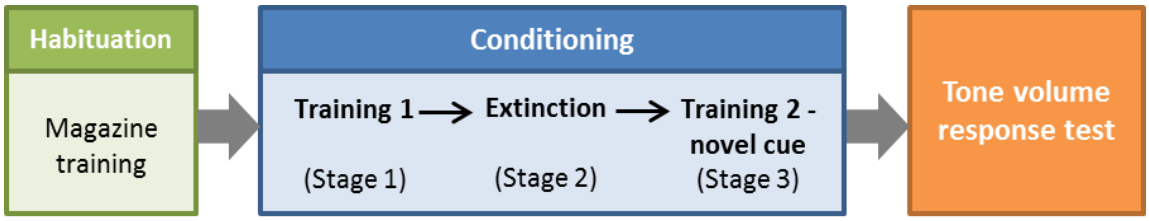


Figure 4-1. Overview of the order of experiments presented in this chapter. *All experiments were performed on the same cohort of mice. For details on the stages comprising the tone volume response test, see text below.*

Auditory stimuli. White noise (WN) and a 3kHz pure tone (Tone, T) auditory stimuli were used as conditioned cues in Stages 1-3 of the experiment. The volume of each was set to 75dB (6-7dB above background noise levels). Auditory cues were randomised and counterbalanced between subjects (Table 4-1).

Table 4-1. Cue-reward contingencies and subject numbers in single-cue Pavlovian auditory conditioning

Group		**Training 1** (32 sessions)	**Extinction** (6 sessions)	**Training 2** (7 sessions)
Group 1: "WN first"[2]	WT n = 4 Met n = 7	WN+	WN-	T+
Group 2: "T first" [2]	WT n = 5 Met n = 5	T+	T-	WN+

WN: white noise; T: 3kHz pure tone

[2]Numbers only include mice that were also included into data analysis (see Results)

Single-cue Pavlovian auditory conditioning

Figure 4-1 provides an overview of the experiments performed in this chapter.

Magazine training: Mice were habituated to the test chambers and trained to retrieve rewards from the food magazine as described in Chapter 2.

Stage 1 (Training 1): Following magazine training, the mice underwent 32 appetitive Pavlovian conditioning sessions. Each session consisted of 15 rewarded cue presentations (CS+) lasting 8s each. There were no unrewarded cue presentations. The average inter-stimulus interval was 120s.

Stage 2 (Extinction): After Pavlovian training, cue-reward associations were extinguished for six sessions. Extinction sessions were identical to those of Stage 1, except that no rewards were delivered.

Stage 3 (Training 2 – Novel Cue): In Stage 3, sessions were identical to Stage 1 except that animals were retrained using the auditory stimulus type that was not experienced in Stage 1 (i.e., if a Tone-reward pairing had been acquired in Stage 1, Stage 3 would use WN-reward).

Conditioned reinforcement tests: Conditioned reinforcement tests (see Chapter 2.2) were performed after training session 28, at the end of Stage 2, and at the end of Stage 3 (data not presented). Each test lasted for 30min.

Data analysis. Data were averaged across trials within each session and, for training Stage 1 only, grouped into two-session blocks for data presentation as well as statistical analysis. Normalised magazine responding during cue presentation was analysed using repeated measures ANOVA as described in Chapter 2.2. Separate ANOVAs were performed for each experimental Stage.

In order to directly test whether the learning experience in Training 1 affected cue-reward learning in Training 2, responding at the end of Training 1 (average of last 2 sessions) and that at the end of Training 2 (average of last 2 sessions) was compared using repeated measures ANOVA with genotype and

learning history (i.e., the order of stimuli used for Training 1 and 2) as between-subjects factors and Stage as a within-subject factor. According to the hypothesis at the core of this experiment, learning of Tone-reward pairings would be enhanced after previous learning with WN, and this effect will be stronger in COMT-Met mice. To test this prediction, each stimulus type was examined separately using planned comparisons (independent samples t-test) to compare responding to a stimulus type when it was used in Training 1 (i.e. during initial learning in naïve animals) to when it was used in Training 2 (i.e. in animals that had previously experienced the other stimulus).

Tone volume response test

Procedures. Following Stage 3 training (see above), the respective cue-reward contingencies were extinguished in both groups of animals (Table 4-1) for 3 sessions and all mice were re-trained on Tone-reward for 4 sessions (identical to Stage 1 training sessions) until stable performance was reached. The tone volume response test was then carried out in 2 phases, each consisting of two sessions. Each test session consisted of 20 rewarded Tone presentations, with cue duration and ITI identical to previous training sessions. However, this time the volume at which the stimuli were presented was varied on individual trials within a session. In test phase 1, Tones were presented at 55dB, 65dB, 75dB, and 85dB; in test phase 2, Tones were presented at 35db, 45dB, 55dB, and 75dB. Each volume was presented five times during one session and trials with different volumes were randomly intermixed. Magazine responding during cue presentation was assessed as usual.

Data analysis. Magazine responding during the cue was normalised to the 8s pre-cue period on a trial by trial basis and averaged across same-volume trials of the two

sessions of each test phase. Separate repeated measures ANOVAs were performed on data of each phase with stimulus volume as a within-subject factor (4 levels) and genotype and training history (i.e., "WN first" vs "T first") as between-subjects factors. Significant main effects and interactions ($p < 0.05$) were followed up with pairwise comparisons with Bonferroni correction.

4.3. Results

4.3.1. Acquisition of single-cue Pavlovian auditory learning (Training 1)

Twenty-one (COMT-Met n = 12, WT n = 9) of the 25 mice trained on the single cue Pavlovian conditioning task successfully acquired a conditioned response as measured by an increase in magazine head entry behaviour during cue presentation. Four mice (one of each cue type and genotype) did not show any changes in cue responding across the 32 conditioning sessions and were excluded from analysis.

The average rate of baseline responding, as assessed by time spent in the food magazine during the 8s period before cue onset (Figure 4-1A), fluctuated across training (main effect of session block: $F_{4.5,76.7} = 6.4$, $p < 0.01$) but was generally similar in both genotype and cue groups (main effect of genotype: $F_{1,17} = 0.2$, $p = 0.63$; cue type: $F_{1,17} < 0.1$, $p = 0.9$; genotype × cue typeinteraction: $F_{1,17} = 0.5$, $p = 0.50$; Figure 4-2A). The only exception to this was the group of WT mice trained on WN, whose baseline responding on block 14 was significantly higher than in COMT-Met mice trained on WN, as well as WT mice trained on Tone (genotype × block × cue type interaction: $F_{4.5,76.7} = 3.1$, $p = 0.02$, and pairwise comparisons $p < 0.05$; see Supplementary Figure S4-1).

Both genotypes learned the cue-reward associations, exhibiting increased time spent in the magazine during cue presentation over sessions (main effect of session: $F_{4.9,82.6} = 35.0$, $p < 0.01$; Figure 4-2B). There was no main effect of genotype or genotype × session interaction ($F < 0.8$, $p > 0.42$).

Nevertheless, as in the discrimination learning experiment described in Chapter 3, there was a significant main effect of stimulus type ($F_{1,17} = 6.9$, $p = 0.02$) and, importantly, a significant stimulus type × genotype interaction ($F_{1,17} = 5.4$, $p = 0.03$; Figure 4-2C). Again, stimulus type significantly affected cue-reward learning in COMT-Met ($p < 0.01$) but not WT mice ($p = 0.85$). The difference in responding between COMT-Met and WT mice was marginally significant when mice were trained on WN ($p = 0.05$) but did not reach significance in the group of mice trained on Tone ($p = 0.24$), although there was a numerical attenuation between the COMT-Met mice compared to WT controls in the early sessions (Figure 4-2C).

Overall, these results replicate major aspects of those obtained during discrimination learning (Chapter 3) and suggest that the stimulus type-dependent effects of COMT on associative learning persist in the absence of stimulus competition.

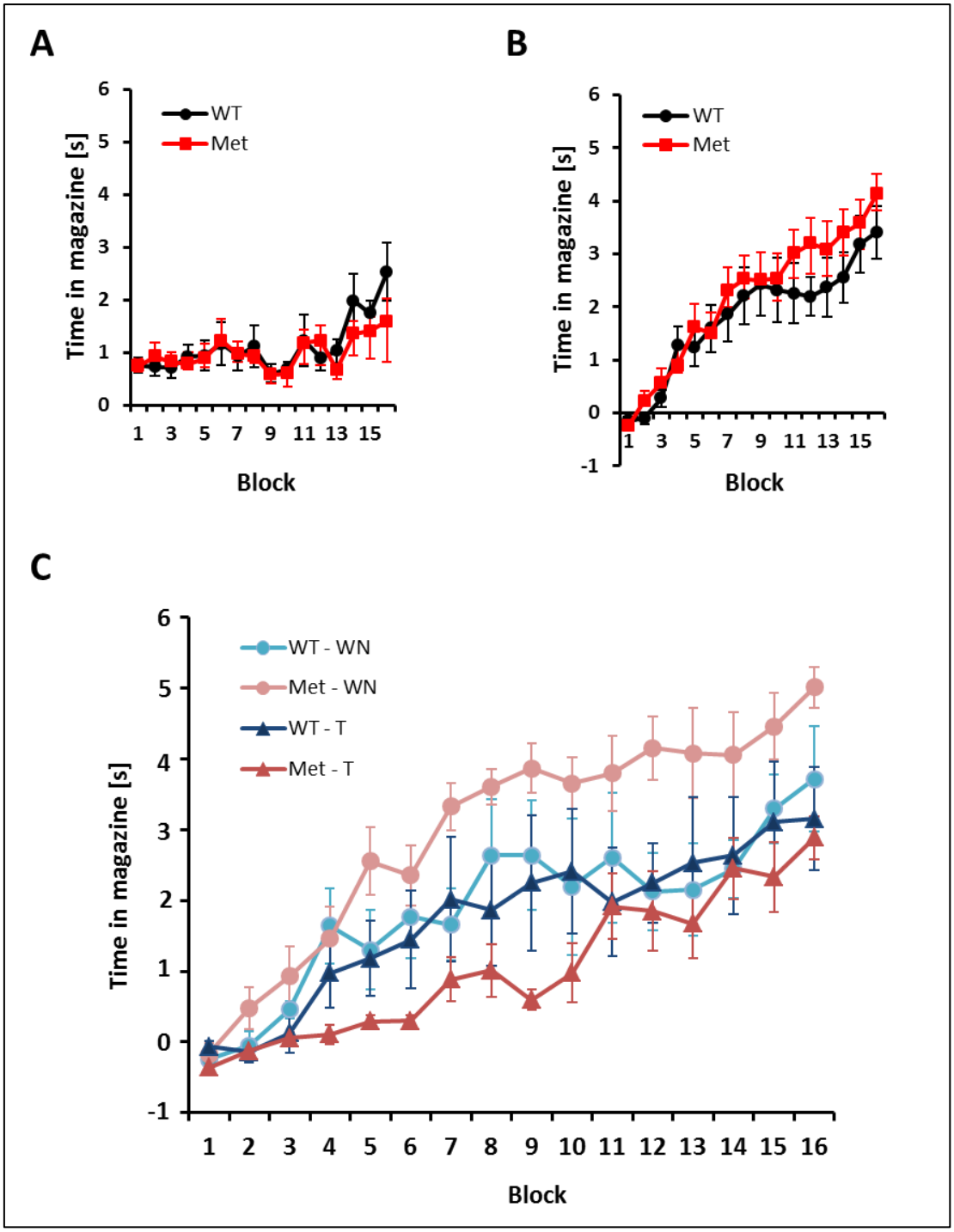


Figure 4-2. Overview of initial acquisition of single cue-reward associations (Training 1). A. *Average time spent in magazine during the 8s preceding auditory cue onset.* ***B-C.*** *Average time spent in the magazine, normalised to the 8s pre-cue period during cue presentation, presented as an average of all COMT-Met and WT mice (B), as well as separated by stimulus type (C; WN: white noise; T: Tone). Data points represent the means of blocks of 2 sessions ± SEM.*

4.3.2. Extinction of cue-reward associations

Following 32 conditioning sessions, mice were exposed to 6 extinction sessions during which cues were presented as before, but this time without being followed by reward. As expected, mice reduced magazine responding during cue presentations across the extinction sessions (main effect of session: $F_{2.7,46.0} = 37.4$, $p < 0.01$; Figure 4-3). Genotype did not affect extinction of conditioned responding in either cue group (no significant main effect of genotype: $F_{1,17} = 1.0$, $p = 0.34$; no interactions involving genotype: all $F < 1.3$, $p > 0.26$).

4.3.3. Acquisition of novel cue-reward association (Training 2)

After extinction, mice were again exposed to cue-reward pairings, but this time with the cue they had not yet experienced, i.e. mice initially trained on a rewarded Tone (Training 1) were now exposed to WN-reward pairings and vice versa. Both genotypes quickly acquired the new cue-reward association (main effect of session: $F_{3.0,52.0} = 15.6$, $p < 0.01$) with magazine responding plateauing by session 4 (pairwise comparisons after session 4, $p > 0.05$; Figure 4-3). There were no statistically significant stimulus type or genotype effects when all 7 sessions of Training 2 were analysed (genotype: $F_{1,17} < 0.1$, $p = 0.82$; stimulus type: $F_{1,17} = 1.8$, $p = 0.20$; interactions involving genotype: $F < 2.2$, $p > 0.15$).

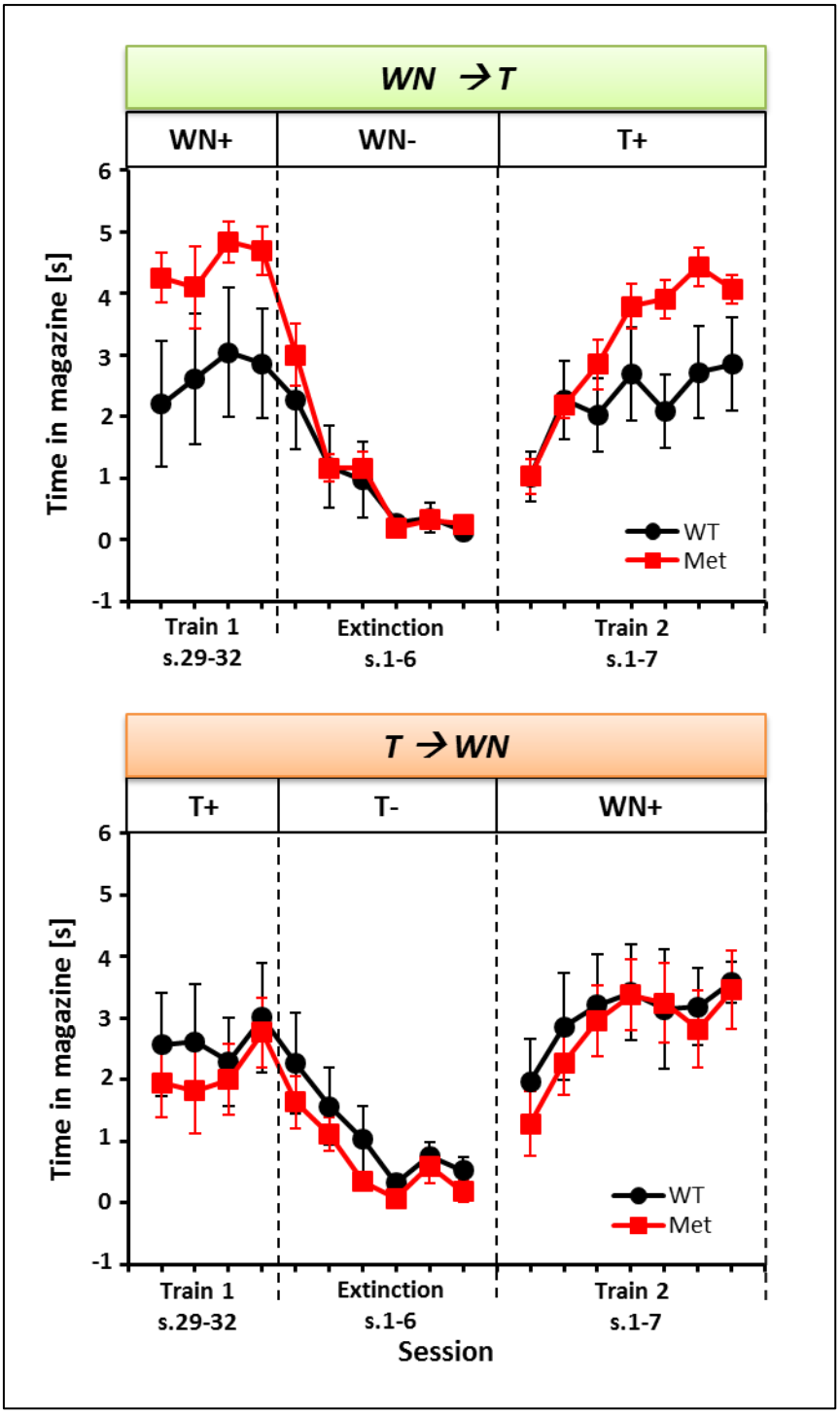


Figure 4-3. Overview of extinction and Training 2 results. *Graphs depict normalised magazine responding during cue presentation on the 4 final sessions of the initial cue-reward acquisition stage (sessions 29-32 of Training 1), 6 extinction sessions, as well as the 7 sessions of Training 2, where animals were trained to associate a novel stimulus with reward. Top: Data from "WN first" group. Bottom: Data from "T first" group. Data points represent averages of all trials per training session ± SEM.*

To directly test whether the learning experience in Training 1 affected cue-reward learning in Training 2, magazine responding at the end of Training 1 (average of sessions 31-32) was compared to that at the end of Training 2 (average of sessions 6-7 of Stage 2; Figure 4-4). Interestingly, there was a main effect of learning history (i.e., of the order of stimulus types in learning; $F_{1,17} = 7.3$, $p = 0.02$), as well as an interaction between learning history and genotype ($F_{1,17} = 5.0$, $p = 0.04$). Mice that had learned to associate WN with reward in Training 1, showed overall higher magazine responding rates than mice whose initial training was with Tone. This effect was significant in COMT-Met ($p < 0.01$) but not WT mice ($p = 0.76$).

The question as to whether the influence of stimulus salience on learning rate in COMT-Met mice was an inherent property of the stimulus (and should therefore be expressed regardless of an animal's experience with other stimuli), or whether it was subject to an animal's rudimentary model of the environment (which could be changed, based on previous experience with cues, possibly in a cue salience dependent manner), was at the core of this experiment. One possible interpretation of the results of the repeated measures ANOVA described above was that in COMT-Met mice, acquisition of T+ was enhanced *if* it was preceded by training with WN+. To confirm this picture, each stimulus type was examined separately and planned t-test comparisons were used to compare responding to each stimulus type when it was used in Training 1 as opposed to when it was used in Training 2 (i.e., in animals that had previously experienced the other stimulus). Interestingly, learning experience in Training 1 affected performance in Training 2 in COMT-Met but not WT mice, in a bi-directional manner. Response rates to Tone were significantly higher in COMT-Met mice at the end of Training 2 when compared to Tone-

responding at the end of Training 1 (independent samples t-test, $t_{10} = 4.1$, $p < 0.01$; Figure 4-4). By contrast, responding to WN at the end of Training 2 was attenuated as compared to WN-responding at the end of Training 1 ($t_{10} = 2.6$, $p = 0.03$, Figure 4-4). No such effects were evident in WT mice (all comparisons: $t_7 < 0.4$, $p > 0.73$).

Taken together, these results suggest that in COMT-Met mice, cue-reward learning with a highly salient cue (WN) facilitates subsequent formation of cue-reward associations with a less salient cue (T).

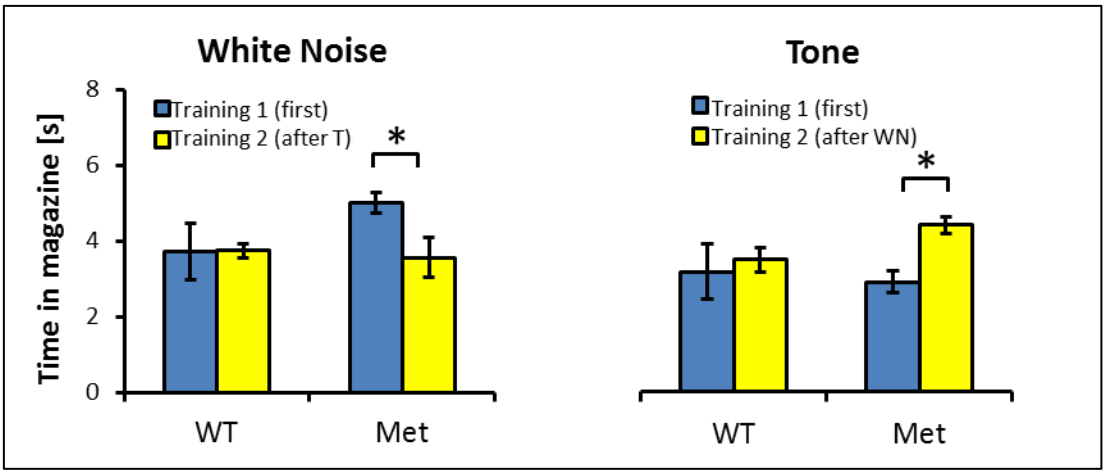


Figure 4-4. Effect of learning history on cue-reward learning with novel stimuli. *Figure compares normalised magazine responding during White Noise (left) and Tone (right) when used as stimulus during Training 1 (blue) as opposed to Training 2 (yellow). Bars represent averages of the 2 final sessions of Training 1 as well as the 2 final sessions of Training 2. *, independent samples t-test, $p < 0.05$. Error bars are ± SEM.*

4.3.4. Tone volume response test

In order to determine a hearing threshold for the volume at which the mice would not respond to presentation of the Tone, cue-reward associations were extinguished (3 extinction sessions) and all mice were re-trained on Tone for four sessions until magazine responding during Tone presentation reached plateau. On the subsequent four test sessions, mice received Tone-reward pairings as before,

however this time the volume at which the stimuli were presented was systematically varied on individual trials within a session: In test phase 1 (sessions 1 and 2), Tone volumes were varied in 10dB steps between 85dB and 55dB; in test phase 2 (sessions 3 and 4) volumes were varied between 75dB and 35dB (see Figure 4-5).

The interaction between genotype and learning history described in the sections above (i.e. COMT-Met mice originally trained on WN showed greater responding here than those trained on Tone) persisted in both phases of the volume test ($F_{1,17} > 5.0$, $p < 0.04$), although none of the pairwise comparisons exploring those interactions reached statistical significance (all $p > 0.05$).

More importantly, as expected, there was a main effect of stimulus volume on magazine responding in both phases of the test ($F_{3,51} > 3.1$, $p < 0.04$). Pairwise comparisons between the four different volumes of test phase 1 showed that there were only negligible differences in responding to stimuli in the range of 85-65dB as the only trend-wise significance emerged between 75dB and 55dB ($p = 0.06$). However, when volumes were decreased further in test phase 2, there were significant reductions in magazine responding between each of the volumes tested (pairwise comparisons: all $p < 0.01$). Most importantly, however, there was no main effect of genotype, nor any interaction between genotype and stimulus volume in either test phase 1 or phase 2 (all $F < 1.6$, all $p > 0.22$). Whilst both genotype groups still exhibited some magazine responding to 45dB Tone stimuli (one-sample t-tests against 0, $t > 4.5$, $p < 0.01$), responding decreased to baseline levels in both genotype groups when the volume was further reduced to 35dB (one-sample t-tests against 0, $t < 0.4$, $p > 0.71$), suggesting that the hearing threshold for Tone in these groups

of mice was between 45dB and 35dB, i.e., significantly below the cue volumes used in the Pavlovian conditioning experiments described in this thesis (75dB and 88dB).

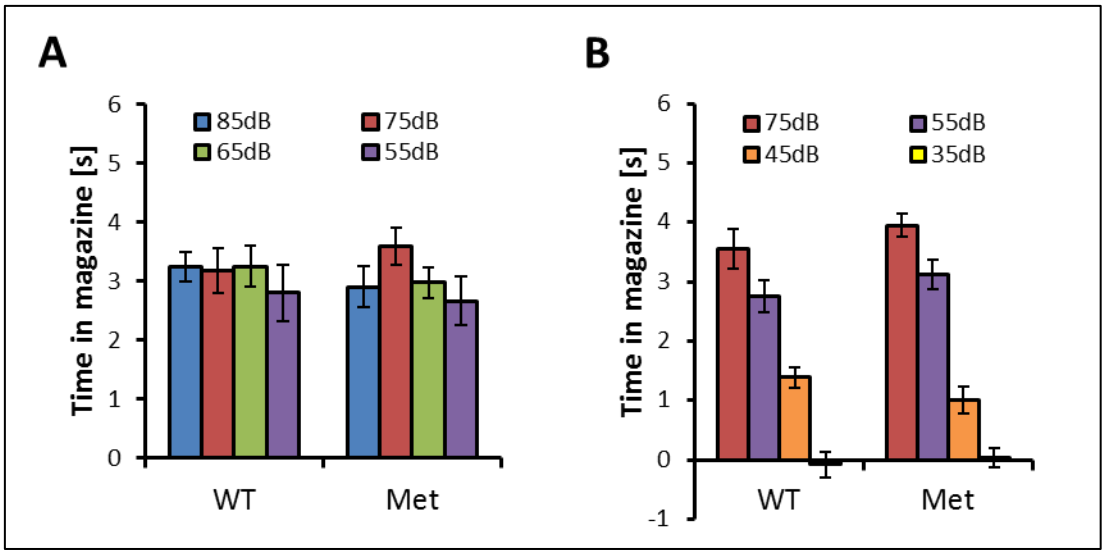


Figure 4-5. Normalised magazine responding during Tone presented at different volumes. *Bars represent averages (± SEM) of the 2 sessions of test phase 1 (A) and test phase 2 (B).*

4.4. Discussion

The present study assessed the effect of COMT on the acquisition of a cue-reward association in a single-cue (WN or Tone) Pavlovian conditioning task. Consistent with the results of the 2-cue discrimination study described in Chapter 3, this experiment revealed a significant effect of cue salience on learning in COMT-Met but not WT mice: in COMT-Met mice, the initial acquisition of the cue-reward association proceeded significantly faster when the cue was the WN, as compared to when it was the Tone. This could not be attributed to a basic impairment in hearing of the 3kHz Tone in COMT-Met mice (although see discussion of caveats below). More importantly, however, conditioned responding to the Tone was

significantly facilitated in COMT-Met mice that had previously experienced WN-reward pairings.

The first question addressed by the present study was whether the bi-directional effect of stimulus salience on learning observed in COMT-Met mice in a discrimination conditioning paradigm (Chapter 3) was dependent on an interaction between CS+ and CS- or whether it was driven by the absolute salience of the CS+ alone. In the case of the former scenario, learning rates between COMT-Met and WT mice should not differ in the absence of a CS-. On the other hand, if learning in COMT-Met mice was solely affected by the absolute, rather than relative, salience of the CS+, then the genotype by stimulus type interaction – faster learning about the WN *and* slower learning about the Tone, compared to WT – would be expected to persist even in the absence of a second stimulus.

In the present study, learning proceeded significantly faster in the WN condition than in the Tone condition in COMT-Met mice, but not WT mice, thus replicating the previous finding of cue salience having a significant effect on cue-reward learning in COMT-Met mice. This effect was primarily driven by the WN condition, where COMT-Met mice acquired conditioned responding significantly faster than WT mice. Whilst this was the most robust finding statistically, inspection of the learning curves presented in Figure 4-2 showed that there was also an initial attenuation in learning in COMT-Met compared to WT mice in the Tone condition. This effect was not statistically robust, presumably due to the relatively small sample size (n = 5 in each genotype group) and high variance in the WT group. It should be noted, however, that the parallels between the results of this study and those of the discrimination conditioning study presented in Chapter 3 are striking

(see Supplementary Figure S4-2). It could therefore be suggested that the observed numerical difference between genotypes in learning rates in the Tone condition might indeed be meaningful, albeit in the present study underpowered. It will be important to replicate these results with a larger sample size in the future.

It should be noted that the caveat described above does not weaken the main result, which clearly demonstrates that stimulus type, and thus salience (see Chapter 3), strongly affects learning in COMT-Met but not WT mice. Crucially, these results extend the findings of Chapter 3 by demonstrating that the cue salience effect persists in the absence of a second stimulus, and suggest that the main factor driving the cue by genotype interaction in the discrimination conditioning study was indeed the *absolute* salience of the CS+, rather than an interaction between CS+ and CS-.

Before focussing on the potential cognitive processes underlying these findings, it is important to address a simpler explanation for the finding, namely that of impaired Tone hearing in COMT-Met mice. Although tyrosine-hydroxylase positive cells have been identified in the auditory brainstem as well as midbrain (Fyk-Kolodziej et al., 2015; Tong, Altschuler, & Holt, 2005), little is known about the role of dopamine in subcortical auditory processing. Most certainly, there has not been any evidence for effects of COMT in the brain on basic hearing processes[1]. Moreover, of importance for the current study is the fact that learning with the Tone was retarded, whilst learning with WN was *enhanced* in COMT-Met mice, relative to WT. A *general* impairment in hearing could therefore not easily explain this bi-directional effect. An alternative explanation would be that hearing of the 3kHz tone

[1] Whilst there is catechol-O-methyltransferase expressed in hair cells of the ear, that specific form of the enzyme is coded by a different gene, which is not targeted in our transgenic model (Du et al., 2008).

is selectively impaired in COMT-Met mice. Indeed, 3kHz lies at the lower end of the hearing range of mice (Heffner & Heffner, 2007). A shift in the hearing range towards higher frequencies could therefore have a greater impact on the perception of the Tone than it would do on WN, which covers a wider range of frequencies (Ison, Allen, & O'Neill, 2007).

While the most direct and accurate way to examine frequency-specific effects of COMT on hearing would be to measure auditory brain stem responses to stimuli of different frequencies and amplitudes (Ison et al., 2007), there are a number of reasons to doubt this as the primary cause of the pattern of data observed here. First, it would be expected that a basic hearing impairment would impact on all Tone-reward learning. However, COMT-Met mice that were trained on Tone associations in Training 2 (i.e., after learning WN associations in Training 1) showed, if anything, *enhanced* responding to the Tone compared to initial sessions. Second, when the volume of the learned Tone stimulus was systematically varied in 10dB steps between 85dB and 35dB, there was a striking overlap in the responses of both the COMT-Met and WT mice. In both genotypes, responding was similarly attenuated with decreasing volumes and both genotypes ceased responding above baseline when stimulus volume was reduced to < 45dB, marking the animals' hearing threshold. Even if there were subtle differences in hearing threshold that this test might not have resolved, note that the volume of the Tone cue used in the present behavioural tasks (75dB) was significantly above the response threshold (35-45dB). Finally, pre-pulse inhibition and startle response tests performed in naïve animals did not reveal any differences between COMT-Met and WT mice (Stumpenhorst and Tunbridge, *unpublished observations*). The rest of the discussion will therefore

proceed under the assumption that any interactive effects between cue type and genotype on cue-reward learning were mediated by COMT effects on higher order processing rather than basic hearing differences at the level of the brainstem.

One way to approach potential neural mechanisms underlying the observed bi-directional effect of absolute stimulus salience on learning in COMT-Met mice is by referring to Durstewitz and Seamans' theory of dopamine-dependent prefrontal activity states (Durstewitz & Seamans, 2008). According to this framework (which is presented in more detail in Chapter 1), a D1-dominated state favours stable representations of task-relevant information in working memory, whilst D2-dominated states lower the energy barrier among activity states and therefore allow easier access of new information and/or noise to PFC networks (O'Reilly, 2002). Due to reduced COMT enzyme activity, and thus increased PFC dopamine levels, Met-allele carriers are thought to have a higher D1/D2 activation ratio than Val homozygotes. As a result, the energy barrier that incoming activity needs to overcome in order to be able to disrupt the current active state is higher in Met-allele carriers than Val homozygotes (Durstewitz & Seamans, 2008).

The implications of this theory for the present study would be that only stimuli above a certain salience threshold would be able to overcome this barrier and gain access to cortical resources, and that this threshold would be higher in COMT-Met than WT mice. However, once stimulus-related neuronal activity surpasses that threshold, cortical representation of that stimulus will be more stable in COMT-Met than WT mice. This in turn would lead to increased allocation of cognitive resources towards the stimulus and might therefore speed up learning about it. A less salient stimulus, however, will be less likely to overcome the energy

barrier. It will therefore be less likely to enter an active state that will enhance learning about it. Increased PFC dopamine levels conferred by the COMT-Met allele therefore enhance the contrast between stimuli with difference in *absolute* salience[2], which might lead to the bi-directional effect of cue type on learning observed in COMT-Met mice in the present study.

On the other hand, due to lower PFC dopamine levels, relative to COMT-Met mice, cortical activity networks in WT mice are closer to the D2-dominated state, thereby lowering the salience threshold that a stimulus must reach in order to enter into an active state. It could be hypothesised, that as a result of a lower energy barrier, both the Tone and the WN would be able to disrupt ongoing states and gain access to cognitive resources in WT mice. As a result, WT mice would be predicted to learn more readily about the Tone than COMT-Met mice. However, because their representations are less stable than in COMT-Met mice, they would be expected to learn more slowly than COMT-Met mice in the case of WN. Taken together, according to the predictions from the dual-state hypothesis of PFC dopamine (Durstewitz & Seamans, 2008), differences in stimulus salience would therefore be expected to impact more strongly on learning in COMT-Met than in WT mice, as was indeed found in the present study.

It should be noted that in the above discussion the term "stimulus salience" was, in the broadest sense, equated to the capacity of a stimulus to elicit excitation in the PFC. On a behavioural level, multiple factors can contribute to the "summed"

[2] The term "absolute" salience is used here to emphasise that the observed cue effects on learning are present in the absence of a second auditory stimulus (and therefore in the absence of relative differences between task-relevant stimuli). However, it should be noted that even the "absolute" salience of the task-relevant stimulus used here is *relative* to other contextual stimuli as well as to current internal states and goals of the animal.

salience of a stimulus, including its unconditioned salience, novelty, ability to predict reward, and relevance to current goals (Anderson, Laurent, & Yantis, 2011; Esber & Haselgrove, 2011; Schultz, 2013). At the start of the present study, the WN and Tone shared all of these properties, except for their unconditioned salience, leading to the hypothesis that interactions of cue type and genotype were driven by differences in that factor. The summed salience of a stimulus can, however, change with an animal's experience with the stimulus or with other learning experiences (e.g. Anderson et al., 2011).

Stage 3 of the present experiment therefore tested whether differences in unconditioned cue salience would exert lasting differential influences on behaviour (i.e., whether learning Tone-reward associations would always be slower in COMT-Met mice) or whether, instead, the initial speed of learning would affect subsequent learning rates with novel stimuli (i.e. whether Tone-reward associations would be acquired more rapidly after WN-reward associations). Interestingly, the second of these hypotheses most accurately describes the pattern of data found in the present study. Specifically, acquisition of Tone in Training 2 was not impaired relative to WN in the COMT-Met mice, suggesting that the influence of unconditioned stimulus salience on learning in COMT-Met mice can be overcome by an animal's previous experience with cue-reward learning. In fact, COMT-Met mice that had acquired the WN-reward association first, showed higher rates of conditioned responding in *both* stages of the experiment, as compared to COMT-Met mice whose first experience with cue-reward association was with the less salient Tone. One caveat of the current experiment is that it was not fully balanced in that it did not include groups that learned with one cue and were retrained with the *same* cue in the later stage.

Nonetheless, it is tempting to speculate that, in COMT-Met mice, unconditioned stimulus salience not only influences the learning of the current association, but also the acquisition of a rudimentary model of the environment (i.e. the notion that auditory cues in general may predict reward), and therefore the speed at which future associations with other cues are acquired.

Conclusions

The present study replicates previous results (Chapter 3), revealing an interactive effect between cue salience and COMT genotype in associative learning. It furthermore extends the findings by demonstrating that learning in COMT-Met mice is significantly affected by the absolute salience of the rewarded cue, rather than the relative salience of a rewarded and an unrewarded cue. This result might be explained in the framework of the dual-state theory of PFC dopamine (Durstewitz & Seamans, 2008), according to which COMT-Met mice are predicted to learn significantly faster than WT mice with cues above a certain salience threshold but worse with cues below this threshold. This study further provides some data for the speculation that previous learning experience changes future learning in a manner that is dependent on the interactive effect between genotype and cue salience.

Chapter 5: Effects of COMT on NAc dopamine transmission evoked by unconditioned reward

5.1. Introduction

The previous chapters focussed on establishing the effects of COMT genotype on cue-reward learning. A striking, replicable influence of cue salience on associative learning has been demonstrated in COMT-Met mice. These results were considered from the perspective of what is known about cortical dopamine and networks involved in attributing salience to environmental cues. On the other hand, a related possibility is that there are also effects mediated by an influence of COMT genotype on subcortical dopamine transmission, which is known to influence acquisition and performance of Pavlovian conditioning. However, little is currently known about whether and how COMT affects phasic dopamine release in the NAc core. In the present study I therefore used fast-scan cyclic voltammetry (FSCV) to provide a *direct* measure of phasic dopamine release in the core of the NAc in COMT-Met and WT mice, evoked by unsignalled delivery of reward, which reliably causes phasic increases in the activity of midbrain dopamine neurons (Fiorillo et al., 2003a; Schultz et al., 1997) and dopamine release in the NAc (Day et al., 2007; Parker et al., 2010).

COMT and striatal dopamine transmission

Little is currently understood about the influence of COMT on NAc dopamine release, or how any effects might be mediated. The fact that COMT is expressed in

the striatum (Kastner et al., 1994; Matsumoto, Weickert, Akil, et al., 2003; Matsumoto, Weickert, Beltaifa, et al., 2003) allows, at least in theory, for the possibility of local modulation of dopamine signalling in the NAc. However, its *direct* influence is likely to be limited given that uptake rather than transmitter metabolism has been shown to be the main regulator of extracellular levels of dopamine here (Budygin et al., 1999; Cass, Zahniser, Flach, & Gerhardt, 1993). Nonetheless, given the reported inverse relationship between cortical and striatal dopamine transmission, COMT might also *indirectly* modulate striatal function down-stream of its effects in the PFC (Carr & Sesack, 2000; Jackson et al., 2001; Kolachana et al., 1995). Bilder and colleagues have extended these ideas to suggest that the effects of COMT on subcortical dopamine signalling might be better understood in a framework that differentiates between tonic ('background') and phasic ('burst') dopamine release, and their interaction, in limbic regions (Bilder et al., 2004; Grace, 1991). Within this framework, it is proposed that the higher cortical dopamine levels conferred by the COMT-Met allele should lead to greater tonic release of dopamine in the striatum mediated by corticostriatal glutamatergic afferents. In addition, tonic levels might be further increased due to the decreased capacity to metabolise tonic dopamine by COMT-Met directly in the striatum. Overall, this is proposed to result in *suppression* of phasic dopamine release subcortically in COMT-Met allele carriers.

While this idea has been influential, there has been little research to test the tonic-phasic hypothesis of COMT to date. The tonic dopamine component of the hypothesis has been partially addressed by a number of microdialysis studies. However, these failed to show any effect of either pharmacological inhibition or

genetic ablation of COMT on extracellular dopamine levels in the striatum (Acquas, Carboni, de Ree, Da Prada, & Di Chiara, 1992; Huotari et al., 2002; Käenmäki et al., 2010; Napolitano, Zürcher, & Da Prada, 1995). A number of studies have used FSCV to measure the effect of COMT on phasic release of dopamine in the striatum evoked by the stimulation of the medial forebrain bundle in anaesthetised rodents. These experiments have shown mixed results. For instance, a recent study by Simpson and colleagues showed increased stimulated phasic dopamine release in dorsomedial striatum in a transgenic mouse model of COMT hyperactivity (Simpson et al., 2014). In contrast, however, Yavich and colleagues failed to show any effect of genetically-ablated COMT activity in mice on electrically-evoked phasic dopamine release in the caudate nucleus using FSCV with slightly modified parameters (Yavich et al., 2007). Similarly, acute pharmacological inhibition of COMT did not have any effects on stimulated dopamine release in the caudate-putamen of anaesthetised rats (Budygin et al., 1999).

There could be at least two reasons why these studies might not be able to reveal the full extent of the effects of COMT on striatal dopamine signalling. First, although COMT does not seem to impact dopamine levels in the striatum *under basal conditions* (i.e. in anaesthetised or behaviourally inactive animals), its effects might become evident under conditions of elevated transmission, such as after a pharmacological challenge of the dopamine system or during behavioural tasks that rely on dopamine release. Indeed, several microdialysis studies have shown potentiating effects of the COMT inhibitor tolcapone when striatal dopamine levels were elevated pharmacologically (e.g. Budygin et al., 1999; Kaakkola & Wurtman, 1993). More importantly, Lapish and colleagues demonstrated an effect of tolcapone

on PFC dopamine, as measured by microdialysis, when dopamine levels were increased by the anticipation of food rewards in the context of a behavioural task. No such effects were observed when the animals were not involved in any behavioural task, suggesting that task-relevant increases in dopamine transmission might unmask roles for COMT not detected at baseline (Lapish et al., 2009).

Second, it should be noted that all studies investigating the effects of COMT on phasic dopamine to date have measured dopamine following electrical stimulation, which leaves the question of the role of COMT on phasic dopamine release in the striatum under more naturalistic conditions, such as during the performance of reward-related tasks.

Indeed, as described in more detail in Chapter 1, recent functional neuroimaging studies have demonstrated an effect of the Val158Met polymorphism on ventral striatal activity during reward learning, as measured by the blood-oxygen-level dependent (BOLD) signal (reviewed in Tunbridge et al., 2012). In these studies, the Met allele has been repeatedly linked to increased striatal activity during the anticipation of rewards and to reduced differential coding of positive and negative reward outcomes (Camara et al., 2010; Krugel et al., 2009; Marco-Pallarés et al., 2009; Schmack et al., 2008; Yacubian et al., 2007). Whilst it cannot be ruled out that the observed differences in BOLD signal were driven by changes not dependent on dopamine transmission, it should be noted that the direction of the BOLD effect in these studies (Met-allele leads to *increased* ventral striatal activity) was opposite to that predicted by the tonic-phasic hypothesis (Met-allele leads to *reduced* striatal phasic dopamine). This apparent dichotomy has not been addressed in the literature to date. Moreover, these studies highlight a role for COMT in ventral

but not dorsal striatum, whilst animal studies investigating dopamine release have almost entirely focused on dorsal striatum (Käenmäki et al., 2010; Simpson et al., 2014; Yavich et al., 2007).

To increase our understanding of the influence of COMT on dopamine release in awake, behaving animals, I therefore monitored phasic dopamine release in the NAc core of COMT-Met and WT control mice during unsignalled delivery of reward. Rewards presented in this manner are known to cause increases in firing rates of midbrain dopamine neurons and dopamine release in NAc core (Day et al., 2007; Schultz et al., 1997). According to the hypothesis proposed by Bilder and colleagues, a *reduction* of reward-evoked phasic dopamine release in COMT-Met mice was expected when compared to WT mice (Bilder et al., 2004).

Second, given the evidence from functional neuroimaging studies linking the COMT Val158Met polymorphism with striatal activity during reward-guided learning and behavioural adaptation (Tunbridge et al., 2012), there was a special interest in investigating the effect of COMT genotype on NAc dopamine evoked by *different magnitudes* of reward. In particular, I wanted to test whether COMT genotype would affect the scaling of dopamine reward prediction error signalling to rewards of different sizes (Tobler et al., 2005). Therefore, mice were first trained with a "standard" reward before being switched to a schedule where standard reward trials were intermixed with reduced and increased reward magnitude trials.

5.2. Methods

Animals and surgery

Detailed descriptions of the surgical procedures can be found in Chapter 2. In short, 25 male mice (COMT-Met n = 10; WT n = 15), 16-19 weeks of age, were anaesthetised with isoflurane and chronically implanted with carbon fibre microelectrodes targeted either unilaterally (left hemisphere, n = 15) or bilaterally (n = 11) to the NAc core (coordinates from Bregma: AP 1.1 mm, ML 1.0 mm, DV 4.0 mm from skull surface), along with a unilateral Ag/AgCl reference electrode placed above posterior cortex in the right hemisphere (coordinates from Bregma: AP -3.0 mm, ML: 1.5 mm). Twenty-one mice (COMT-Met n = 7; WT n = 14) successfully recovered to allow recording to be attempted. After a two-week recovery period, mice were food restricted and maintained at 85-90% of their post-surgical *ad libitum* weight throughout testing.

Experimental design

Habituation and magazine training. All behavioural and recording sessions were carried out in operant chambers (Med Associates, VT), modified for FSCV-recordings as described in Chapter 2. Mice were habituated to the experimental setup (see Chapter 2) and then underwent magazine training sessions during which they obtained reward (40μL of 20% sucrose solution, delivered in two successive boli of 20μL separated by 20ms) on a variable interval (VI) schedule. Rewards became available with a mean VI of 60s (VI60, range: 35-83s) in session 1 and 120s (VI120, range 95-143s) on session 2. The delivery of reward was contingent upon the first head entry the animal made into the food magazine after the VI had elapsed. The session ended after mice had received 24 rewards or 60min had elapsed. If a

mouse failed to collect at least 20 rewards in session 2, they were given an additional magazine training session before voltammetric recording.

Variable reward recording session. On the next session, dopamine release was monitored using FSCV (for details of the procedures followed during FSCV recording sessions, see Chapter 2) as animals again obtained rewards on a VI120 schedule (due to experimental error, one COMT-Met mouse received rewards on a VI with a mean of 110s). However, in this session, standard trials (40μL) were intermixed with trials where reward was reduced (20μL) or increased (80μL). Reward delivery was again contingent upon head entry after the elapsed VI and was delivered in discrete boli of 20μL, separated by 20ms, with each bolus accompanied by an auditory click. Total delivery times for reduced, standard, and increased rewards were 0.11s, 0.25s, and 0.50s respectively. The session always commenced with a standard reward size trial, but subsequently each reward size was presented 8 times according to a pseudorandom Latin Square design. Mice were given a maximum of 80min to complete the session.

Data analysis

Behavioural data. A total of 19 mice (COMT-Met n = 7; WT n = 12) successfully completed all trials of the variable reward session. The number of head entries into the food magazine and the duration of these magazine entries were recorded throughout the session. Due to technical problems, head entry duration measures could not be calculated for one WT mouse and this mouse was excluded from any analysis relating to duration.

Genotype groups were compared on basic metrics of task performance (session length, total number of head entries, and head entry duration) using

independent samples t-tests. As a basic measure of exploration, locomotion and motivation to obtain rewards, response latencies were computed, defined by the time between the end of the VI and the first head entry into the magazine (i.e., the head entry triggering reward delivery). In order to test whether motivation to obtain rewards changed across the session, for instance as a result of increasing satiation, I also computed latency averages across three consecutive 8-trial blocks and analysed these using repeated measures ANOVA with genotype as the between-subjects factor and block as the within-subject factor (3 levels: blocks 1-3). Given that dopamine prediction error signalling scales both with the size of a reward and its temporal predictability (Clark, Collins, Sanford, & Phillips, 2013; Fiorillo, Tobler, & Schultz, 2003b), it was also important to verify that response latencies did not differ between trials of different reward sizes. Latencies were therefore averaged within each trial type and analysed using repeated measures ANOVA with genotype as the between-subjects factor and trial type as a within-subjects factor (3 levels: standard, reduced, increased sizes). Finally, to determine how reward delivery motivated subsequent behaviour, changes in head entry behaviour in the course of a trial were assessed across fourteen 7s time bins (one prior to, and thirteen following reward delivery). A 7s window for magazine responses was chosen to match the time window used for analysis of the dopamine data (see below). The number of time bins was chosen such that it covered the entire duration of the minimum VI used in the session (i.e., 91s). Genotype effects and interactions with time and/or reward size were tested using repeated measures ANOVAs with genotype as the between-subjects factor and reward size and time bin as within-subjects factors (3 and 14 levels respectively). Significant interactions ($p < 0.05$) were followed up with pairwise comparisons with Bonferroni corrections.

FSCV data. Of the 19 animals that successfully completed the variable reward session, only 10 passed inclusion criteria for FSCV data analysis (see Chapter 2). Final FSCV data analysis was conducted on data from a total of 8 electrodes from 6 WT mice and 6 electrodes from 4 COMT-Met mice (COMT-Met n = 6; WT n = 8 individual electrodes). A detailed description of the processing of FSCV data can be found in Chapter 2. In summary, changes in dopamine levels relative to a 0.5s baseline before each reward delivery were extracted using principal component analysis. Data were smoothed using a 0.5s moving average window. Any trials where the principal component regression model was violated on >30% of time points in the 10s following reward delivery, were excluded from analysis. In addition, the first trial of the session was excluded from analysis because its temporal unpredictability was maximal and dopamine release was therefore disproportionally large compared to the following trials of the session. Moreover, it might have been perceived by the animal as a cue for the start of the session and therefore carried additional information which would be conflated with a purely reward-evoked response.

Trial-by-trial peak dopamine (magnitude and time post reward delivery), as well as the area under the curve (AUC) in a time window of 7s post reward delivery, were calculated for each electrode. A 7s time window for the AUC was chosen based on the average time it took the dopamine signal evoked by the smallest reward to return to pre-reward baseline levels. In a preliminary analysis of the effect of COMT on the decay of the dopamine signal, I also calculated the time it took for the dopamine signal to decay to 50% of peak height.

For statistical analysis, these measures were log-transformed to correct for skewing in the distribution of the data caused by very large signals recorded on a small number of electrodes and analysed using repeated measures ANOVAs with genotype as the between-subjects factor and reward size as the within-subject factor (3 levels: reduced, standard, increased).

In order specifically to examine dopamine release when receiving a smaller than average reward (i.e., negative prediction error) or a larger than average reward (i.e., positive prediction error), we computed within-subject measures of signal change on the reduced or increased reward trials relative to standard size trials ('% change'). Separate one-sample t-tests were performed to test whether % changes were significantly different from 0 and genotype effects were tested using an independent samples t-test.

5.3. Results

5.3.1. Characterisation of magazine head entry behaviour

In order to obtain greater statistical power, all animals that successfully completed the variable reward schedule (COMT-Met n = 7, WT n = 12) were included into the analysis of behavioural performance on the variable reward session, irrespective of whether or not they contributed to the dopamine data presented below (for corresponding figures showing data selectively of mice that contributed dopamine, see Supplementary Figure S5-2). The mice completed the session within an average time of 57.3min (± 8.7min SD), and made a response with an average latency of 2.8s (± 3.0s SD; median latency: 1.0s) after the variable interval between trials had elapsed. There were no genotype differences in either the length of the session, response latency, or the number of total magazine head entries made during

the session (all $t_{17} < 0.7$, $p > 0.48$, Figure 5-1A). Importantly, a more detailed analysis showed that response latencies were similar on all trial types (no main effect of trial type: $F_{2,34} = 2.3$, $p = 0.11$, Figure 5-1B), although overall, there was a trend towards an increase in latencies towards the end of the session (main effect of block: $F_{1.5,25.6} = 3.2$, $p = 0.07$, Figure 5-1C). There were no effects of, or interactions with, genotype in either of these measures (all $F < 2.5$, $p > 0.10$).

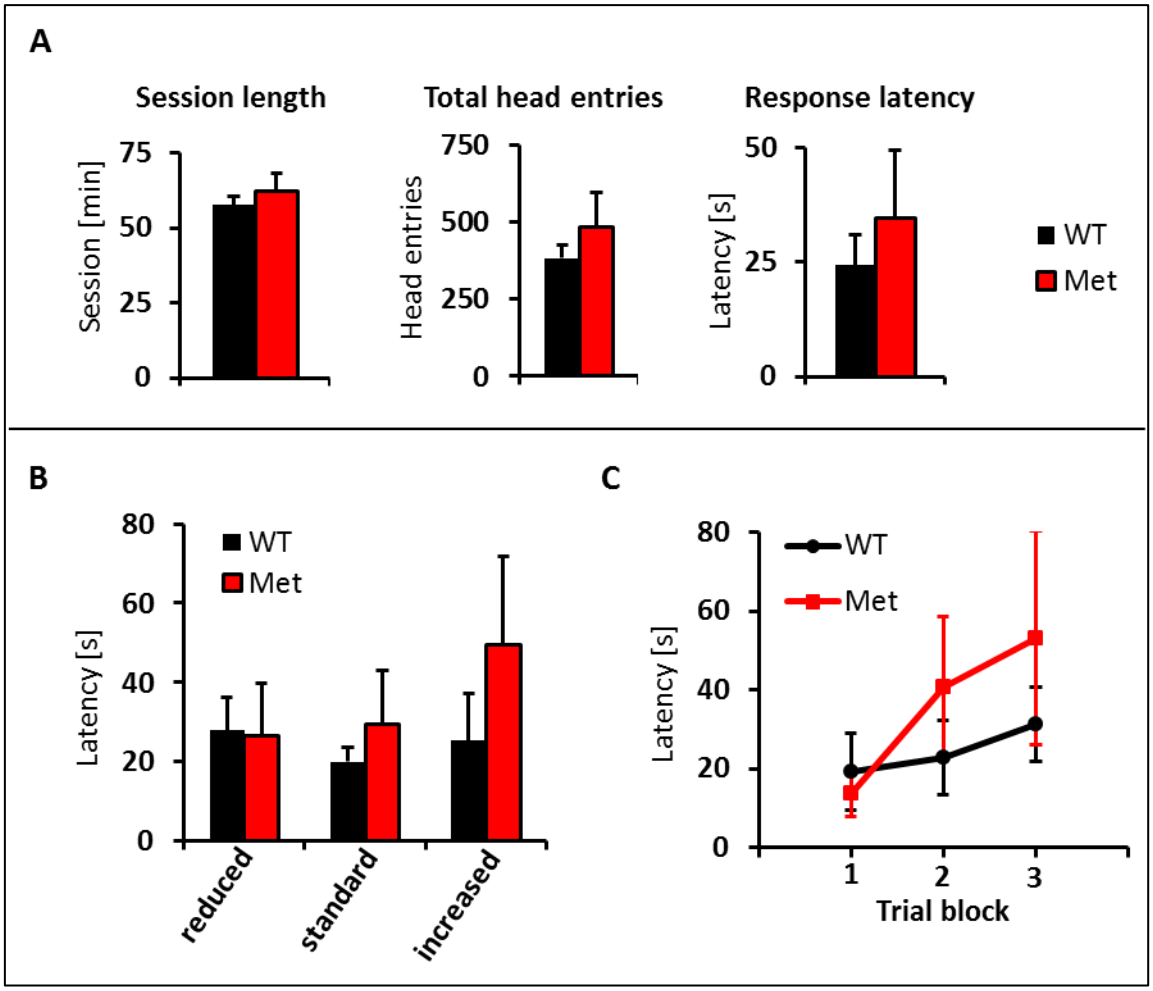


Figure 5-1. Summary of head entry behaviour in the variable reward session. A. *Bar graphs show session length (left), total head entries into the magazine (middle) and latency to respond after the VI had elapsed (right).* ***B.*** *Latency to respond after the VI had elapsed, split by reduced, standard, and increased reward size trials.* ***C.*** *Latency to respond after VI had elapsed across the three 8-trial blocks of the session. Data are group means ±SEM.*

Having investigated the behaviour just *prior* to reward delivery (i.e., head entries into the magazine after passage of the VI), I next asked whether behaviour immediately *following* reward delivery was affected by the size of the reward received, and whether this was modulated by genotype. One possibility would be, for instance, that a bigger reward had a more arousing/motivating effect on the animal, causing increased magazine responding after receipt of reward and/or during the VI following it. I was therefore also interested to find out how far into the following VI the impact of reward size would stretch. To address these questions, I analysed magazine entry numbers and duration in thirteen 7s time bins following reward delivery, and one 7s time bin preceding it (Figures 5-2 and 5-3).

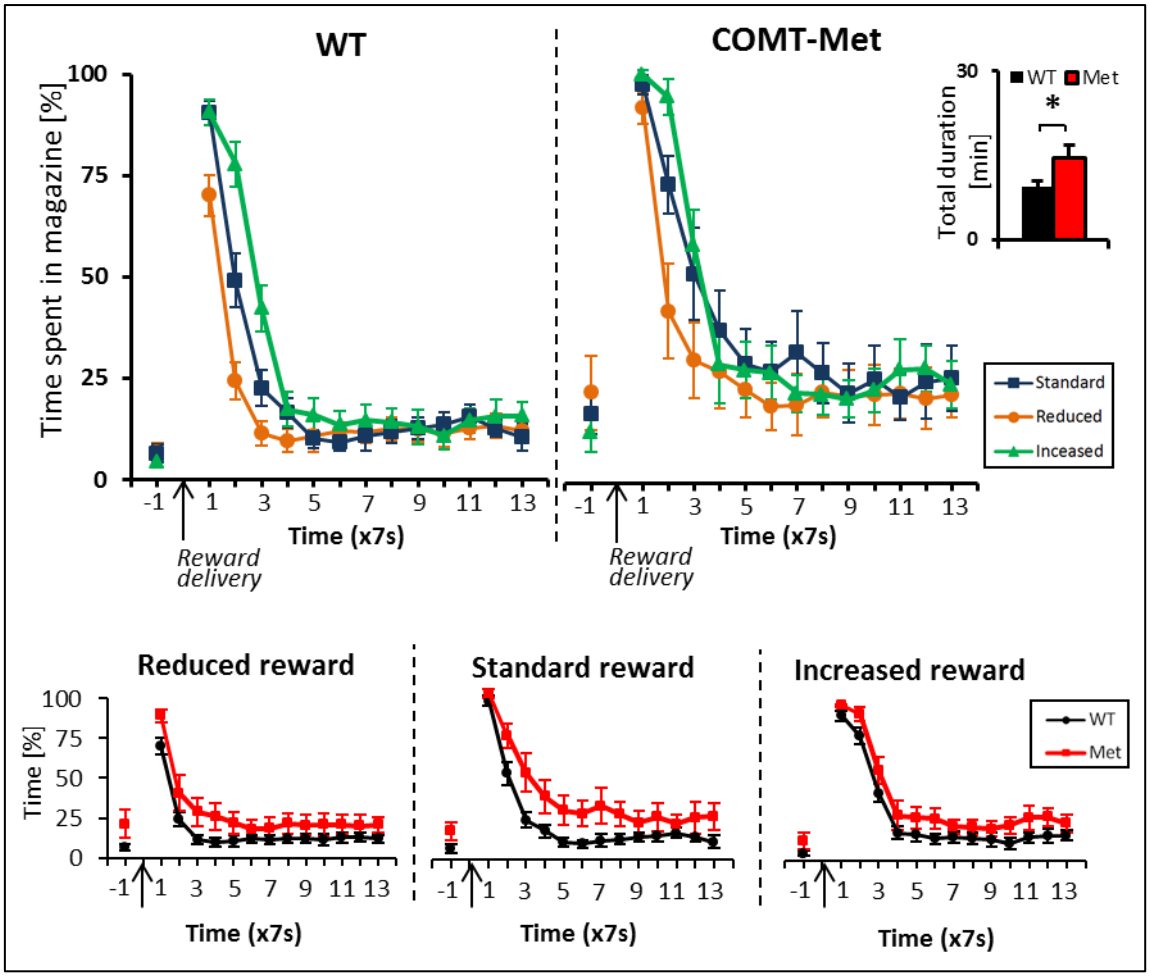


Figure 5-2. Time spent in the food magazine: within-trial dynamics and effect of reward size. *Top panel: Overview of time spent in the food magazine (as % of time bin) across thirteen 7s-time bins after delivery of standard, reduced or increased reward, as well as in the 7s preceding reward delivery in COMT-Met (right) and WT mice (left). Inset shows the total time mice spent in the magazine in the session (group mean +SEM, *, independent sample t-test $p < 0.05$). Bottom panel shows direct comparison of the above data between WT and COMT-Met mice. Arrows indicate time of reward delivery. Data are group means ±SEM*

Reward size significantly affected the amount of time mice spent in the food magazine overall (main effect of reward size: $F_{2,32} = 21.6$, $p < 0.001$), with magazine time after reduced reward delivery being significantly lower compared to standard or increased reward trials ($p < 0.01$). In addition, there was a significant reward size × time bin interaction ($F_{26,416} = 15.9$, $p < 0.01$). Importantly, overall, COMT-Met mice spent more time in the magazine than WT mice, both when analysing the summed duration of the session ($t_{16} = 2.2$, $p = 0.04$) and when analysing duration across time

bins (main effect of genotype: $F_{1,16}$ = 5.1, p = 0.038; but no genotype × time bin interaction: $F_{2.8,44.8}$ = 1.0, p = 0.41).

However, whilst COMT-Met mice spent more time in the magazine overall, genotype did not significantly affect magazine *checking* behaviour (i.e., number of magazine head entries; no significant main effect of genotype: $F_{1,17}$ = 1.0, p = 0.33; Figure 5-3). There was only a significant main effect of time bin ($F_{4.2,71}$ = 10.8, p < 0.01), caused by variations in checking behaviour during reward consumption and then temporal expectation of the next reward delivery (Figure 5-3), but no interaction with genotype.

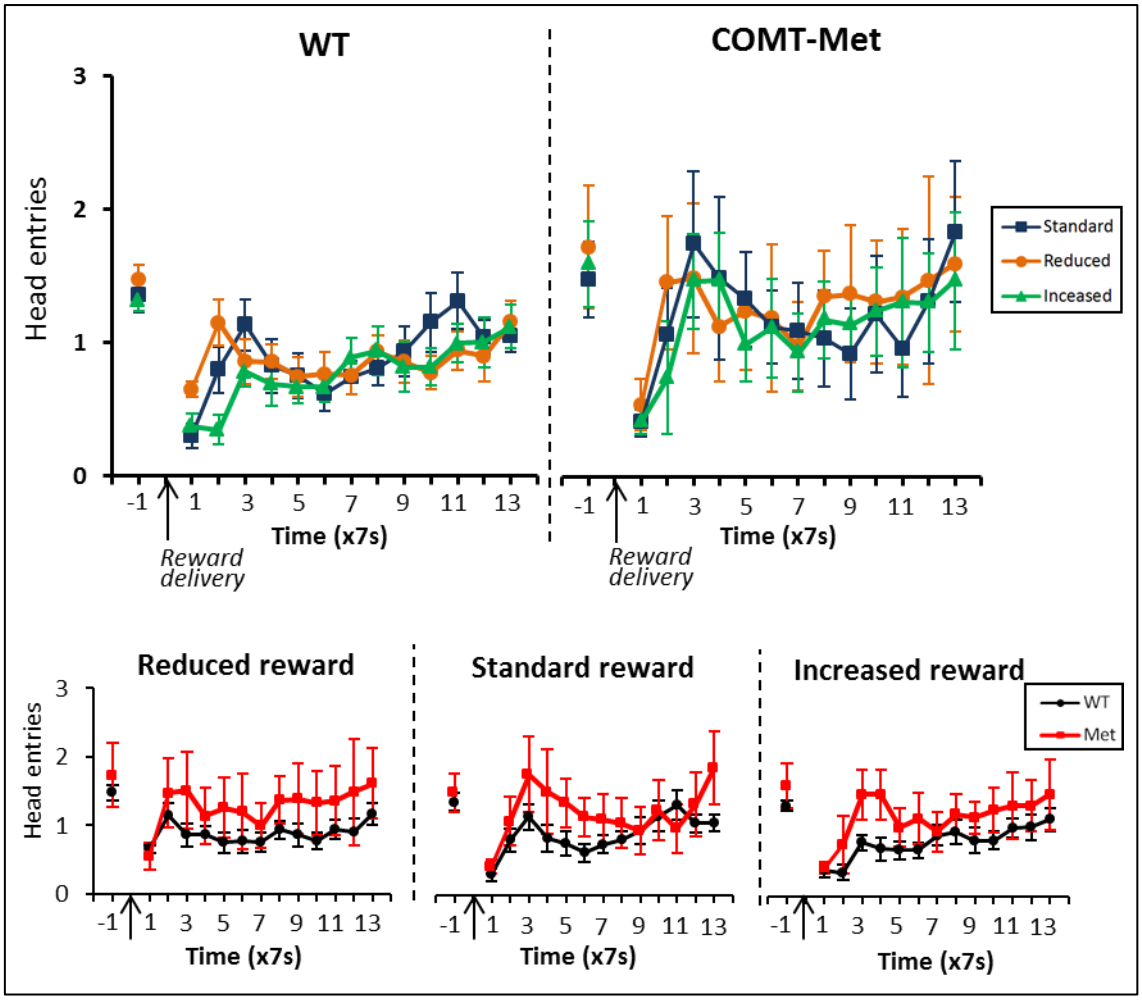


Figure 5-3. Magazine checking behaviour: dynamics across a trial and effect of reward size. *Top panel: Overview of magazine checking behaviour across 7s time bins after delivery of standard, reduced or increased reward, in COMT-Met mice (right) and WT controls (left). Bottom panel shows direct comparison of the above data between WT and COMT-Met mice. Arrows indicate time of reward delivery. Data are group means ±SEM*

5.3.2. Dopamine data

Effect of COMT genotype on phasic dopamine transmission on standard reward trials

Dopamine release recorded in response to unsignalled reward delivery was analysed (WT n=6 mice / 8 electrodes, 5 in left NAcc; Met-COMT n=6 electrodes, 4 in left NAc core; for histology showing electrode placements see Supplementary Figure S5-1). To assess basic effects of COMT on dopamine levels, I first focused my analysis on standard reward size trials.

As can be observed in Figure 5-4A, there was a rapid rise in dopamine within 200ms of reward delivery on all trials in both genotypes, peaking at on average 3.2s (±0.9s SD) after reward delivery. COMT genotype did not significantly affect dopamine release as measured by dopamine peak height and time to peak (both $t_{12} < 0.5$, $p > 0.59$; Figure 5-4B). However, there was a trend towards an effect of genotype when the area under the curve (AUC) was considered, with COMT-Met mice having greater AUC than WT mice ($t_{12} = 2.0$, $p = 0.07$). I also conducted a preliminary analysis of dopamine decay times which suggested more sustained dopamine levels post-reward in COMT-Met mice (Supplementary Figure S5-3), although additional formal modelling of the kinetics of dopamine elimination from the extracellular space will be required to confirm this finding (Wightman et al., 1988).

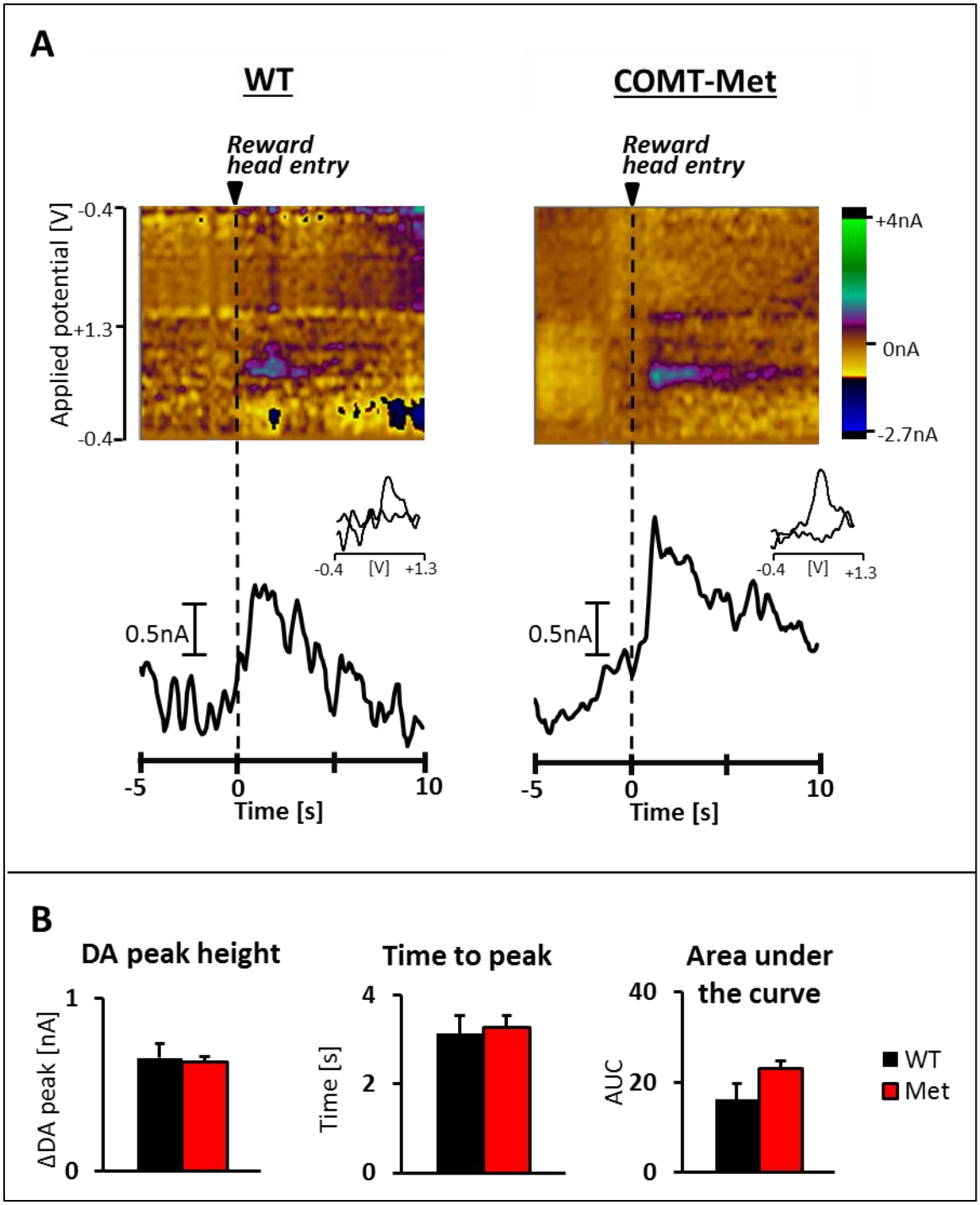


Figure 5-4. Overview of dopamine release on standard reward size trials. A. *Representative examples of FSCV recordings on a standard reward size trial in a WT (left) and a COMT-Met mouse (right). Pseudocolour plots (top panel) show changes in redox current as a function of applied potential (y-axis) plotted over time (x-axis). Current-by-time plots (bottom panel) depict changes in dopamine current. Insets depict cyclic voltammograms taken after reward delivery.* ***B.*** *Dopamine peak height, latency to peak, and area under the curve in COMT-Met mice and WT controls averaged across all standard reward size trials. Data in B are group means +SEM.*

Effect of COMT genotype on dopamine response to rewards of varying sizes

As expected, dopamine release scaled with reward size in both genotype groups (Figure 5-5A). Repeated measures ANOVAs performed on dopamine peak height and time to peak following reward delivery revealed a significant main effect of reward size, with significantly lower dopamine peak heights measured on reduced compared to standard and increased reward size trials (both $F_{2,24} > 10.3$, $p \leq 0.01$, Figure 5-5B), although there was no reliable difference in peak height between standard and increased reward trials. As with the analysis of standard reward size trials only, genotype did not significantly affect dopamine peak height or time when all trial types were included (no significant main effects of genotype: $F_{1,12} < 0.7$, $p > 0.6$; no genotype × reward size interaction: $F_{2,24} < 1.9$, $p > 0.1$).

To obtain a more comprehensive measure integrating both peak height and the temporal persistence of the dopamine signal, the AUC over the 7s following reward delivery was calculated. Again, there was a strong effect of reward size ($F_{1.2, 14.2} = 25.3$, $p < 0.01$). However, here the difference in AUC was significant between each of the three reward sizes (Figure 5-5B). More importantly, COMT-Met mice displayed increased levels of dopamine compared to WT mice as measured by AUC (main effect of genotype: $F_{1,12} = 5.7$, $p = 0.04$). Moreover, there was a genotype × reward size interaction ($F_{1.2,14.2} = 4.8$, $p = 0.04$), caused by a significant difference between the groups only on *reduced* reward trials, where the mice received a smaller-than-average reward size (post-hoc pairwise comparisons: $p < 0.05$).

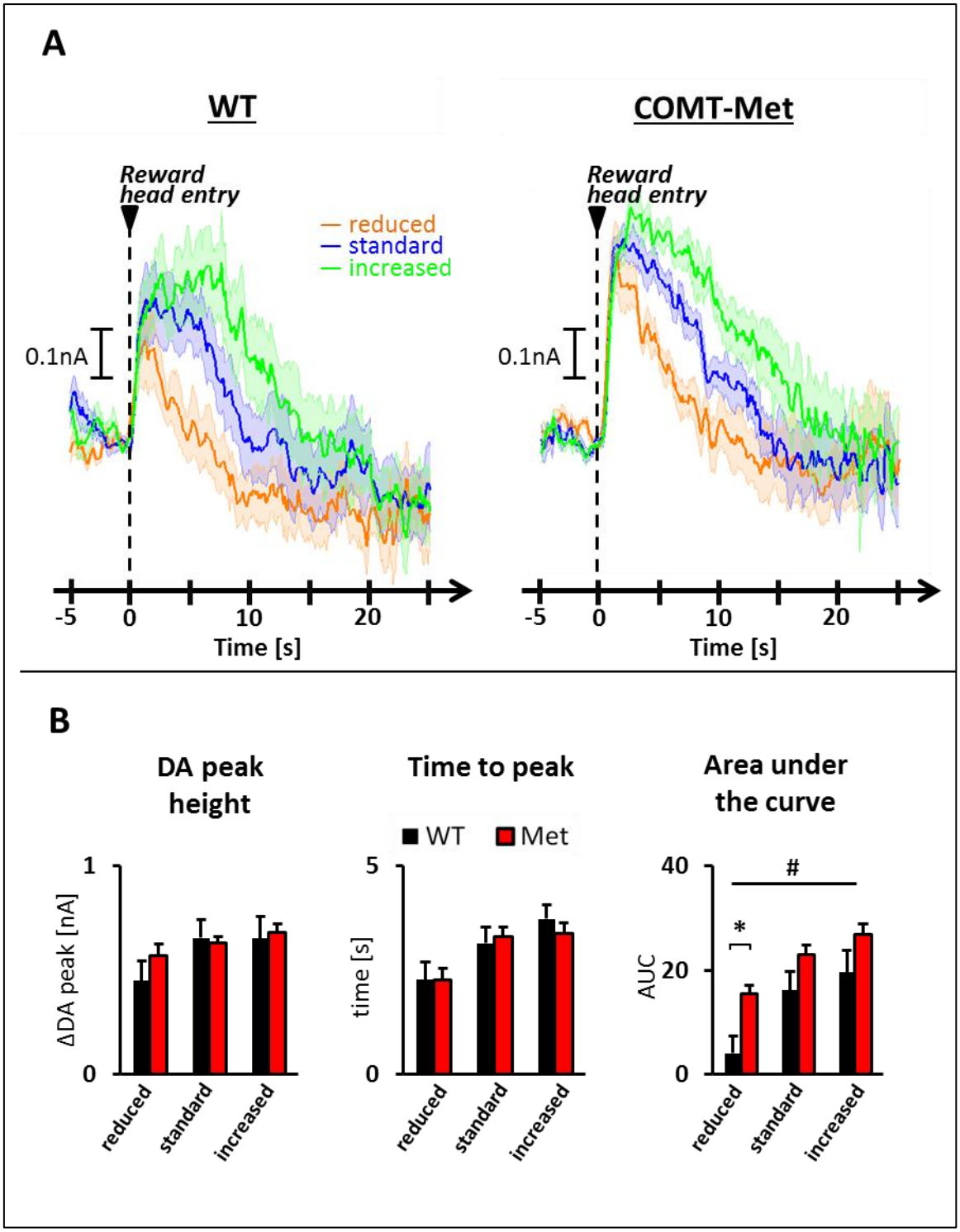


Figure 5-5. Dopamine release on trials of different reward sizes. A. *Traces depict mean dopamine signal (y-axis) over time (x-axis) in WT (left) and COMT-Met (right) mice, evoked by standard, as well as reduced and increased reward sizes. Data was averaged across all trials within each trial type. Data are group means ±SEM.* ***B.*** *Dopamine peak current height (left), time to peak (middle) and total release (area under a 7s window after reward deliver; right). There was significantly more dopamine release in COMT-Met mice compared to WT mice overall (#, main effect of genotype: $p < 0.05$ on RM ANOVA; *, significant pairwise comparison, $p<0.05$).*

Given that the genotype differences observed in absolute measures of reward-evoked dopamine release were most apparent on reduced reward trials (Figure 5-6A), we hypothesised that COMT might differentially affect how dopamine responds to outcomes that are smaller or larger *than expected* (i.e., deviating from the standard or average reward size). In order to determine this effect independently of any change in overall reward-related dopamine release, I therefore assessed dopamine release recorded on each electrode on reduced or increased reward trials *relative to* dopamine recorded on standard reward trials (Figure 5-6B).

While there was a significant decrease in dopamine signal in both genotypes following the reduced relative to the standard reward size (one-sample t-tests against 0: both $t > 3.9$, $p < 0.01$), this decrease was significantly greater in WT compared to COMT-Met mice ($t_{12} = 2.2$, $p < 0.05$). By contrast, there was no significant difference between genotypes when comparing normalised responses to the increased reward size ($t_{12} = 0.7$, $p = 0.78$).

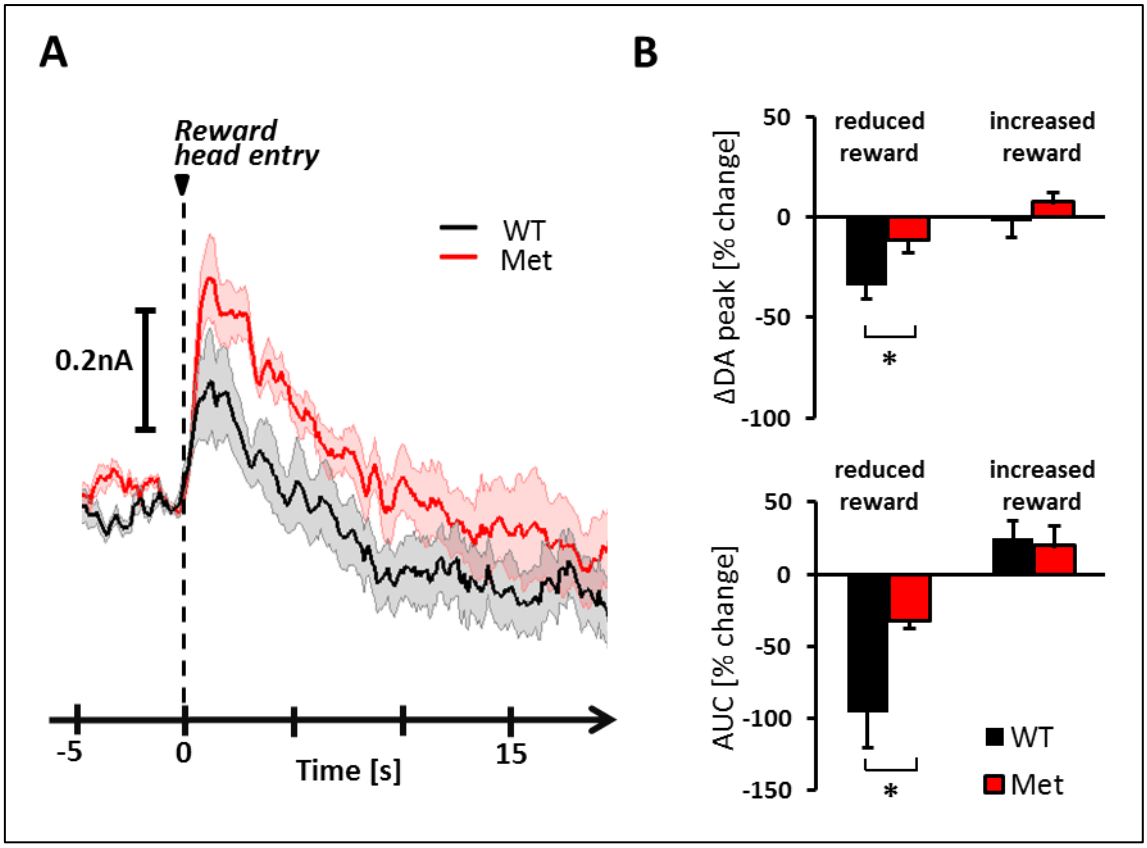


Figure 5-6. Effect of COMT on dopamine release to relative changes in reward size. A. *Traces depict mean dopamine signal (y-axis) over time (x-axis) in WT and COMT-Met mice on trials with reduced reward size, where the biggest genotype effect was observed. Shaded error bars are ±SEM.* ***B.*** *Dopamine peak current height (top) and overall release, as measured by the area under the curve in a 7s window following reward delivery (bottom), expressed as % change from standard reward size trials (*, significant genotype difference, $p < 0.05$). Data are group means ±SEM.*

5.4. Discussion

This study provides the first experimental investigation into the effect of a genetic modulation of COMT on phasic dopamine signalling in behaving mice. By directly measuring phasic dopamine release in the NAc core as mice obtained rewards, it was possible to (a) test the hypothesis that reduced COMT activity conferred by the Met-allele attenuates phasic dopamine signalling in the striatum, and (b) test the effect of COMT-Met on the responsiveness to rewards of varying size.

In this study, COMT genotype did not significantly affect the time or height of dopamine peaks evoked by unpredicted reward delivery, but a preliminary analysis suggests an effect of COMT on dopamine elimination from the extracellular space, with COMT-Met mice showing slower decay. In line with this, COMT effects reached significance when both the peak and the temporal persistence of the dopamine signal were taken into account by using the AUC measure, with COMT-Met mice showing greater AUC than WT controls. Intriguingly, the most consistent effects were found on trials with smaller than expected reward, where WT mice showed greater reduction in dopamine signal than COMT-Met mice.

5.4.1. COMT-Met effects on phasic dopamine release in the context of existing literature from COMT transgenic mice

The prominent "tonic-phasic hypothesis" of COMT postulates that reduced COMT activity conferred by the Met-allele leads to increased tonic levels of dopamine in the striatum, which in turn suppresses phasic dopamine signalling (Bilder et al., 2004). The results from the current study, however, suggest *increased*

phasic dopamine in the NAc core to rewards in COMT-Met mice. While this contradicts the predictions from the tonic-phasic hypothesis (Bilder et al., 2004), it should be noted that, to date, there has been little experimental evidence in direct support of the proposal by Bilder and colleagues: both microdialysis studies measuring tonic dopamine levels and some FSCV studies of electrically evoked phasic dopamine release have found little effect of ubiquitous COMT knockout on dorsal striatal dopamine (Käenmäki et al., 2010; Yavich et al., 2007).

The primary piece of direct experimental evidence in support of the phasic dopamine side of this hypothesis was provided by a study by Simpson and colleagues, who showed increased dopamine release in the dorsomedial striatum upon electrical stimulation of the medial forebrain bundle in a mouse model of increased forebrain COMT activity (Simpson et al., 2014). Our mouse model of *reduced* COMT activity also exhibited increased phasic dopamine release (albeit in the NAc core), in contrast to what might have been expected based on the results of Simpson and colleagues. However, there are a number of potential ways to reconcile these differences, addressed in turn below.

First, Simpson and colleagues used a mouse model where COMT transgene expression was limited to neurons and primarily targeted to the forebrain, whilst the genetic modification used in our model was ubiquitous, to model the human Val158Met polymorphism. This difference might constitute an important factor in comparing between our experiments, as in addition to being expressed in the forebrain, COMT is expressed fairly ubiquitously, including in the cell bodies of identified dopamine neurons of the midbrain (Kastner et al., 1994; Matsumoto, Weickert, Akil, et al., 2003). Thus, it is plausible that COMT might affect dopamine

signalling by modulating the availability of intracellular dopamine for release by dopamine neurons.

Second, Simpson – as well as Yavich and colleagues (Yavich et al., 2007) – recorded dopamine in the dorsal striatum, while the current study targeted the NAc core. Previous FSCV recording studies in brain slices as well as in freely-moving animals have shown that the kinetics of dopamine transmission differ between subregions of the striatum for a number of reasons, including intrinsic differences in fibres innervating the striatum and the expression of transporters and dopamine receptors at the terminal sites (Garris & Wightman, 1995; Zhang et al., 2009). Importantly in the context of COMT, analysis of *post mortem* human brains has shown that COMT expression in midbrain neurons is restricted to dopaminergic neurons of the VTA, with no expression in the SNc, emphasising the importance of COMT in neurons projecting to the ventral striatum (Kastner et al., 1994).

Finally, and most importantly, it should be noted that the dynamics of dopamine regulation at the nerve terminal observed in stimulation studies in anaesthetised animals are likely to be altered compared to the dynamics of dopamine release evoked by natural stimuli in awake behaving animals. Electrical stimulation of the MFB would be expected to stimulate *all* dopamine fibres, irrespective of their projection targets, thereby affecting dopamine release in multiple brain regions, which might, in turn, have downstream effects on dopamine release recorded in the region of interest. Moreover, in addition to mimicking burst firing activity, it would also increase population activity due to synchronous stimulation across the entire population of axons, potentially offsetting the balance between tonic and phasic dopamine levels present at a more natural physiological

state. Perhaps unsurprisingly, the choice of stimulation frequency affects dopamine kinetics at the terminal, and therefore also the contribution of COMT to the dopamine signal observed: This was clearly demonstrated in a study by Garris and Wightman, where the COMT inhibitor tolcapone was found to increase evoked dopamine release in the rat caudate-putamen by nearly 50% at a stimulation frequency of 20 Hz but not at higher frequencies (Garris & Wightman, 1995), which are often used in evoked dopamine studies (including those of Simpson and colleagues, and Yavich and colleagues). Caution should therefore be exercised in comparing directly our results of increased reward-evoked phasic dopamine in the NAc of freely moving mice with those of phasic dopamine in the dorsal striatum obtained by electrical stimulation of anaesthetised mice, in the studies of Simpson or Yavich and colleagues.

In summary, the results of the present study do not meet the predictions of the tonic-phasic hypothesis of subcortical COMT function proposed by Bilder and colleagues (Bilder et al., 2004). It should be noted, however, that Bilder and colleagues did not make any claims about any potential differences between striatal subregions. The present study only provides data from the core of the NAc, and different results might be found in the dorsal striatum, given the potential for differences in dopamine release and re-uptake kinetics, as well as in the balance of tonic vs phasic dopamine levels between the ventral and the dorsal striatum (Garris & Wightman, 1995; Zhang et al., 2009).

5.4.2. Is the effect of COMT-Met on NAc dopamine pre- or post-synaptically mediated?

One of the unanswered questions with respect to the role of COMT in striatal dopamine transmission is whether its effects are mediated pre-synaptically, post-synaptically, or both. A purely presynaptic effect should manifest itself as changes in dopamine *release* (i.e., peak magnitude), whilst a purely post-synaptic effect should mainly affect the signal *decay* times (Wightman et al., 1988).

Unfortunately, the current data do not permit any strong conclusion in favour or against either possibility. Genotype did not significantly affect dopamine peak magnitude on standard reward size trial, which could be an indication for a lack of effect on dopamine release; instead, genotype differences started to emerge when the area under the curve was analysed, which integrates both peak size and the decay of the signal. One potential caveat of the experimental design was the fact that reward delivery was split up in multiple, rapidly delivered but discrete boli of sucrose solution. The rationale for this was based on experiments performed with solid pellet delivery in rats, where differences in reward size (i.e., in the number of pellets) were immediately obvious. Analogous to the rat experiments, we conjectured that splitting up reward delivery into multiples of a minimum delivery unit would improve the ability of animals to discriminate between rewards of different sizes. Although the timing difference between standard and large reward sizes was very small (250ms), it is nonetheless possible that this influenced the dynamics of dopamine release. This could potentially be the reason for the observation that dopamine responses to larger rewards did not show significantly higher peaks than standard rewards, but were instead more temporally extended.

This complicates the comparison of peak dopamine amplitudes between genotypes and might contribute to the fact that a more temporally extended measure of dopamine release was a better representation of the data. Small reward trials, where reward was a single bolus of sucrose solution, are an exception to this caveat, and indeed, the strongest differences in dopamine *release* were observed on these trials. However, the "purely" physiological effect of COMT on dopamine release in these trials might be confounded with more complicated factors such as reward expectation (see below), which might be mediated by separate neural systems interacting with COMT.

5.4.3. Relationship to functional neuroimaging studies in humans

A second aim of the present study was to address the question whether COMT affected NAc dopamine release to rewards of varying sizes. This aim was motivated by a range of human neuroimaging studies which have linked the COMT Val158Met polymorphism to ventral striatal activity, as measured by the fMRI BOLD response, during the anticipation or receipt of monetary rewards (reviewed in Tunbridge et al., 2012). The two main emerging findings from these studies suggest that the Met-allele leads to (1) increased ventral striatal BOLD activity during anticipation of rewards (Dreher et al., 2009; Yacubian et al., 2007), and (2) attenuated differential coding of gains versus losses in striatal activity, especially when the size of the gain or loss is larger than usual (Camara et al., 2010; Krugel et al., 2009; Marco-Pallarés et al., 2009). While there are several lines of evidence that suggest that the BOLD signal might be related to dopamine release and/or dopamine D1 receptor activation in the striatum (Knutson & Gibbs, 2007; Schott et al., 2008), there is currently no direct evidence for this conjecture. Therefore, the present data,

which shows that COMT-Met mice have greater dopamine responses elicited by rewards overall and attenuated differential coding of reward size when reward was smaller than expected, is an important piece of evidence in line with this idea.

Whilst the behavioural correlates of the enhanced striatal activation in Met-allele carriers during reward anticipation in human studies are far from being sufficiently explored, two studies by Lancaster and colleagues showed that subjects homozygous for the Met allele developed a greater response bias towards rewarded stimuli in a line discrimination task with asymmetric reinforcement (Lancaster, Heerey, Mantripragada, & Linden, 2015; Lancaster, Linden, & Heerey, 2012). In a separate study, Wichers and colleagues found a positive correlation between Met-alleles and an increase in positive affect experienced in response to pleasant daily life events (Wichers et al., 2008). Studies as these have lead some to propose that the Met allele increases "reward responsiveness" (Lancaster et al., 2012). Interestingly, increased reward-evoked dopamine signalling in the present study was accompanied by increased time spent in the food magazine in COMT-Met mice relative to WT controls. Importantly, this is less likely to be attributable to general, non-task specific locomotor activity, as magazine head entry behaviour did not differ between COMT-Met and WT mice in any of the other paradigms presented in this thesis, where reward delivery was not contingent on magazine head entry behaviour (see experiments in Chapters 3, 4, and 6). Additional analyses, such as correlating dopamine signal with head entry behaviour, to be conducted on a larger data set, will bring some insight into how exactly NAc dopamine and behaviour are related in this task.

The second finding of the present study, namely that of attenuated differential coding of reward size on the small reward trials in COMT-Met mice is, again, in line with the findings of the human neuroimaging literature. Most notable in this context is a study by Camara and colleagues (Camara et al., 2010) where participants played a gambling task during which the amounts of gains and losses were known – with the exception of a few (10%) so called "boost trials", where gains and losses were unexpectedly large. A COMT genotype effect on striatal activity (as measured by the BOLD signal) only emerged on such boost trials. Specifically, Met-allele homozygotes showed attenuated differential coding of gains versus losses - an effect that was driven entirely by the loss trials, where Val homozygotes showed greater reduction in striatal activity, interpreted as greater negative prediction error, than Met homozygotes. The results of the present study, showing a smaller change in NAc dopamine release following smaller-than-expected rewards compared to standard rewards therefore closely parallels this result.

Importantly, it might be expected that changes in negative reward prediction errors would be related to changes in learning and behavioural flexibility. One interpretation would be that the reduced negative prediction errors might reflect a *downstream effect of improved learning* (i.e., a faster reduction in the discrepancy between expectation and outcome). If so, then it would seem that COMT-Met mice had learned *faster* from the occurrence of negative outcomes than WT mice – a hypothesis supported by some researchers (Frank, Moustafa, Haughey, Curran, & Hutchison, 2007). However, a contrasting hypothesis would be that negative prediction error signals are in fact not a result of *adaptation to* learning, but are instead crucial for *driving* learning themselves. In such a scenario, it would appear

that the reinforcement learning system in COMT-Met mice functions at a lower gain than in WT mice and learning from negative outcomes would therefore be expected to be *impaired* – a hypothesis that has also found some experimental support (Krugel et al., 2009).

These opposing views cannot be resolved with the behavioural paradigm used in the present study. A more suitable task for addressing the above questions would be one where animals would be required to flexibly adjust behaviour based on changing reward contingencies.

Conclusions

According to the influential tonic-phasic hypothesis by Bilder and colleagues, phasic dopamine release in the striatum was expected to be attenuated in COMT-Met mice. However, direct recordings of phasic dopamine in the NAc core of awake, behaving mice in the present study revealed an *increase* in reward-evoked dopamine signals in COMT-Met, relative to WT, mice. Although this is at odds with the above hypotheses, it is in line with a growing number of human neuroimaging studies which have demonstrated increased ventral striatal activity in Met-allele carriers during some aspects of reward-related tasks. One possible explanation for this dichotomy could be that genotype effects on dopamine release in the striatum are in fact mediated by COMT's action in upstream structures, which in turn might be differentially recruited depending on the task at hand. As a result, the way COMT affects striatal dopamine release might be dependent on the particular requirements of a task. Further experiments will be required to confirm this hypothesis.

Chapter 6: Effects of COMT-Met on cue-reward learning and dopamine release in the NAc

6.1. Introduction

Chapters 3 and 4 established a complex, cue-dependent role for COMT in associative learning. One of the main questions arising from the behavioural studies presented in those chapters was as to whether the main locus for these effects was in the cortex or whether there might have been any influence of COMT on subcortical dopamine signalling. The aim of the present study was therefore to provide an insight into the latter question by directly recording phasic dopamine release in the NAc core in COMT-Met and WT mice during the earliest stage of Pavlovian auditory discrimination conditioning.

The studies presented in Chapters 3 and 4 showed that appetitive auditory conditioning was significantly affected by the salience of the rewarded cue in COMT-Met mice – learning proceeded faster than in WT mice when the rewarded stimulus was WN and slower when it was the Tone – whilst the influence of stimulus type on associative learning was negligible in WT mice. Both chapters proposed that these findings might be driven by genotype differences in attentional processes, primarily mediated by differences in PFC dopamine levels. Although not explicitly formulated, this interpretation tapped into the theoretical frameworks of learning that fall into the CS-processing category (see Chapter 1), which emphasise the importance of the attention a cue receives for learning (Mackintosh, 1975; Pearce & Hall, 1980). Some

studies suggest to have found the neural correlates for such attentional signals in the basolateral amygdala (BLA) and prefrontal regions (Bryden, Johnson, Tobia, Kashtelyan, & Roesch, 2011; Roesch, Calu, Esber, & Schoenbaum, 2010; Roesch, Esber, Li, Daw, & Schoenbaum, 2012). However, it is possible that COMT might affect learning of factors that are better characterised by US-processing models such as the Rescorla-Wagner or the temporal difference model (Rescorla & Wagner, 1972; Sutton & Barto, 1990; Sutton, 1988). Indeed, there is a large number of studies demonstrating that firing in midbrain dopamine neurons as well as phasic dopamine release in the striatum correlates with the reward prediction error term conceptualised in these models (Bayer & Glimcher, 2005; Matsumoto & Hikosaka, 2009; Schultz et al., 1997; Tobler et al., 2005). Given the tonic-phasic hypothesis of COMT (Bilder et al., 2004), as well as what is known from the reciprocal relationship between prefrontal and striatal dopamine (see Chapter 1) COMT could therefore have an influence on dopamine release in the NAc and therefore on learning by affecting reward prediction error signalling.

Moreover, the role of phasic dopamine in associative learning may extend beyond encoding reward prediction errors, as suggested by an extensive literature that shows burst firing in midbrain dopamine neurons in response to novel, unconditioned stimuli (Dommett et al., 2005; Horvitz, Stewart, & Jacobs, 1997; Horvitz, 2000; Schultz, 2013). It has been proposed that this novelty signal in itself might act as a motivational signal that drives exploration of novel stimuli and therefore provides the opportunity for learning (Kakade & Dayan, 2002). However, it is currently not understood how these novelty-driven dopamine signals interact with the signals typically recorded during associative learning.

To investigate the possibility that COMT might affect subcortical dopamine signalling, phasic dopamine release in the core of the NAc was monitored in COMT-Met and WT mice during the first session of Pavlovian auditory discrimination learning, using WN as the rewarded (CS+), and the Tone as the unrewarded (CS-), cue. The core of the NAc was chosen as a target for recordings because dopamine release in this striatal subregion has been shown to be involved in associative learning processes. In particular, dopamine release here parallels more closely a prediction error-like signal than that recorded in other parts of striatum such as the NAc shell or the dorsomedial striatum (Brown et al., 2011; Saddoris, Cacciapaglia, Wightman, & Carelli, 2015).

Novel, salient stimuli are known to elicit firing of midbrain dopamine neurons (Dommett et al., 2005; Horvitz et al., 1997; Horvitz, 2000; Redgrave & Gurney, 2006). It was therefore predicted that initial presentations of both the WN and the Tone would elicit phasic dopamine release in the NAc core in the present study, which would scale with the unconditioned salience of the cues. A possible mechanism for the cue-dependent differences in Pavlovian learning observed in COMT-Met mice could be greater dopamine release to the presentation of the WN, compared to the Tone. The first part of the analysis in the present study therefore focused on dopamine release to stimulus presentations in the early trials of the session, before the animals had acquired the stimulus-reward associations.

Another factor affecting learning is dopamine released in response to rewards. The second part of the present analysis therefore focused on reward-evoked dopamine in the early trials of the session, i.e., prior to learning. Given the cue-dependency of the genotype effect on behaviour, it was not believed likely that

differences between COMT-Met and WT mice in reward-elicited dopamine responses could be the principal factor underlying the behavioural effects observed. However, given the increased levels of dopamine release reported in Chapter 5 to instrumentally contingent rewards in COMT-Met mice, I was interested to determine whether there was a similar increase to reward delivery in a Pavlovian task and if that related to changes in learning rates.

The final analysis performed in the present study tracked the evolution of cue- and reward-evoked responses across the session. It was predicted that, overall, dopamine responses to CS- would decline across the session, as the cue would progressively lose its novelty with repeated presentations. Responses to the CS+ would initially be expected to follow a similar pattern, which would, however, change when the animals started associating the cue with reward. In that case, dopamine responses elicited by cue presentations would be expected to increase with the increase of the reward-predictiveness of the cue. At the same time, according to the classical temporal difference models, reward responses were expected to decline (Schultz et al., 1997). If faster learning to a more salient cue in COMT-Met mice directly reflected the earliest NAc dopamine-dependent associative processes, I expected to see more rapid changes in dopamine signalling (i.e., reduction in CS– -elicited dopamine, larger increases in CS+-elicited dopamine and/or faster reductions in reward-elicited dopamine) in this group than in WT mice.

In summary, the aim of the present study was to provide some insight into the question whether COMT genotype affected phasic dopamine release in the NAc

core to (1) novel stimuli with differences in unconditioned salience, (2) habituation to unrewarded cues, and (3) reward prediction error signalling.

6.2. Methods

Animals

The experiment outlined in this chapter was carried out on the same mice (COMT-Met n = 7; WT n = 14) that had undergone the experimental procedures described in Chapter 5.

Experimental design

Habituation and magazine training. All behavioural and recording sessions were carried out in operant chambers (Med Associates, VT), modified for FSCV-recording as described in Chapter 2. The mice used in this experiment had undergone habituation to the chambers as well as magazine training as part of the procedures described in Chapter 5.

Pavlovian discrimination learning. The mice underwent appetitive Pavlovian discrimination learning as outlined in Chapter 2. Each session consist of 15 rewarded (CS+) and 15 unrewarded (CS-) cue presentations lasting 8s each. Trials were separated by an ITIwith a mean of 120s (range 90-155s). The experiment followed the same design as described in Chapter 2, with two exceptions. First, the assignment of auditory cues to CS-type was *not* counterbalanced; instead, the CS+ was always signalled by the white noise (WN) and the CS- was signalled by a 3 kHz tone (T) in all animals. However, the order in which cues were presented within a session was counterbalanced between animals (i.e., half of the animals experiences WN on trial 1). Second, as in the previous behavioural experiment, the volume of

the auditory stimuli was set to be 6-7 dB above the background noise emitted by an exhaust fan fixed to the sound-attenuating cubicle. However, background noise levels in the FSCV recording setup were lower and therefore the absolute level of the cues was less than in the previous experiment (on average 66-69 dB).

Reward delivery coincided with the termination of the CS+ cue and consisted of 40µl of 20% sucrose solution delivered in two discrete boli of 20µl, separated by 10ms (total delivery time: 0.23s). Delivery was accompanied by two auditory clicks. Mice were given 16 sessions of training. All sessions were identical, with the exception of sessions 6 and 10 which each included an additional four probe trials, interspersed amongst the standard 15 CS+ / CS- trials, where the CS+ (white noise) was presented but then either not followed by reward (session 6) or followed by an increased reward size (80µl, session 10). Data from these sessions are not presented in this thesis.

FSCV recordings. NAc core dopamine release was monitored at chronically-implanted carbon fibre electrodes using FSCV on Pavlovian training sessions 1-3, and subsequently on sessions 6 and 10, following the procedures for recording described in Chapter 2. Although recordings were only made on a subset of sessions, conditions were kept constant across all sessions, i.e., all mice were attached to a recording headstage and experienced a similar lag (~45 minutes) between being placed into the recording chamber and session start throughout training.

Analysis of behavioural data

Both the number of head entries into the food magazine and the duration of these magazine entries were recorded throughout training. However, learning

effects were quicker to appear in the number of head entries, likely caused by modification to the food magazine in the FSCV operant chambers (the food cup protruded into the chamber rather than being recessed). Analysis was therefore focused on the number of head entries. For the corresponding duration data, see Supplementary Figure S6-1.

For each mouse, magazine responses were first averaged across trials within each session, and then grouped into two-session blocks for data presentation as well as statistical analysis.

Before examining cue-elicited responding, magazine responding was first assessed in the 8s period *prior* to cue onset to determine whether there was any overall change in magazine responding in the two groups ('baseline'). Analyses was then focused on cue-reward learning across sessions. Response rates during cue presentation were normalised by subtracting the responses made in this pre-cue baseline period on a trial-by-trial basis.

Unless otherwise stated, all behavioural data were analysed using repeated measures ANOVAs with genotype as the between-subjects factor and session or trial block and, where appropriate, CS type (CS+ / CS-) as within-subject factors. Analysis of behavioural performance was conducted on mice that successfully completed all training sessions, including mice that later did not pass criteria for inclusion into dopamine data analysis. 'Dopamine group' was therefore included as an additional between-subjects factor in order to verify that mice contributing dopamine data followed the same behavioural pattern as the entire cohort.

Behaviour of mice included in dopamine analysis. Two additional analyses were performed using only mice that contributed dopamine data in order to be able to

match behaviour and dopamine data on the first Pavlovian conditioning session more closely. First, within-session acquisition of a conditioned response was assessed using normalised magazine head entry data (see above) across five 3-trial blocks of the session. Second, I examined the latency to make a magazine head entry after the end of cue presentation. On trials where an animal had entered the magazine during the cue and was still in the magazine when the cue ended, latency was scored as 0s; on trials were an animal did not make any response during the ITI following cue presentation, latency was assigned a maximum value of 60s. Median latencies of five 3-trial blocks were analysed using repeated measures ANOVA with genotype as the between-subject factor, and trial block (5 levels) and CS type as within-subject factors (2 levels).

Analysis of dopamine data

Note that only data collected during the first Pavlovian discrimination learning session is presented here. Dopamine release recorded on 12 electrodes passed criteria for inclusion into analysis (WT n = 5 mice / 7 electrodes, 4 in left NAcc; COMT-Met n = 4 mice / 5 electrodes; 4 in left NAcc; for recording locations, see Supplementary Figure S6-2) (see Chapter 2). Due to experimental error, for one animal (COMT-Met mouse, 1 electrode, left NAcc) data was only recorded during the first two blocks of session 1. Whilst this electrode was included in analyses concerning initial cue-and reward responses, it was excluded from analyses of the evolution of dopamine signals across blocks.

FSCV data processing. A detailed description of the processing of FSCV data can be found in Chapter 2. In brief, cue- and reward-evoked dopamine responses were extracted separately using principal component regression, relative to a 0.5s

baseline taken either before cue onset or the time of retrieval of reward respectively. Trials where the principal component regression model was violated on >20% of time points in the 10s following the event of interest (i.e. cue onset or reward retrieval) were excluded (<0.5% of trials).

Data were smoothed using a 0.5s moving average window and then trial-by-trial peak dopamine as well as area under the curve (AUC) were obtained during the cue period and an equivalent 8s window following reward delivery / omission (i.e., cue offset) or reward retrieval for each electrode.

Initial response to auditory cues. One possible reason for the difference in learning rates in COMT-Met compared to WT mice could relate to the degree to which the stimuli initially evoke dopamine release, akin to a novelty bonus (Kakade & Dayan, 2002) or a salience tag (Matsumoto & Takada, 2013; Redgrave & Gurney, 2006). We therefore assessed whether the ability of the auditory cues to evoke increases in phasic dopamine release prior to the acquisition of cue-reward associations was different between the two groups. Dopamine peak currents on the first trial block of the session (average of the first three CS+ and first three CS- trials, respectively) were analysed using a repeated measures ANOVA on log-transformed data with genotype as the between-subject factor and cue type as a within-subject factor (2 levels).

Finally, to determine relative dopamine release in each animal to the different cue types, a within-subject metric was computed by dividing the difference between WN- and Tone-evoked peak dopamine by their sum, according to:

$$(WN - Tone) / (WN + Tone)$$

Using this ratio, scores of >0 indicate greater dopamine release to WN while scores of <0 indicate greater dopamine release to the Tone. The one sample t-test against 0 was used to test whether either of the genotype groups responded more strongly to either cue type and genotypes were compared using the independent samples t-test.

Initial response to rewards. In order to obtain a measure of reward-evoked dopamine release prior to the acquisition of cue-reward associations, I focused analysis on dopamine release upon reward retrieval (first magazine head entry after CS+ termination) on the first trial block of the session (average of the first three CS+ trials). Independent samples t-tests were used to compare peak dopamine currents and AUC between genotypes.

Evolution of cue-evoked dopamine release across session. Peak dopamine currents within 4s of cue onset were computed on a trial-by-trial basis and then binned into five blocks of three trials of each cue type. The data were analysed using repeated measures ANOVA with genotype as the between-subject factor and cue type (2 levels) and trial block (5 levels) as within-subject factors.

While a number of studies have used the magnitude of dopamine peak currents immediately elicited by cues to track changes in dopamine signalling during Pavlovian learning (Flagel et al., 2011; Parker et al., 2010), some recent studies have demonstrated the development of more sustained or increasing dopamine levels during relevant periods of a behavioural task (Hart, Clark, & Phillips, 2015; Howe et al., 2013). In order to assess any changes in the persistence of dopamine levels during the 8s cue presentation between the first and last trial block, dopamine currents were averaged across 1s bins, resulting in eight time bins

describing the dopamine levels over the 8s of cue presentation in each block. Repeated measures ANOVA with genotype as the between-subjects factor and time bin (8 levels), trial block (2 levels) and CS type (2 levels) as within-subject factors was used to compare changes in the dynamics of the dopamine signal in the course of cue presentation between the first and the last block of the session.

Evolution of outcome-evoked dopamine release across session. Outcome-evoked dopamine release was assessed by analysing the AUC in the 8s following termination of CS+ and CS- respectively (for additional analysis showing dopamine data time-locked to *retrieval* of reward from food magazine on CS+ trials, see Supplementary Figure S6-4). These data were collapsed across all trials of CS+ and CS-, respectively, and analysed using repeated measures ANOVA with genotype as the between-subjects factor and CS type (2 levels) as a within-subjects factor. One-sample t-tests against 0 were used to test whether there was any significant elevation or suppression in dopamine signal from baseline, which would indicate the presence of a positive and negative prediction error signal, respectively. Finally, outcome-evoked dopamine on CS+ and CS- trials was analysed in five 3-trial blocks using repeated measures ANOVA with genotype as the between subject and trial block as the within-subject factor (5 levels).

6.3. Results

6.3.1. Behaviour

Two mice (COMT-Met n = 1; WT n = 1) did not display any conditioned response to the CS+ within 10 sessions of Pavlovian discrimination learning, likely the result of accidental damage to their ear canal during surgery, and were therefore excluded from analysis. Of the remaining 19 mice 14 (COMT-Met n = 5; WT n = 9)

completed all 16 training sessions (1 animal lost a headcap during later training and 4 developed unusably noisy electrodes and so their testing was stopped).

The average rates of responding, as assessed by magazine head entries during the 8s period before cue onset, were very similar between genotype groups, with a decrease across sessions (main effect of session: $F_{2.2,26.9} = 16.6$, $p < 0.01$) but no effect of genotype ($F_{1,12} = 0.2$, $p = 0.65$) or genotype × session interaction ($F_{2.2,26.9} = 0.2$, $p = 0.87$; Figure 6-1A). This indicates that there was no evident difference in the general patterns of responding in the operant chambers between the two groups.

Both genotype groups rapidly learned the cue-reward association, exhibiting increased magazine responding during presentation of the CS+, but not the CS-, over sessions (CS × session interaction: $F_{3.3,39.8} = 23.1$, $p < 0.01$), with pairwise comparisons showing that the mice exhibited significantly higher responding to the CS+ over the CS- from session block 1 onwards (all $p < 0.01$).

Importantly, as observed in Chapter 3, the COMT-Met mice responded more strongly to the CS+ than the WT mice (Figure 6-1 B). This resulted in a main effect of genotype ($F_{1,12} = 5.1$, $p = 0.04$) and an interaction between CS type and genotype ($F_{1,12} = 6.3$, $p = 0.03$).

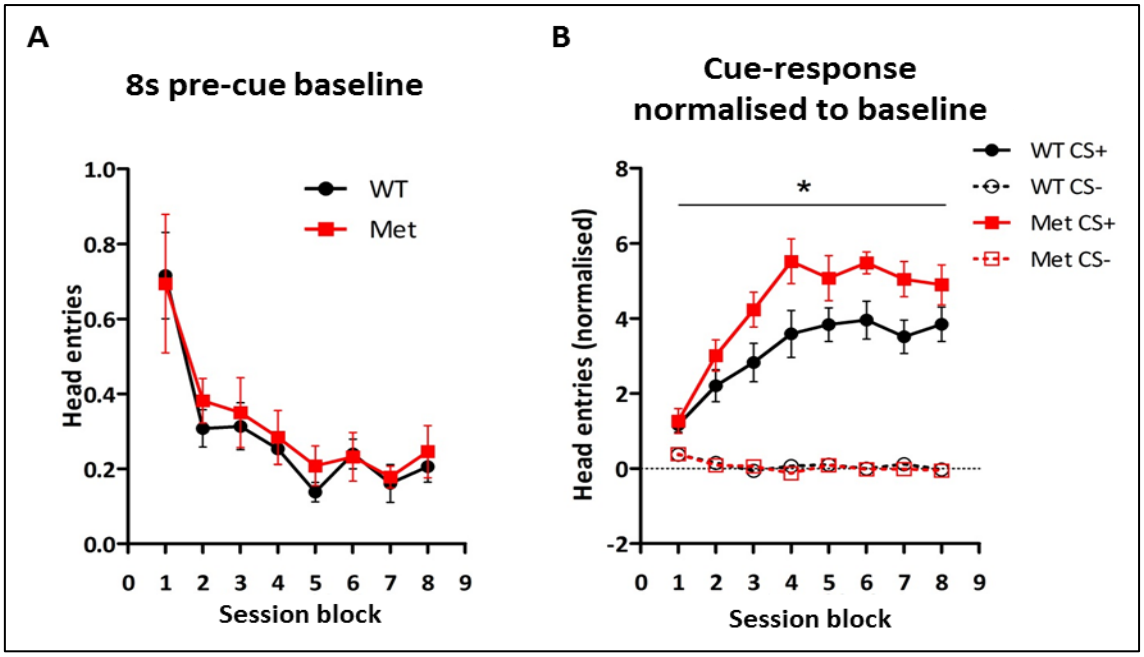


Figure 6-1. Average number of magazine head entries during (A) pre-cue baseline and (B) cue presentation (normalised to the pre-cue period) *during Pavlovian discrimination learning in mice that had undergone surgery for FSCV. COMT-Met mice made significantly more head entries during CS+ presentation (*, RM ANOVA with main effect of genotype and genotype × CS interaction, $p < 0.05$). Data points represent blocks of two sessions. Error bars are ± SEM.*

The subset of mice that contributed dopamine data ("Dopamine group") displayed patterns of behaviour that were analogous to those described above for the whole group of mice (no significant main effects or interactions with Dopamine group in any of the analyses above: all $p > 0.05$; see Supplementary Figure S6-3 for depiction of behavioural performance exclusively of 'Dopamine group' mice).

6.3.2. Dopamine Release during initial learning

Patterns of dopamine release recorded in the NAc core (Supplementary Figure S6-2) during the *first* Pavlovian conditioning session were examined. Figure 6-2 shows a representative example of a WN CS+ and a Tone CS- trial recorded from the same mouse (WT) on the first block of the first Pavlovian discrimination learning session. Both auditory cues and reward delivery elicited a phasic rise in dopamine

release throughout the session and the sections below will deal with both of these drivers of dopamine release in turn.

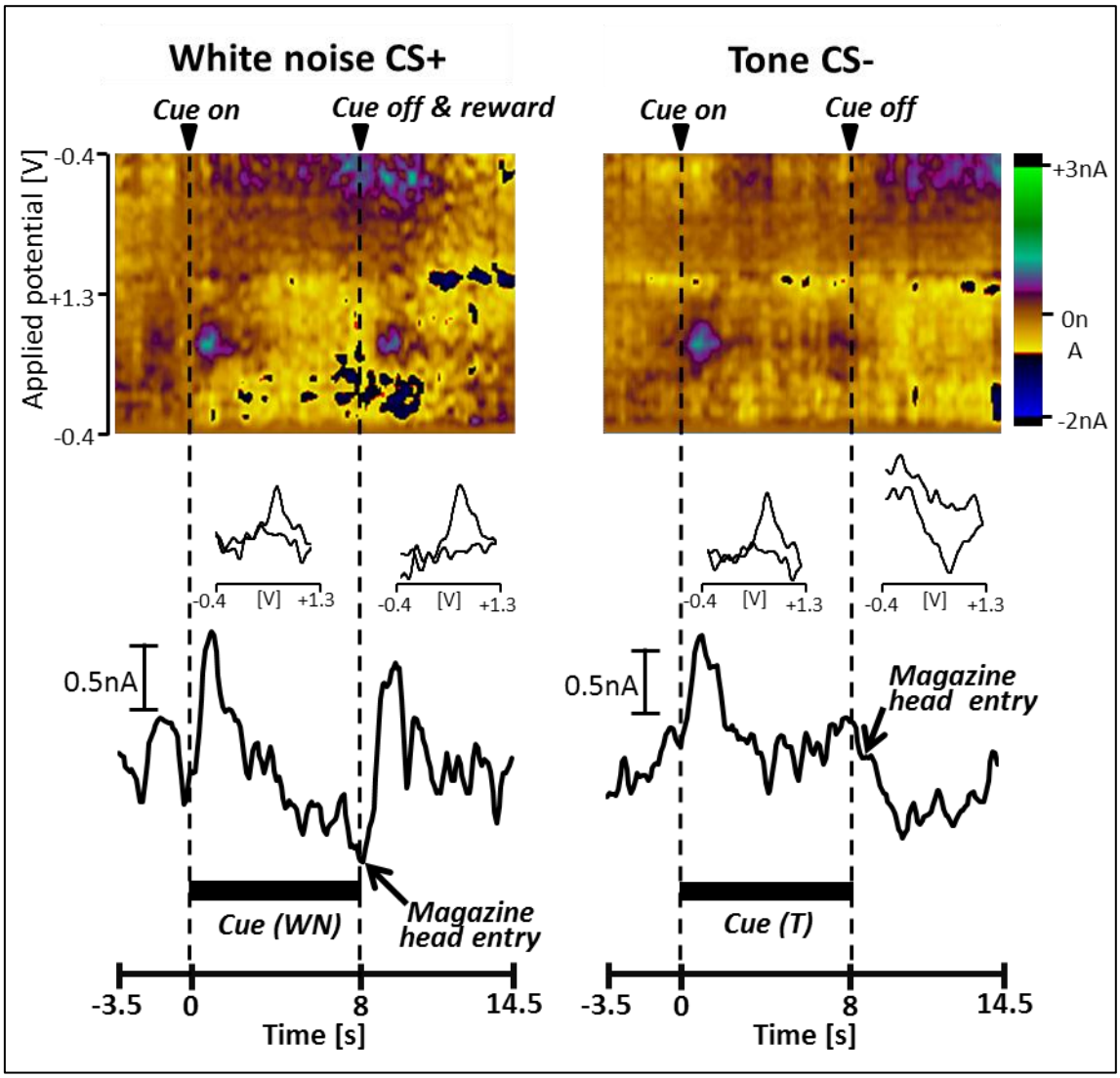


Figure 6-2. Examples of cue- and reward-evoked dopamine release*. Examples of dopamine release on a white noise (WN, left) and a Tone (T,right) trial during trial block 1 of the first Pavlovian discrimination learning session from one mouse (WT). Pseudocolour plots (top panel) show changes in baseline-subtracted redox current as a function of applied potential (y-axis) plotted over time (x-axis). Bottom panel depicts dopamine current over time. Insets depict cyclic voltammograms taken at the time of cue presentation and following the first head entry after cue termination (indicated by arrows). Dotted lines indicate cue onset and end.*

Dopamine response to initial exposure to novel auditory cues

First, I asked whether presentation of novel auditory stimuli at the start of the session (first block of 3 trials), before any reward associations would have been formed, elicited differential patterns of dopamine release in the two groups. Presentation of the cues elicited a rapid rise in dopamine in both genotype groups, peaking at a mean latency of 1.8s following cue onset (Figure 6-3A). While both the WN and the Tone elicited increases in dopamine, peak heights were significantly larger after the WN (main effect of cue type: $F_{1,10} = 6.4$, $p = 0.03$, Figure 6-3B). Mean dopamine peak heights were numerically higher in WT than in COMT-Met mice, although this did not reach statistical significance (main effect of genotype: $F_{1,10} = 1.7$, $p = 0.22$; genotype × cue type interaction: $F_{1,10} = 0.01$, $p = 0.91$).

To confirm this picture and to determine whether the overall higher dopamine release in WT mice could be masking any genotype effect, a within-subject cue discrimination score was computed using peak dopamine size recorded on WT and Tone trials (Figure 6-3C). Both genotype groups showed discrimination scores of >0, indicative of greater dopamine release evoked by the WN than the Tone, although this effect only reached statistical significance in COMT-Met mice (one sample t-test against 0: $t_4 = 3.9$, $p = 0.02$ in COMT-Met and $t_6 = 1.3$, $p = 0.25$ in WT mice). There was no statistically significant difference between genotype groups (independent samples t-test: $t_{10} = 0.5$, $p = 0.66$).

The foregoing analysis implies that, while novel cues elicited a sharp increase in dopamine, this cue novelty signal was not evidently increased in COMT-Met over WT mice..

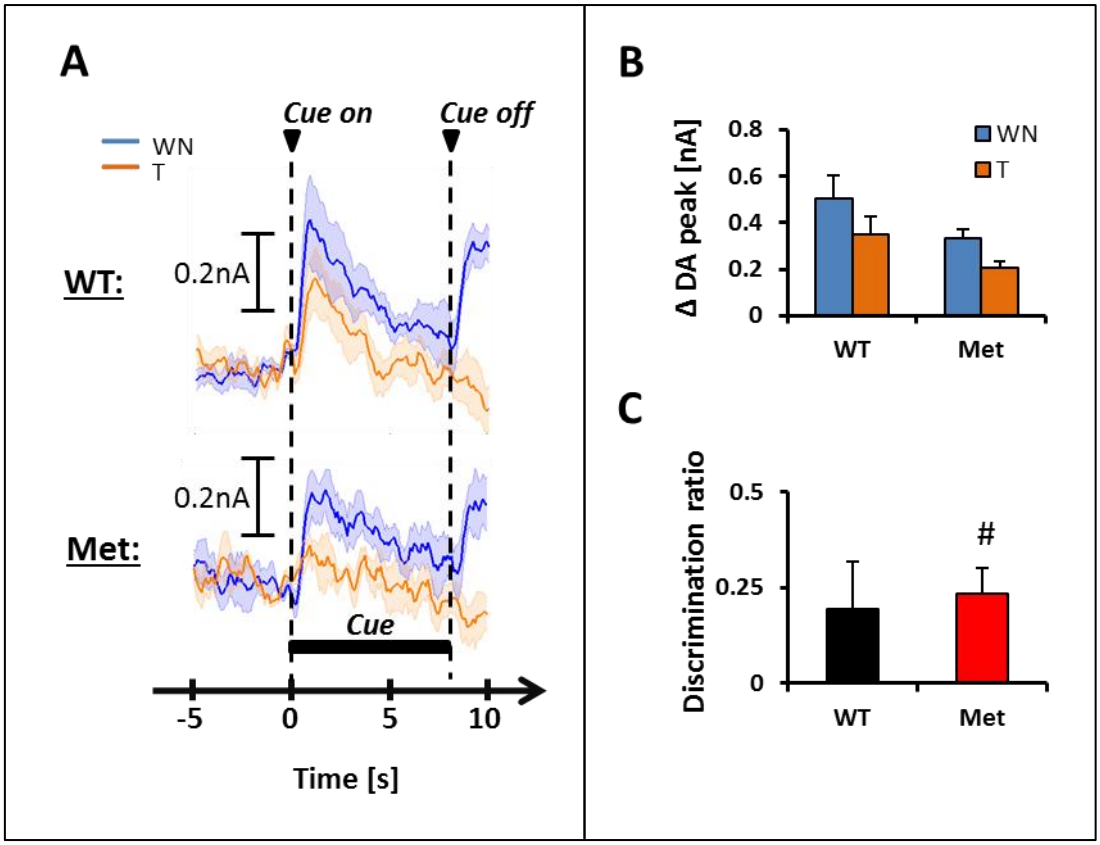


Figure 6-3. Dopamine evoked by initial presentation of auditory stimuli. A. *Traces depict mean dopamine signals in WT (top) and COMT-Met (bottom) mice, averaged across the three trials of the first trial block of the session. Dotted lines indicate the times of start and termination of the white noise CS+ (WN, blue) or Tone CS- (T, orange). Reward is delivered at the end of CS+.* ***B.*** *Mean dopamine peak currents measured during WN and Tone trials on the first block of training.* ***C.*** *Cue discrimination ratio, calculated by dividing the difference of WN-evoked peak dopamine and Tone-evoked peak dopamine, by their sum. Scores of >0 signify greater dopamine release to the WN, whilst scores of <0 signify greater dopamine release to the Tone. (#, one sample t-test against 0, $p < 0.05$). All error bars are ± SEM.*

Dopamine response to initial exposure to rewards

To investigate whether there were differences in reward-evoked dopamine release *prior* to acquisition of the cue-reward associations, changes in dopamine were examined ime-locked to retrieval of reward from the food magazine in the first trial block of the session (Figure 6-4). There was a rise in dopamine signal around reward retrieval which peaked at an average 1.7s (± 0.2 SEM) after magazine head entry. There was no significant difference between genotype groups in either peak magnitude or timing, or area under the curve (all $t_{10} < 1.3$, $p > 0.2$).

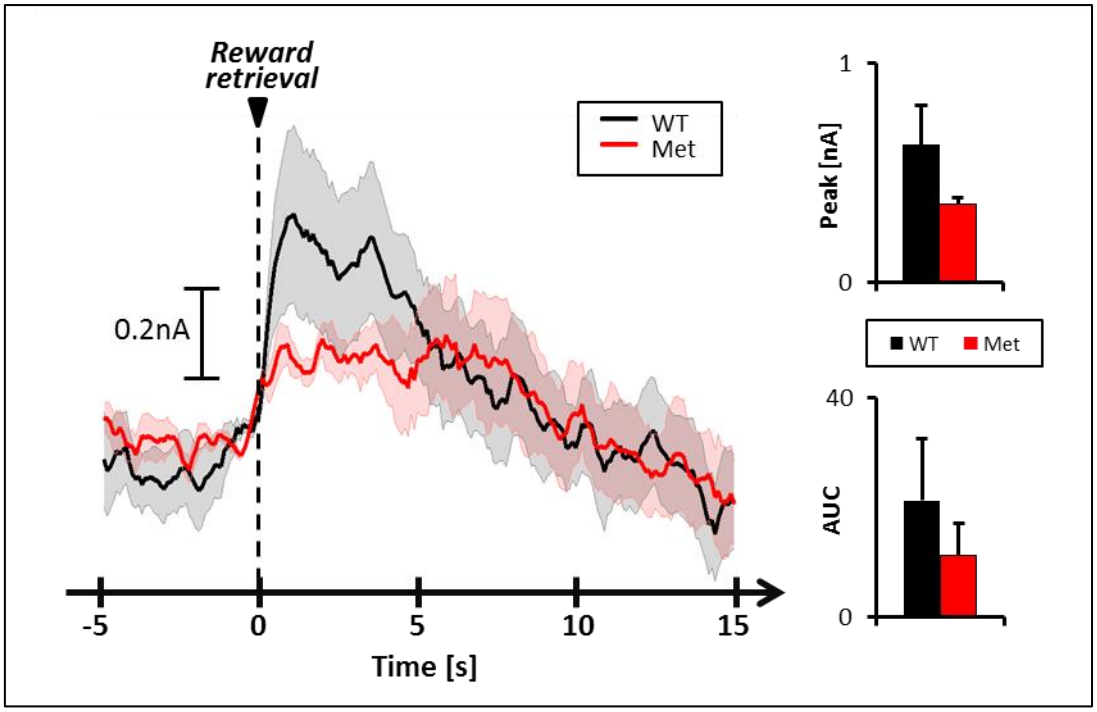


Figure 6-4. Dopamine release to reward retrieval early in learning. *Traces depict dopamine signals in WT (black) and COMT-Met (red) mice in trial block 1. The dotted line indicates the time of reward retrieval from the food magazine. Bar graphs depict mean dopamine peak size [nA] measured within a 4s window of reward retrieval (top) and area under the curve (AUC) measured in an 8s window following reward retrieval (bottom). Error bars are SEM.*

The dynamics of dopamine release across the session

Having established the patterns of dopamine release to cues and rewards prior to the formation of cue-reward associations, I proceeded to analyse changes in behavioural performance as well as dopamine release across the session.

First, I wanted to assess whether the mice showed signs of cue-reward learning across the five 3-trial blocks of the session at a behavioural level. Overall, mice made more head entries to the CS+ than the CS- (main effect of CS type: $F_{1,6}$ = 9.6, p = 0.02), suggesting that a differential response to the cues was present from early in the first session (Figure 6-5A). There was no main effect of, or interaction with, genotype (all p > 0.08).

Animals retrieved rewards at a mean latency of 8.9s ± 4.3s SEM (median: 4.5s) after cue offset on CS+ trials. Latencies to enter the magazine after cue termination were similar for CS+ and CS- trials in the first two trial blocks of the session but began to diverge from the middle of the session onwards (Figure 6-5B). This resulted in a significant CS type × trial block interaction ($F_{4,24} = 5.9$, $p < 0.01$), driven by a significant increase in latency on CS- but not CS+ trials in the second half of the session. These patterns were comparable in both genotype groups and there were no significant effects of, or interactions with, genotype (all $F < 0.8$, all $p > 0.54$).

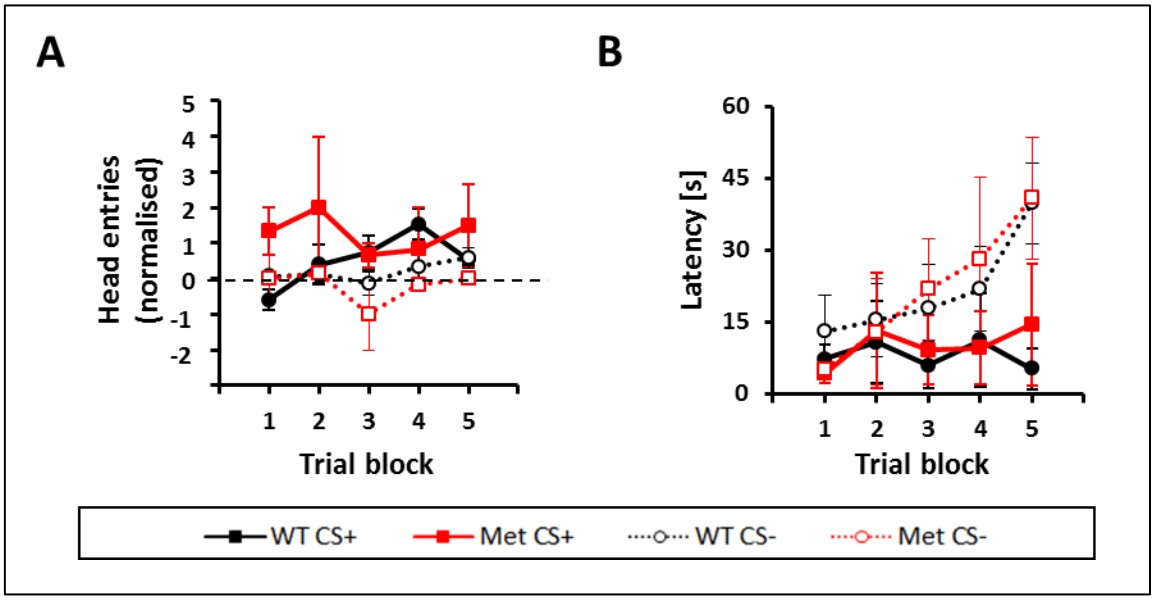


Figure 6-5. Behavioural performance on first Pavlovian discrimination learning session in mice contributing dopamine data. A. *Normalised responding to CS+ and CS- across the five 3-trial blocks of the first session of Pavlovian discrimination learning).* ***B.*** *Latencies to first head entry after cue termination across the five 3-trial blocks of the first Pavlovian discrimination learning session on CS+ and CS- trials. All data are group means ± SEM).*

I next asked whether the ability of cues and rewards to elicit phasic dopamine release changed with repeated exposure to cues and cue-reward pairings across the first Pavlovian discrimination learning session (Figure 6-6).

Analysis of peak cue-evoked dopamine across the five blocks of the session showed that, as expected, dopamine release was higher after presentation of CS+ than CS- (main effect of CS type, $F_{1,9} = 46.0$, $p < 0.01$). There was also a quadratic relationship between trial block and dopamine peak current size (quadratic contrast of block: $F_{1,9} = 6.2$, $p = 0.03$, Figure 6-6B): cue-evoked dopamine levels were larger at the start of the session and, for the CS+ alone, the end of the session relative to the middle three blocks (CS type × trial block interaction at trend-level significance: $F_{4,36} = 2.4$, $p = 0.07$). These patterns were comparable in both the COMT-Met and WT animals (no main effect of, or interaction with, genotype: all $F < 2.6$, all $p > 0.13$).

Finally, outcome-evoked dopamine release was assessed by analysing dopamine levels following cue termination (for dopamine data time-locked to reward retrieval, see Supplementary Figure S6-5). A repeated measures ANOVA revealed significantly greater dopamine release for reward delivery after the CS+ compared to reward omission after the CS- ($F_{1,9} = 30.0$, $p < 0.01$; no main effects of or interaction with genotype ($F < 1.1$, $p > 0.32$); Figure 6-6C insets). Comparisons against pre-cue baseline showed a significant increase in dopamine signal at reward delivery and a significant suppression in dopamine signal below baseline after reward omission in both genotype groups (one sample t-tests, all $t > 3.2$, $p < 0.05$).

Again, there were no significant effects of block, genotype, or block × genotype interactions when reward delivery-evoked dopamine release was analysed across the five 3-trial blocks of the session (all $F < 2.0$, all $p > 0.11$), suggesting that reward signalling did not change across the first Pavlovian discrimination learning session in either group (Figure 6-6C left).

Interestingly, however, there was a quadratic contrast of trial block when an analogous analysis was performed on CS- trials at the time of reward omission ($F_{1,9}$ = 7.3, p = 0.03). In accordance with this, when AUC on each of the trial blocks was tested against pre-cue baseline, the dopamine signal was found to be suppressed in the middle of the session (WT: blocks 2-3: $p < 0.01$, block 4: $p = 0.06$; COMT-Met: block 3: $p = 0.07$, block 4: $p = 0.03$), but not at the start or the end of the session (blocks 1 and 5: all $t < 1.8$, all $p > 0.1$). This result suggests only a transient presence of a negative reward prediction error-like signal in both genotype groups (no main effect of or interaction with genotype: all $F < 0.4$, $p > 0.82$; Figure 6-6C right).

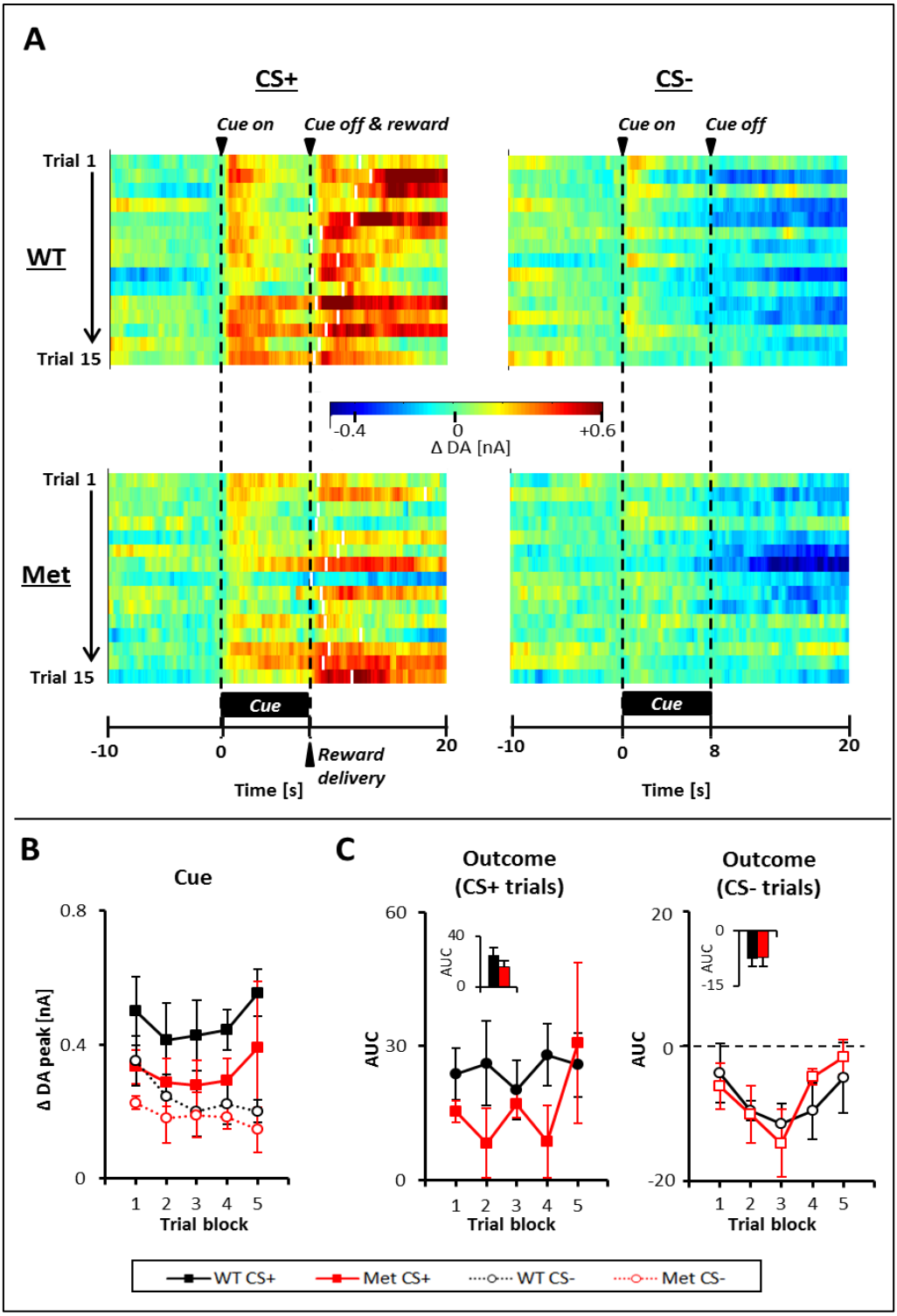


Figure 6-6. Dynamics of dopamine release across the session. A. *Pseudocolour plots depict change in dopamine signal (nA, colour coded) over the course of a trial relative to a 0.5s baseline prior to cue onset. Each horizontal line depicts one trial, averaged across all animals in the WT (top panel) and COMT-Met (bottom panel) groups. Separate colour plots are shown for the 15 CS+ (left) and 15 CS- (right) trials. Note that whilst reward was delivered at the end of CS+ presentation, the exact timing of retrieval of the reward from the food magazine varied between animals. White marks indicate the median time of reward retrieval on each trial.* ***B-C.*** *Cue-evoked (B) and outcome-evoked (C) dopamine on CS+ and CS- trials binned into five 3-trial blocks. All data are mean ± SEM.*

In summary, while it was possible to observe dopamine release to the presentation of novel cues, reward delivery and collection, and to observe some adaptation of these responses during the first session, these patterns were largely similar in COMT-Met and WT animals on the first Pavlovian discrimination learning session.

6.4. Discussion

The present study investigated the role of COMT on phasic dopamine release in the NAc core during the first session of Pavlovian auditory discrimination learning. The main questions this study wanted to answer were whether COMT affected (1) phasic dopamine responses to unconditioned auditory stimuli (and whether this effect interacted with stimulus type), (2) phasic dopamine responses to unconditioned reward, and (3) the evolution of cue- and reward-evoked responses across the first Pavlovian learning session. Indeed, all of these responses were observed in the NAc core in the present study, however their patterns were found to be largely similar in the COMT-Met and WT groups.

Given the cue-dependency of the observed effect of COMT on learning, the first question addressed in this study was whether COMT affected dopamine release to the presentation of the auditory stimuli prior to learning, and whether this effect interacted with stimulus type. Since only the first three presentations of the WN and Tone were used for this analysis, it was assumed that any cue-elicited dopamine responses recorded were not influenced by the cues' reward outcomes. Indeed, phasic release of dopamine in the NAc core was observed from the first presentation

of both WN and the Tone onwards in the present study, consistent with electrophysiological studies showing increased midbrain dopamine neuron burst firing elicited by novel stimuli in the absence of reward or reward prediction (Dommett et al., 2005; Horvitz et al., 1997; Horvitz, 2000; Steinfels, Heym, Strecker, & Jacobs, 1983). Presentations of WN caused greater dopamine release than presentations of the Tone, consistent with the idea that WN is more salient than the Tone (Chapter 3). Crucially, this pattern was observed in both WT and COMT-Met mice, and there were no genotype differences or interactions with cue type.

It has been proposed that dopamine "novelty signals" might act as motivational signals that drive exploration of novel stimuli and therefore might provide opportunities for learning (Kakade & Dayan, 2002). Interestingly, in the present study, this signal did not only scale with the novelty, but also with the physical salience of the stimuli. It could be speculated that a bigger response to a stimulus might lead to more engagement of the animal with the stimulus. This idea is supported by the results of the unconditioned orienting study presented in Chapter 3, which demonstrated that both genotype groups oriented more to unconditioned presentations of WN than the Tone. Such orienting could be interpreted as increased engagement with, or increased attention to, the stimuli. However, whilst both genotypes showed increased unconditioned responding to WN than to the Tone as measured by behavioural orienting as well as phasic dopamine release in the NAc core, associative learning was affected by stimulus type only in COMT-Met but not WT mice. This suggests that any initial differences in the intrinsic alerting properties of the WN over the Tone might not be the driving factor underlying the observed learning rate differences in later sessions.

As predicted, rewards in the first session of training elicited phasic release of dopamine in the NAc core. Dopamine levels following reward delivery remained constant throughout the session, i.e. there was no decrease in reward-elicited dopamine, as would be expected to happen when rewards are fully predicted (Schultz et al., 1997; Sutton & Barto, 1990). This is not surprising, given that dopamine data were only obtained from the first session of Pavlovian conditioning, whilst behaviourally animals only reached plateau performance after approximately eight sessions.

The strongest adaptations of event-related dopamine release across the recording session were observed on CS- trials. As discussed above, presentation of the CS- cue reliably elicited dopamine release early in the session, which could be interpreted as a novelty signal, scaled by the physical salience of the stimulus (Schultz, 2013). Cue-evoked signals decreased across the session, as animals experienced repeated exposures to the CS-. This could be interpreted as "passive" habituation to the stimulus, i.e., a result of a decrease in stimulus novelty. Analysis of dopamine levels at the outcome stage, i.e., at the time of reward omission, however suggested, that at least part of the attenuation might have been driven by "active" inhibition, as a result of learning of the cue-no reward contingency: there were no prediction errors at the time of reward omission early in the session, consistent with the idea that the animals had no reward expectation associated with the cue at that stage. A suppression of dopamine levels at the time of reward omission was, however, observed in the middle of the session, which could be interpreted as a negative reward prediction error. This would imply that the animals started grasping the concept of a predictive relationship between auditory stimuli and reward, without differentiating between stimulus types (generalisation). The

time of the appearance of negative reward prediction error signals coincided with an increase in magazine head entry latencies on CS- but not CS+ trials, further supporting the idea that the animals might have *learned* to inhibit responding to the cue. Interestingly, even at the end of the session, there was still a small rise in dopamine peak above baseline following CS- cue onset, but no longer any reward omission-related prediction errors. It can therefore be speculated that the cue retained some of its inherent alerting properties, which could be dissociated from its ability to predict reward.

As in the case of CS- trials, there was a slight decrease in peak dopamine responses to the CS+ cue after the first block of the session, but responses rose again towards the end of the session. Although the absolute values of peak CS+ dopamine currents were comparable between the start and the end of the session, there was a change in the dynamics of the dopamine signal throughout the 8s presentations of the cue. Whilst there was an initial increase followed by a rapid decay of the dopamine signal during both CS+ and CS- presentation in trial block 1, in later blocks the increase in dopamine was sustained for the duration of the CS+, but not the CS- (supplementary Figure S6-4). Assessing the dynamics of dopamine release during cue presentation in the different groups might be informative to determine differences in associative learning (Hart et al., 2015).

Conclusion

In summary, this chapter replicated the finding of COMT-Met mice showing faster rates of acquisition of the association between a WN cue and reward. Using fast-scan cyclic voltammetry in the NAc core, it was also possible to observe dopamine release to the presentation of novel cues, reward delivery and omission,

and to observe some adaptation of these responses during the first session. Importantly, however, all of these patterns of dopamine release were similar in COMT-Met and WT mice. The lack of any genotype differences in any of the measures reported here implies that cue-dependency of learning in COMT-Met mice could either be independent of phasic dopamine release in the NAc core or it could evolve at a later stage in learning, in line with the separation in cue-elicited behavioural response rates only in later sessions. Moreover, the lack of any genotype difference in dopamine signalling in the core of the NAc does not necessarily preclude a role for COMT in other sub-regions of the striatum. For instance, there are marked difference between the types of events that evoke dopamine release in the NAc core and shell (Badrinarayan et al., 2012; Brown et al., 2011; Saddoris et al., 2015). Indeed, the NAc shell is known to respond more strongly to unconditioned, salient stimuli than the core (Saddoris et al., 2015) and is therefore a prime candidate region for further investigations of the cue type-dependency of the effect of COMT on associative learning.

Chapter 7: General Discussion

7.1. Summary of findings

7.1.1. COMT modulates associative learning in a cue salience-dependent manner

Catabolism of dopamine by COMT has been shown to be a major route for regulating dopamine transmission in the PFC, but not the striatum (Budygin et al., 1999; Käenmäki et al., 2010; Karoum et al., 1994; Slifstein et al., 2008; Yavich et al., 2007). It is therefore not surprising that most studies to date investigating the behavioural and physiological roles of COMT have focussed on PFC function. However, there are a small number of studies that suggest that COMT may affect reward processing and that this is accompanied by changes in ventral striatal activity (reviewed in Tunbridge et al., 2012), although it is not clear whether these changes are mediated by effects on dopamine signalling in the striatum. Moreover, the particular aspects of reward-related behaviours affected by COMT have not been dissected.

The first aim of this thesis was therefore to investigate the effects of COMT on associative learning, a process known to depend on dopamine transmission (see Chapter 1), using auditory Pavlovian conditioning paradigms in a mouse model of the human Val158Met polymorphism of COMT. Based on the reciprocal relationship between PFC and striatal dopamine, as well as the tonic-phasic hypothesis of COMT, phasic dopamine release in the NAc, and therefore associative learning, were expected to be attenuated in COMT-Met mice. The results of the studies presented

in Chapter 3 and 4 failed to confirm this prediction, but instead revealed a more complex picture for the role of COMT in associative learning.

Using an auditory discrimination learning paradigm, Chapter 3 showed that the direction in which COMT affected associative learning was dependent on cue type (WN or Tone). COMT-Met mice learned significantly faster when WN was the rewarded cue, but significantly slower when the Tone was the rewarded cue. The first question following from this finding related to which features of the cues were driving these effects. I hypothesised that one feature distinguishing the cues in a way that could affect learning was a difference in their unconditioned salience - i.e., their intrinsic ability to attract attention. According to this argument, WN was predicted to be a more salient stimulus than the Tone. Accordingly, performance in COMT-Met mice was improved when the salience of the cues was actively controlled by increasing the volume of the Tone CS+ and decreasing the volume of the WN CS- (Chapter 3, Experiment 2). By demonstrating greater unconditioned orienting to WN than to the Tone, Chapter 3 Experiment 3 further confirmed the working hypothesis that WN might be more salient than the Tone. Interestingly, a similar pattern of orienting was observed in both COMT-Met and WT mice, suggesting that the unconditioned salience of the cues was similar for both genotype groups despite differences in learning when these cues were used for conditioning.

Given the results of Chapter 3, it was not possible to determine whether cue effects on learning were mainly driven by the absolute salience of the CS+, or whether there was an interaction between the salience of CS+ and CS- cues presented within the session. The latter explanation was based on the hypothesis that COMT-Met mice payed more attention to the more salient cue, at the cost of

attention paid to the less salient cue that was presented within the same session. This would lead to facilitation of learning if the more salient cue was rewarded, but attenuation of learning if it was unrewarded, due to a generalised latent inhibition-like effect. The aim of Chapter 4 was therefore to determine whether cue-dependent effects of COMT were mediated by an interaction between cues of different salience and outcomes presented within the same session. The results of the single cue conditioning study in Chapter 4 demonstrated that learning in COMT-Met mice was still significantly affected by cue type, even in the absence of a CS-. This result suggests that the main factor driving differences in learning was indeed the absolute, rather than relative, salience of the CS+. However, it should be noted that the genotype by cue type interaction obtained in the single cue conditioning study was less strong than that observed in the 2-cue discrimination study, especially in the condition where Tone was rewarded. It is therefore possible that latent inhibition-like effects contributed, at least in part, to the effects observed in Chapter 3. A replication of the experiments in Chapter 4 with a greater sample size will be required to refute or confirm this finding. Moreover, classical latent inhibition paradigms (Lubow, 1973) – which are classically thought to be tests of selective attention, although see (Weiner, 2003) for an alternative explanation – could be used to test the effects of cue salience on latent inhibition and their interaction with COMT genotype directly.

Finally, it should be noted that in this thesis, I assumed that WN and Tone were treated as part of the same continuum of salience, with WN occupying a different position on this continuum than Tone. An alternative explanation of the observed effects, however would be that COMT-Met mice process WN in an

inherently different way from Tone (for a discussion touching upon this topic, see section 3.4). A crucial experiment for the future would therefore be to conduct a systematic test on the effect of different WN and Tone volumes (between subjects) on learning in COMT-Met and WT mice. According to the foregoing argument it would be expected that COMT-Met mice would show faster learning to louder CSs, and slower learning to quieter CSs, *irrespective of cue identity*, compared with WTs. The alternative scenario - that COMT-Met mice always show enhanced learning than WTs to WN and diminished learning to tones, irrespective of volume - would imply a qualitative difference in salience between these two types of auditory stimuli. Such a finding would be of great importance, as WN and pure tones are commonly used as equivalent stimuli for a wide range of behavioural tasks. Moreover, additional experiments using, for instance, light stimuli of different intensities should be conducted to investigate whether genotype differences are modality-specific.

7.1.2. Cue-dependency of COMT cannot be explained by differences in NAc dopamine release in early training

One question arising from the results of Chapters 3 and 4 was whether the cue-dependent effect of COMT was mediated by attentional processes, driven by genotype differences in PFC dopamine, or whether COMT might affect the immediate response to auditory stimuli at the level of phasic dopamine release in the NAc.

In Chapter 6, dopamine release in the NAc core was measured during the first session of Pavlovian auditory discrimination (WN+/T- condition only). Consistent with the previous finding of greater unconditioned salience of WN than to the Tone, dopamine responses were higher to WN than to the Tone early in the conditioning

session prior to learning. This pattern was, however, similar in both genotypes, suggesting that differences in learning were not driven by the immediate responses to the cues at the level of NAc core dopamine. It will be important to confirm this finding in future studies by recording dopamine release during the unconditioned orienting task described in Chapter 3. Moreover, recordings in different subregions of the striatum, especially the NAc shell, should be attempted to rule out any regionally-specific effects of COMT on dopamine release (discussed further below).

7.1.3. COMT affects dopamine evoked by rewards of varying sizes

The second goal of this thesis was to investigate whether COMT affected phasic dopamine release in the NAc core. According to the inverse relationship between PFC and striatal dopamine (see section 1.3.6.), increased levels of PFC dopamine conferred by the Met-allele were expected to lead to decreased levels of NAc dopamine in COMT-Met mice. More specifically, according to the tonic-phasic hypothesis (Bilder et al., 2004), tonic levels were expected to *increase* (not directly measurable using FSCV) whilst phasic release were expected to *decrease* (measurable using FSCV) in COMT-Met mice relative to WT mice. In contrast, experimental data from human neuroimaging studies has demonstrated an *increase* in ventral striatal activity in Met-allele carriers during anticipation or receipt of rewards, as measured by the BOLD signal, which might be mediated by an increase in dopamine release (Knutson & Gibbs, 2007; Schott et al., 2008). Chapter 5 therefore addressed this dichotomy by directly measuring the phasic release of dopamine in the NAc core of COMT-Met and WT mice to rewards of varying sizes. In this task, dopamine levels following reward were, overall, larger in COMT-Met than in WT mice. Moreover, whilst both genotype groups showed reduced dopamine

responses when rewards were smaller than expected, this reduction was markedly less pronounced in COMT-Met mice, a finding consistent with the results of human neuroimaging studies using the BOLD signal to assess activity of the ventral striatum to losses (Camara et al., 2010; Dreher et al., 2009). Taken together, the results of this experiment proved to be more in line with the human neuroimaging literature than the predictions made by the tonic-phasic hypothesis.

It should be noted however, that I did not observe any genotype differences in reward-evoked dopamine recorded during Pavlovian conditioning in Chapter 6. How these seemingly discordant results can be reconciled, and what this means for the tonic-phasic hypothesis of COMT, will be discussed below.

7.2. Broader perspective

7.2.1. COMT and CS-processing versus US-processing models of learning and their neural underpinnings

The overarching aim of this thesis was to investigate the effect of COMT on striatal dopamine release and the way it affects reward-guided behaviour. I therefore chose Pavlovian conditioning, a well-studied paradigm shown to depend on NAc core dopamine, as an experimental paradigm to address these questions. Moreover, phasic dopamine release in the NAc core is known to follow a pattern consistent with the reward prediction error term in the TD model of learning, thereby providing the potential for linking neurobiology with distinct aspects of learning theory. Based on the tonic-phasic hypothesis of COMT, COMT-Met mice were expected to have attenuated phasic dopamine release in NAc, which would translate to attenuated prediction error signalling and therefore attenuated learning. However, the cue-dependency of the COMT effect observed in the experiments

described in this thesis suggests that COMT might affect the processing of the CS, rather than the US, and therefore CS-processing models (e.g., those proposed by Pearce and Hall, and Mackintosh and others derived from them (Mackintosh, 1975; Pearce & Hall, 1980), collectively termed "CP-models" from here on) might be more appropriate for the interpretation of the effects of COMT uncovered in this thesis. These two types of models (CP- versus TD-models) have long been treated as mutually exclusive. Recently, Roesch and colleagues showed that neural correlates of TD-type and CP-type learning can be dissociated (Roesch et al., 2012). According to their findings, BLA and ACC mediate CP-like learning while the striatum mediates TD learning (Roesch et al., 2010). In line with this, there were no genotype differences in dopamine release typically ascribed to TD learning in the present experiment (Chapter 6).

It is tempting to speculate that COMT affects learning via modulation of cortical regions or BLA, which is also innervated by dopamine fibres (Andrzejewski & Ryals, 2015; Heath et al., 2015; Larkin, Jenni, & Floresco, 2016). Future experiments will therefore require regional targeting of COMT activity, e.g. by virally-mediated gene transfer, to investigate which brain structures are causally responsible for the observed genotype differences in behaviour.

Recent advances in learning theory have attempted to integrate CP and TD models into one unifying theory of learning (Esber & Haselgrove, 2011; Le Pelley et al., 2012) and whilst the neural structures mediating CP- and TD-like learning processes are dissociable, they are likely to be linked (Roesch et al., 2012). Interestingly, in the experiment presented in Chapter 6, dopamine released to novel cues not only scaled with the novelty of the cue but also with their physical salience,

which implies that NAc core dopamine responds to differences in unconditioned salience. However, it is currently not known how such salience difference impact future TD or CP learning.

Understanding how animals select which cues to learn about is fundamental to a variety of behaviours. Understanding how differences between cues shape this process is essential to developing good theoretical models of learning. Studying COMT might provide an interesting window into further research into this exciting question.

7.2.2. The role of COMT in subcortical dopamine

The second main question of the thesis was whether COMT affected dopamine release in the NAc on a basic level. According to the tonic-phasic hypothesis, COMT-Met was expected to lead to increases in tonic and decreases in phasic levels (Bilder et al., 2004).

Chapters 5 and 6 describe FSCV recordings of phasic dopamine release in the NAc core to two types of stimuli: auditory cues and rewards. Whilst there were no genotype differences in either cue- or reward-evoked dopamine in the Pavlovian conditioning experiment presented in Chapter 6, there was a genotype difference in reward-evoked dopamine release in Chapter 5. In Chapter 5, where reward delivery was contingent upon an action (magazine head entry), reward-evoked dopamine levels were higher in COMT-Met than in WT mice, whilst no such difference was visible in Chapter 6, where reward was delivered irrespective of behaviour. The hypothesis that the differences between reward-evoked dopamine release in the experiments presented in chapters 5 and 6 were dependent on whether the reward

was contingent upon an action can be addressed in future studies. For example, free reward and action-contingent reward could be made available in different blocks within a session, allowing for within-session comparison of the effect of COMT genotype on dopamine release in the two conditions.

The combined findings of Chapters 5 and 6 suggest that the effects of COMT on dopamine release in NAc core cannot be simply explained by one mechanistic hypothesis such as the tonic-phasic hypothesis, but that they are instead dependent on the task at hand, and therefore on other task-relevant brain structures. Indeed, some studies show that non-contingent reward and reward contingent on an action can evoke different patterns of dopamine responses across the striatum (Bassareo, Cucca, Frau, & Di Chiara, 2015; Cacciapaglia, Saddoris, Wightman, & Carelli, 2012). This effect might be mediated by different task-dependent inputs into dopaminergic neurons from upstream structures. It is therefore conceivable that instead of having a direct, local effect on NAc dopamine, COMT might act on input structures upstream.

Conclusion

This thesis investigated the role of COMT in associative learning and phasic dopamine release in the NAc core. The results of this thesis can be summarised in two main findings.

First, the experiments presented in Chapters 3, 4, and 6 demonstrate that COMT affects associative learning in a cue-dependent manner, and put forward the theory that this might be mediated by COMT effects outside of the NAc. This analysis suggests that cortical dopamine might play an underappreciated role in simple

associative learning processes, and highlights the need for integrative models of attention and reward prediction.

Second, the experiment presented in Chapter 5 suggests that COMT affects NAc dopamine, but not in a simple way that could be explained by a mechanistic hypothesis such as the tonic-phasic hypothesis proposed by Bilder and colleagues (Bilder et al., 2004). Instead, the effect of COMT on NAc dopamine release was found to be task-dependent. The data in Chapter 5 aligns with data from human neuroimaging studies, but contradicts what would have been predicted by current anatomy-based theories of fronto-striatal circuitry.

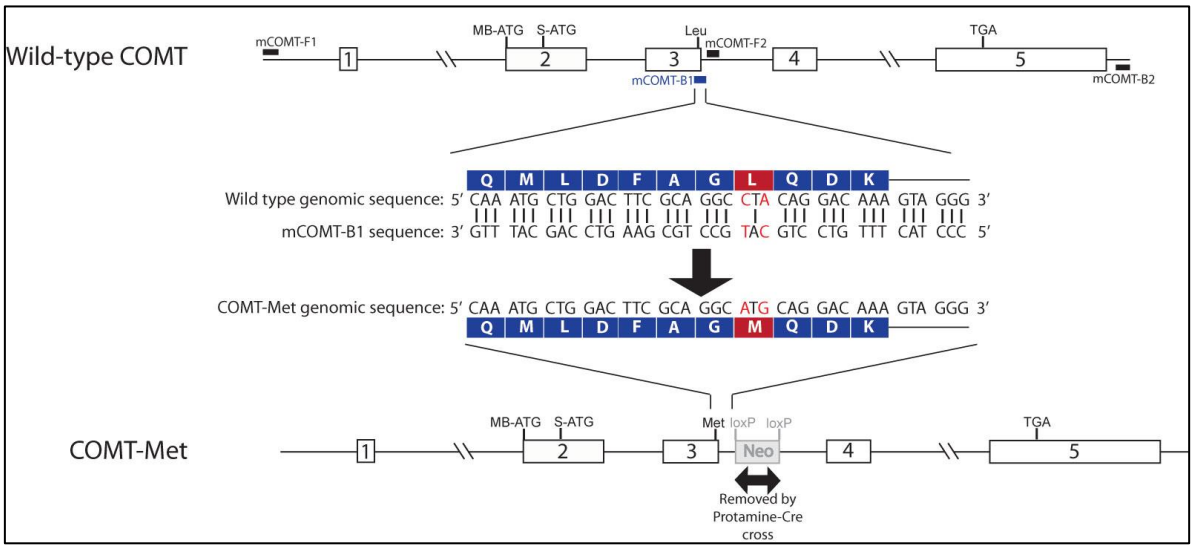
The dichotomy that emerges from the foregoing experiments highlights the insufficiency of present theories of COMT function to explain effects of dopamine on behaviour. By taking into account the cognitive requirements of the tasks used within this body of research, a richer understanding of the role of COMT on reward-guided behaviour, with greater explanatory power, can be reached. In short, there is a lot more research, and many more answers, to COMT.

List of Abbreviations, Acronyms, and Initialisms

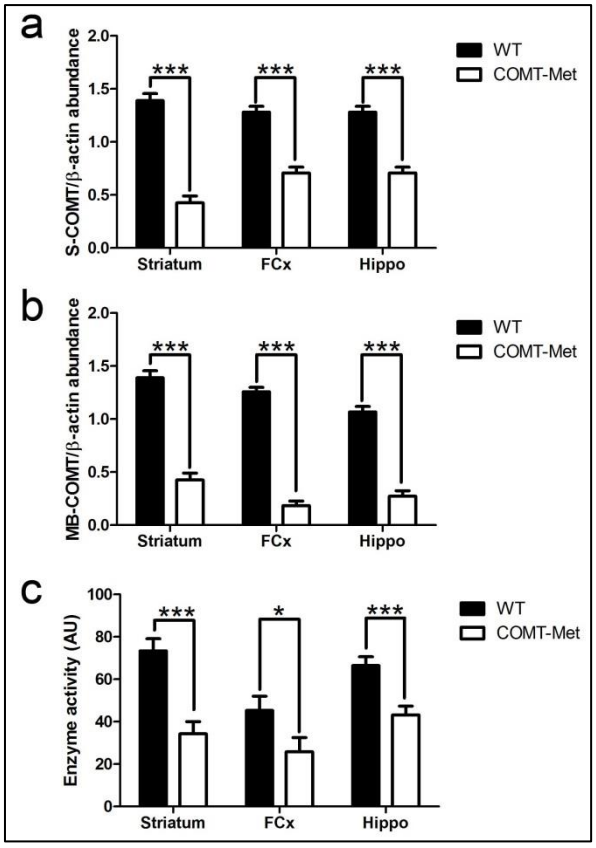
ACC	Anterior Cingulate Cortex
AUC	Area under the Curve
BLA	Basolateral Amygdala
BOLD	Blood-oxygen Level Dependent Signal
COMT	Catechol-*O*-Methyltransferase
CR	Conditioned Response
CS	Conditioned Stimulus
DA	Dopamine
FSCV	Fast-scan Cyclic Voltammetry
GABA	Gamma-aminobutyric Acid
ITI	Inter-trial Interval
LI	Latent Inhibition
L-DOPA	Levodopa (L-3,4-dihydroxyphenylalanine)
MAO	Monoamine Oxidase
MB-COMT	Membrane-Bound COMT

fMRI	Functional Magnetic Resonance Imaging
NAc	Nucleus Accumbens
NMDA	N-Methyl-D-aspartate
PET	Positron emission Tomography
PFC	Prefrontal Cortex
dlPFC	Dorsolateral Prefrontal Cortex
SD	Standard Deviation
SEM	Standard Error of the Mean
SNc	Substantia Nigra Pars Compacta
S-COMT	Soluble COMT
T	Tone
TH	Tyrosine Hydrolylase
US	Unconditioned Stimulus
VI	Variable Interval
VTA	Ventral Tegmental Area
WCST	Wisconsin Card-sorting Task
WN	White Noise
WT	Wild Type

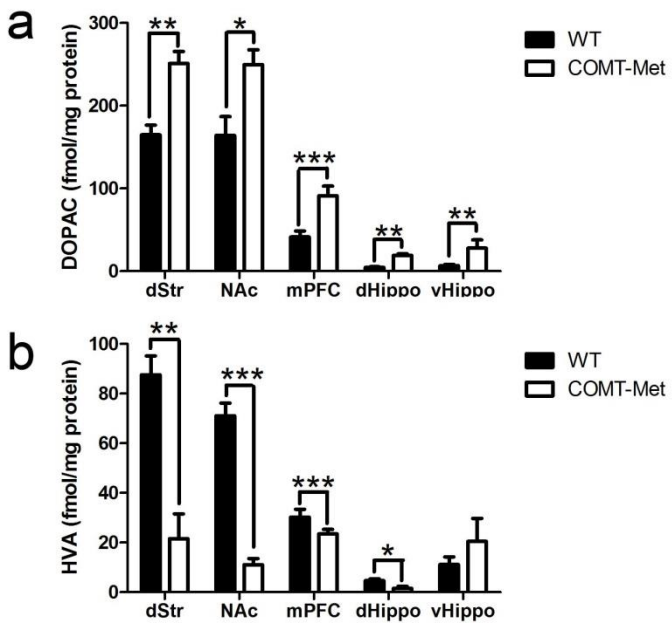
Supplementary Materials



Supplementary Figure S1-1. Generation of COMT-Met mice. *The Met allele was knocked in to the mouse COMT gene using a PCR-based strategy. The mCOMT-B1 primer introduces the Met allele into the mouse COMT gene (mismatched bases are highlighted in red). The final transgene contained the coding region of the mouse COMT gene (Amplified region: chr16:18,407,548-18,415,235, according to Mouse Genome December 2011 GRCm38/mm10 Assembly) with the Met allele, as well as a floxed PGK-neo selection cassette in the intron between exons 3 and 4. The selection cassette was subsequently removed by crossing the COMT-Met mice with a Cre recombinase-expressing line. Taken from Barkus et al., (in preparation).*

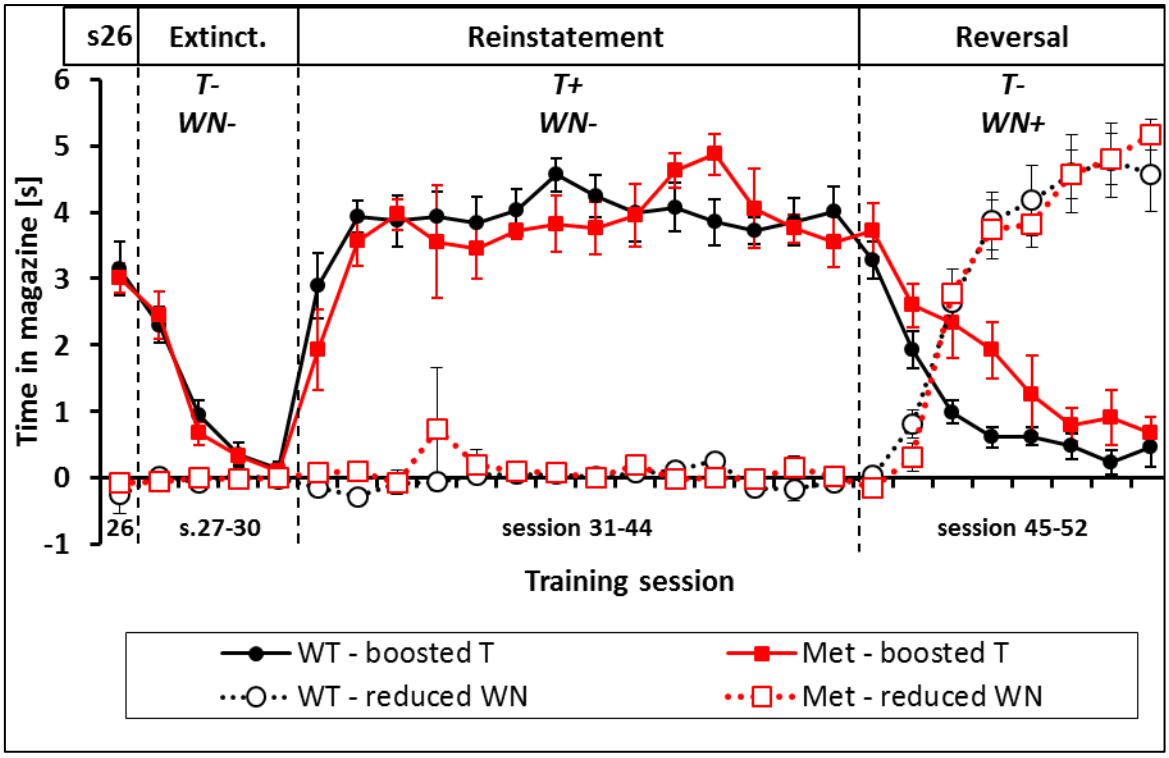


Supplementary Figure 1-2: COMT abundance and activity is reduced in COMT-Met mice, compared to wild-types (WTs). *(a) S-COMT abundance was reduced COMT-Met (open bars) vs. wild-type (closed bars) mice in striatum (n=18 wild-type [9 male]; n=19 COMT-Met [10 male]), frontal cortex (FCx; n=18 wild-type [8 male]; n=19 COMT-Met [10 male]) and hippocampus (Hippo; n=20 wild-type [10 male]; n=20 COMT-Met [10 male]). (b) MB-COMT abundance was reduced in COMT-Met vs. wild-type mice in all brain regions (n's as for S-COMT). (c) COMT activity was reduced in all brain regions in COMT-Met vs. wild-type mice (n=20 wild-type [10 male]; n=20 COMT-Met [10 male]). ***p<0.001; *p<0.05. Taken from Barkus et al. (in preparation)*

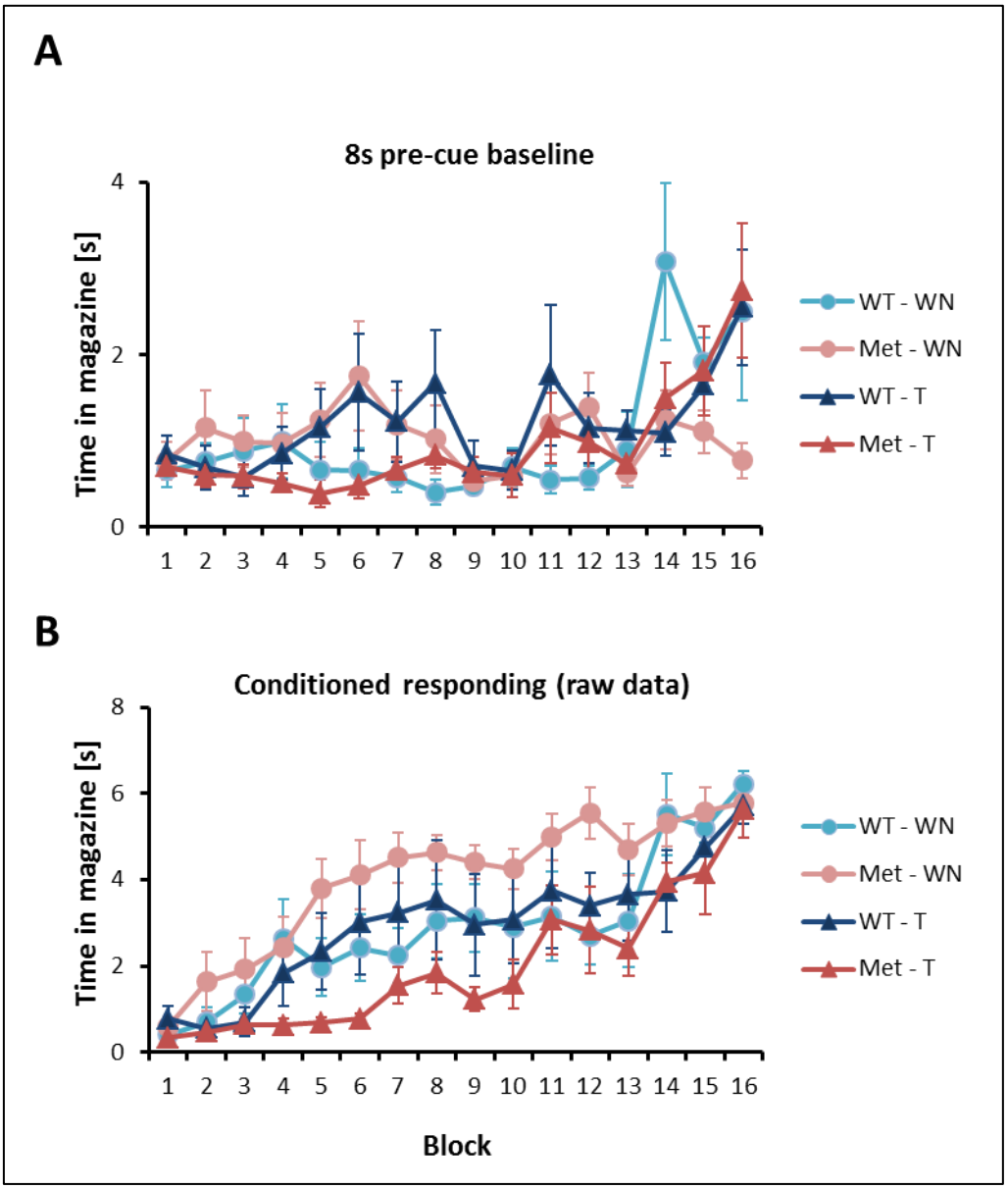


Supplementary Figure S1-3: Dopamine metabolism is reduced in COMT-Met mice, compared to wild-types. *(a) DOPAC is increased in dorsal striatum (dSt), nucleus accumbens (NAc), medial prefrontal cortex (mPFC), dorsal hippocampus (dHippo) and ventral hippocampus (vHippo) in COMT-Met mice (open bars) vs. wild-types (closed bars). (b) HVA is decreased in all regions except ventral hippocampus, in COMT-Met mice vs. wild-types. n=8 wild-type (7 for vHippo); n=12 COMT-Met mice. ***p<0.001; **p<0.01; *p<0.05. Taken from Barkus et al. (in preparation)*

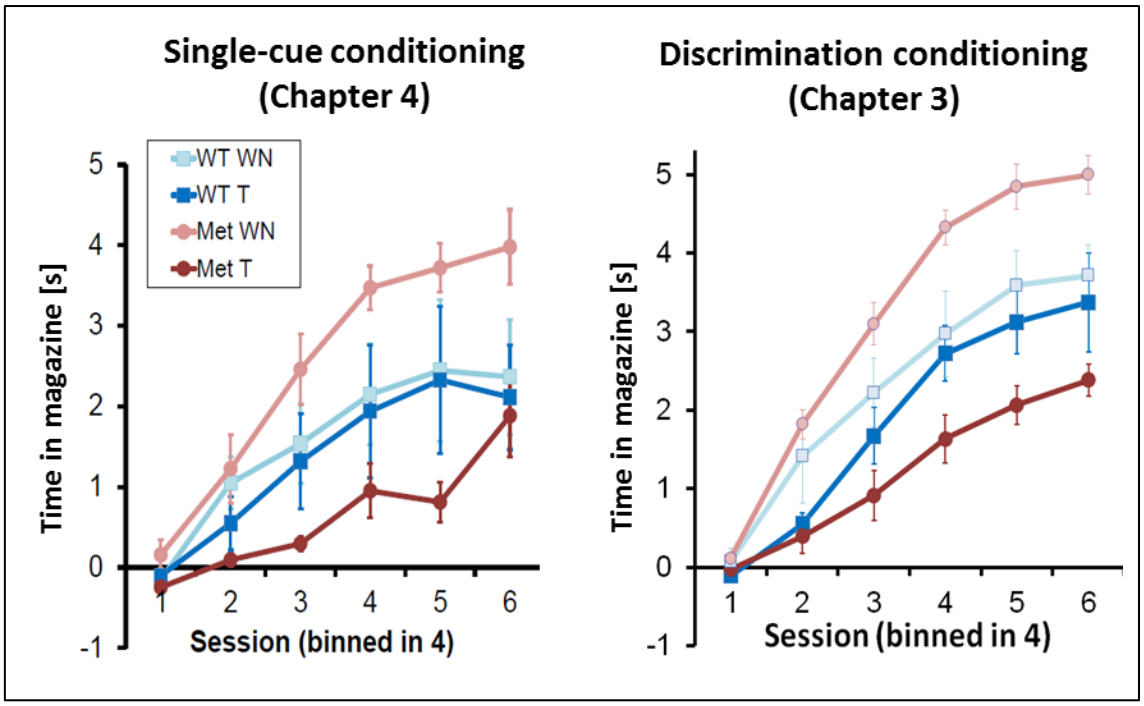
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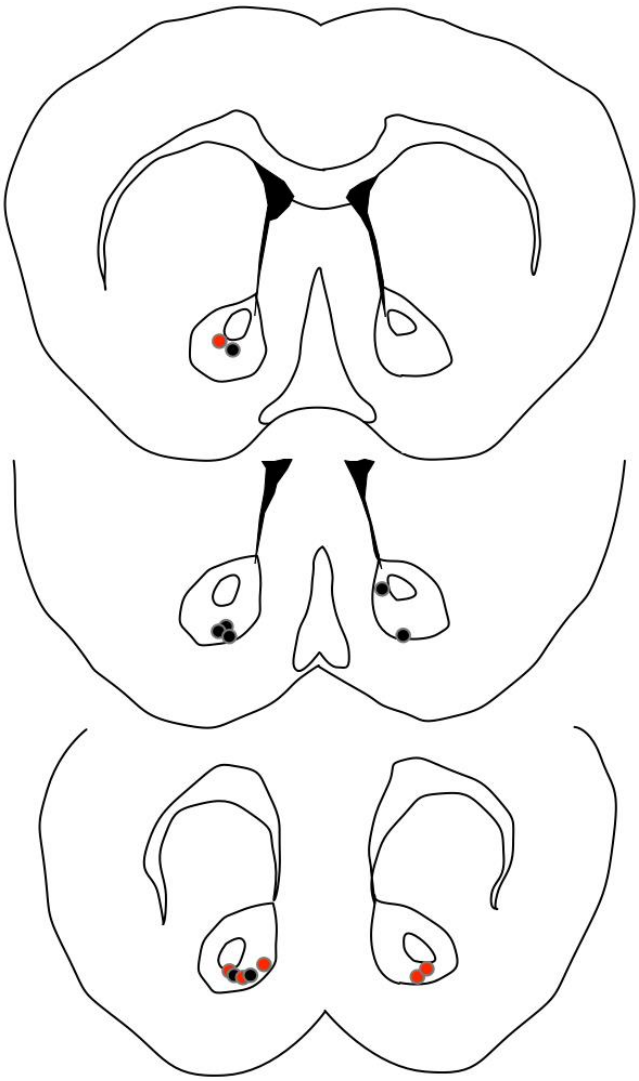
Supplementary Figure S3-1. Results of continuation of Pavlovian training of the boosted Tone group. *The first data point ('s26') depicts normalised magazine head entry duration during auditory stimuli on the final session (session 26) of Pavlovian discrimination learning where 'boosted' Tone was rewarded (T+) and 'lower' WN was unrewarded (WN-). During extinction (sessions 27-30), neither of the cues were rewarded (T-, WN-). During reinstatement (sessions 31-44), initial cue-reward associations were reinstated, with rewarded Tone and unrewarded WN. During reversal (sessions 45-52), cue-reward associations were reversed, such that WN was now rewarded (WN+) and Tone no longer rewarded (T-). There were no significant genotype differences in the initial acquisition (see Figure 3-3), extinction, or reinstatement. During reversal, both genotype groups acquired the new association between WN and reward at similar speeds. However, COMT-Met mice were significantly slower than WT mice to inhibit responding to the no longer rewarded Tone. Data points present session means ± SEM.*



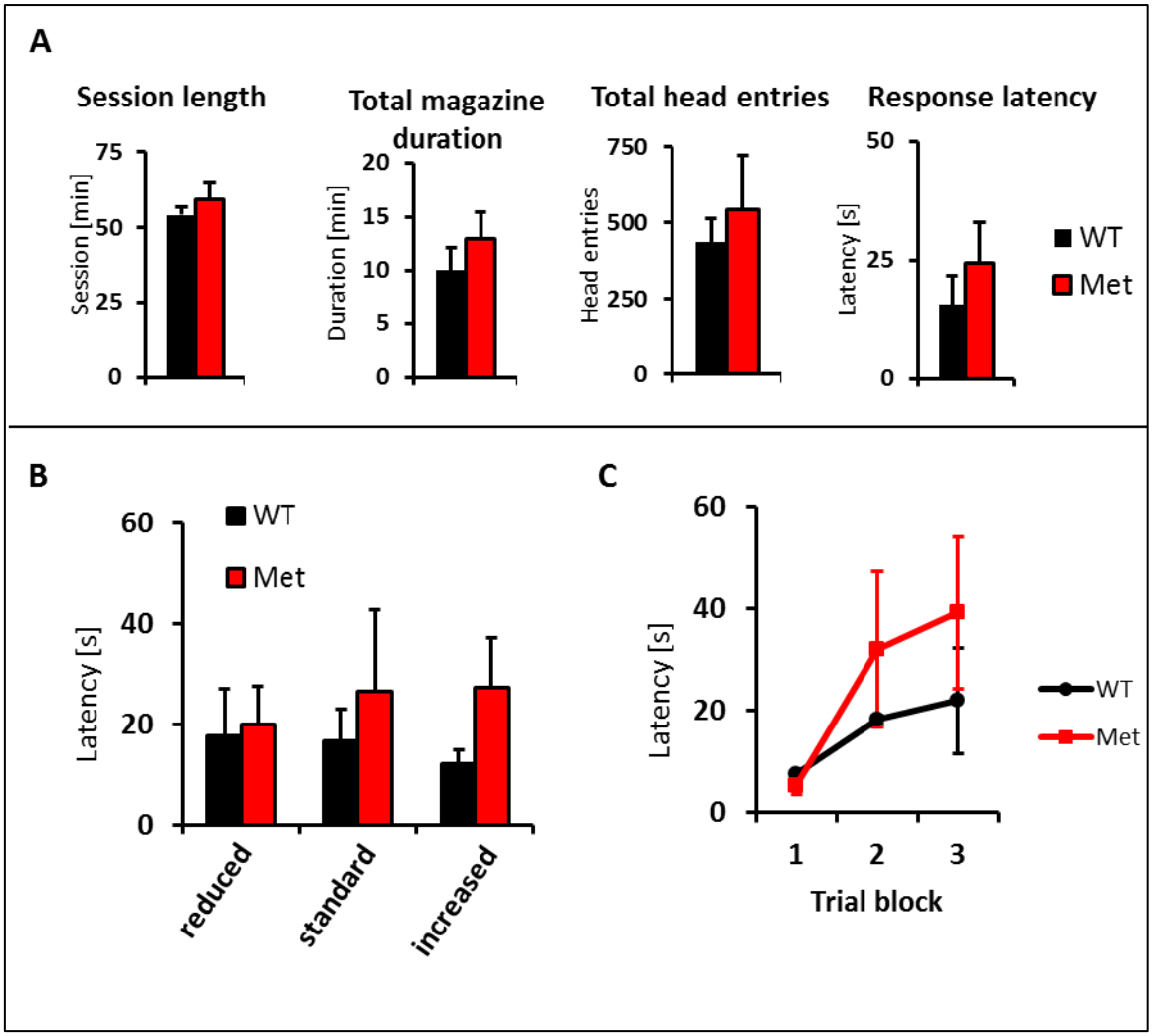
Supplementary Figure S4-1. *Average time spent in magazine during the 8s preceding auditory cue onset (A) as well as during cue presentation (B) in mice trained on White Noise (WN) or Tone (T). Data points represent the means of blocks of 2 sessions ± SEM.*



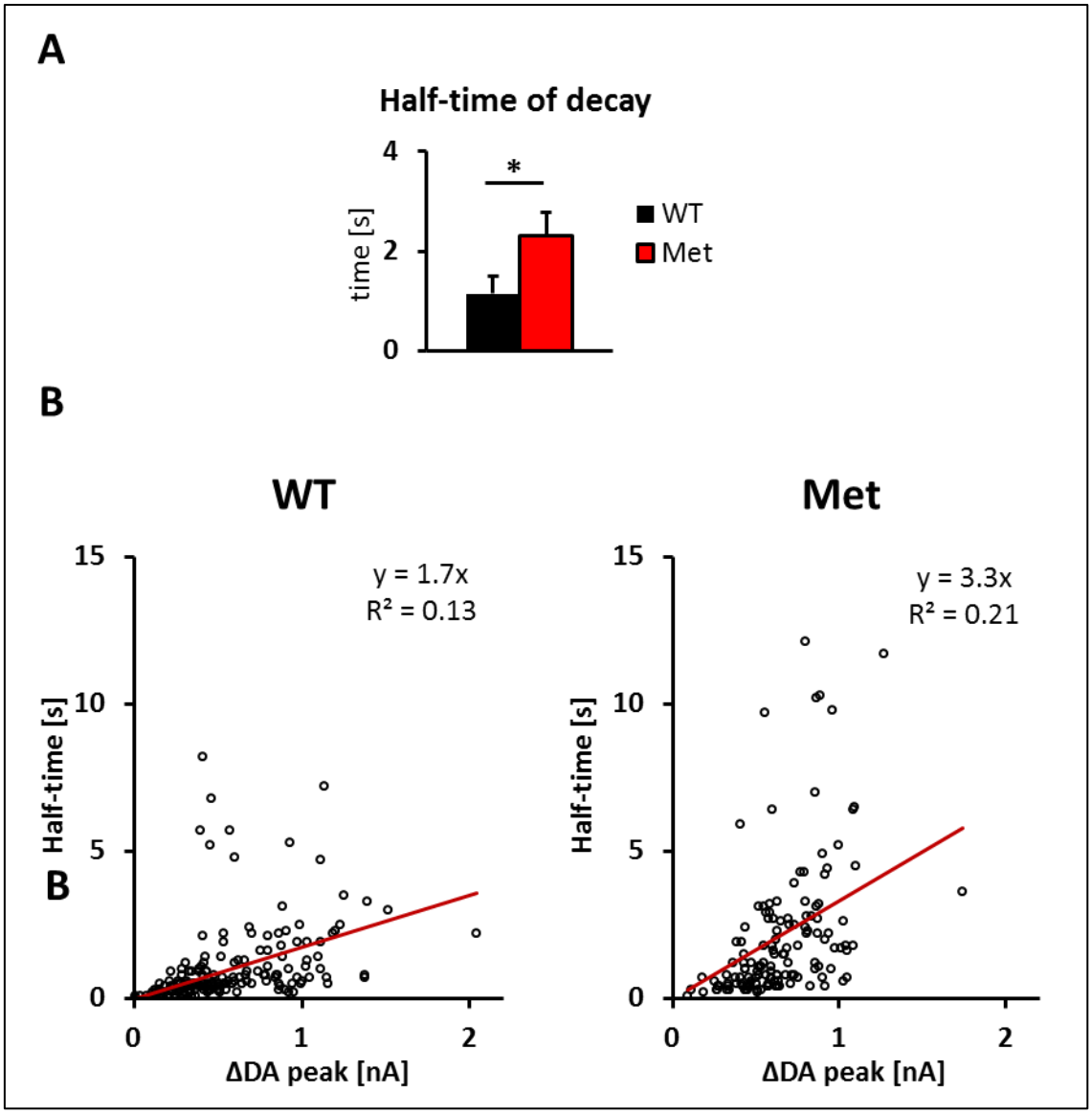
Supplementary Figure S4-2. Comparison of learning curves in single-cue (Chapter 4) and 2-cue discrimination (Chapter 3) learning experiments. *Average time spent in the magazine during cue presentation, normalised to the 8s pre-cue period. Legend: "WN" collectively refers to the WN+ condition in Chapter 4 and the WN+/T- condition in Chapter 3. "T" collectively refers to the T+ condition in Chapter 4 and the T+/WN- condition in Chapter 3. Only data for CS+ trials is shown. Data points represent the means of blocks of 4 sessions ± SEM.*



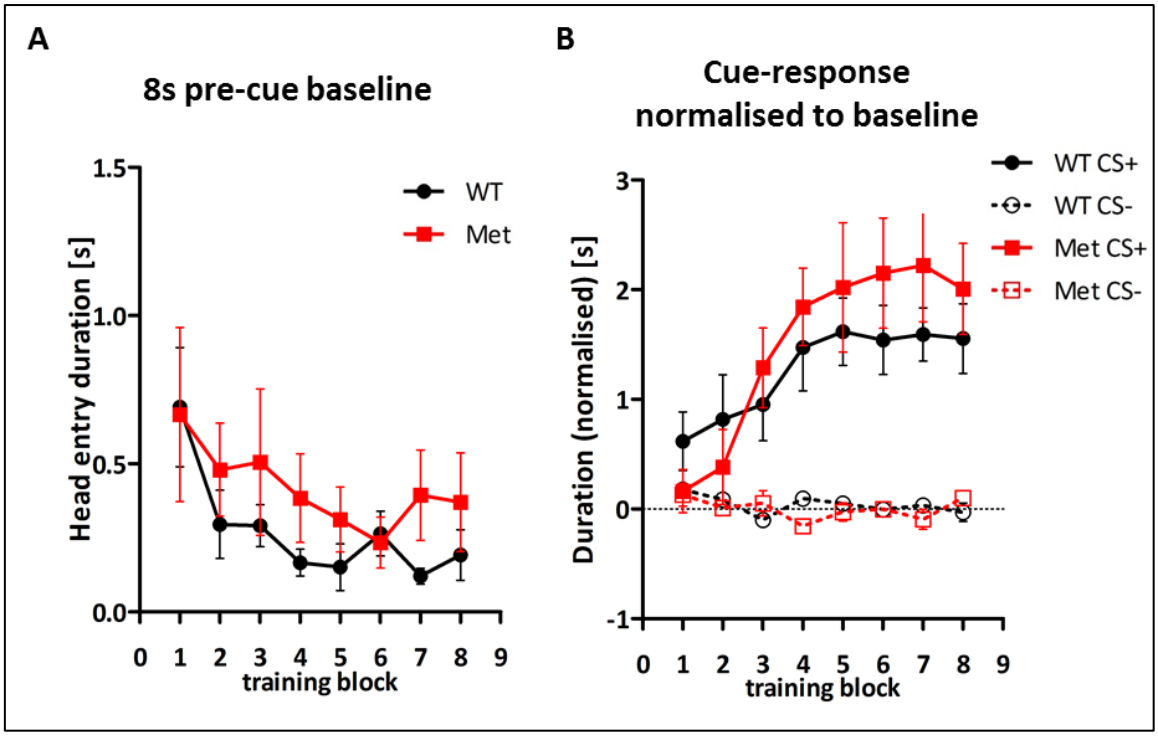
Supplementary Figure S5-1. FSCV recording locations used in variable reward experiment (Chapter 5). *Outlines depict coronal brain sections at 0.98mm (top), 1.18mm (middle), and 1.42mm (bottom) distance from bregma. Placements of recording electrodes are indicated by black (WT) and red (COMT-Met) circles.*



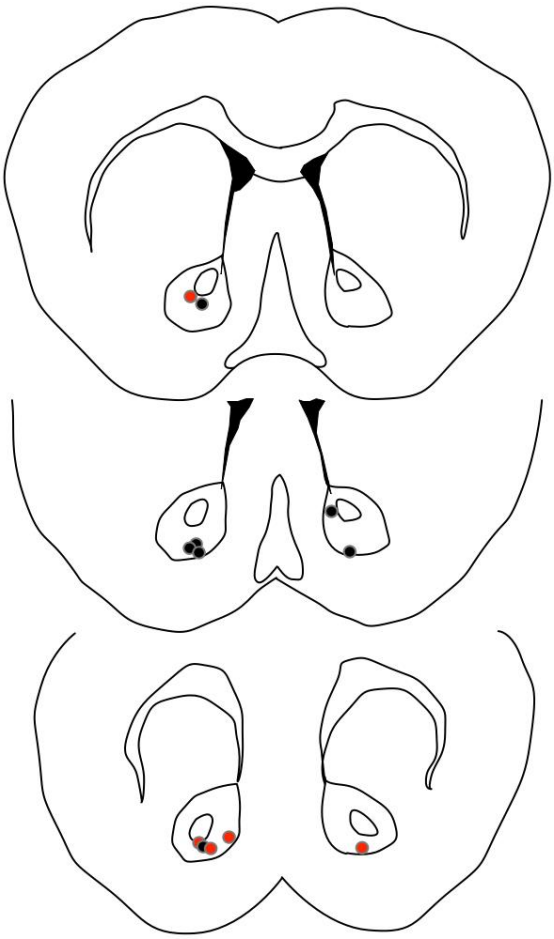
Supplementary Figure S5-2. Summary of head entry behaviour of animals that contributed dopamine data on the unconditioned reward session. A. *Bar graphs show session length, total time spent in the food magazine, total number head entries into the magazine and latency to respond after minimum ITI has elapsed during the session for the WT and Met-COMT mice that contributed dopamine data (mean +SEM).* ***B.*** *Latency to respond after minimum ITI has elapsed, split by reduced, standard and increased reward trials (group means +SEM)* ***C.*** *Latency to respond after minimum variable interval has elapsed across the three 8-trial blocks of the session (group means ±SEM).There were no significant genotype effects on any of the measures.*



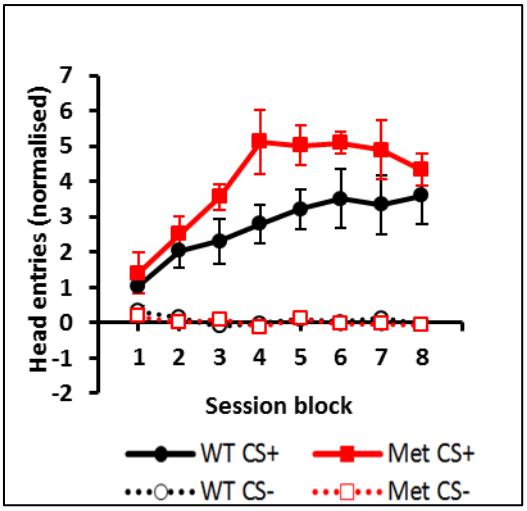
Supplementary Figure S5-3. Preliminary overview of COMT effects on dopamine signal decay. A. *Bar graphs depict the time [s] it took for dopamine signal to decay to 50% of peak on standard reward size trials (*, independent samples t-test, $t_{12} = 2.5$, $p = 0.03$)*
B. *Peak-dependency of half-life of dopamine signal. Scatter plots show time for dopamine signal to decay to 50% (y-axis) against peak height (x-axis). Data was taken from all trial types (all reward sizes). In both genotypes, time to decay to 50% correlated with peak height (Pearson's correlation, $p < 0.001$), however the slope in Met-COMT mice was steeper, suggesting slower decay than in WT mice.*



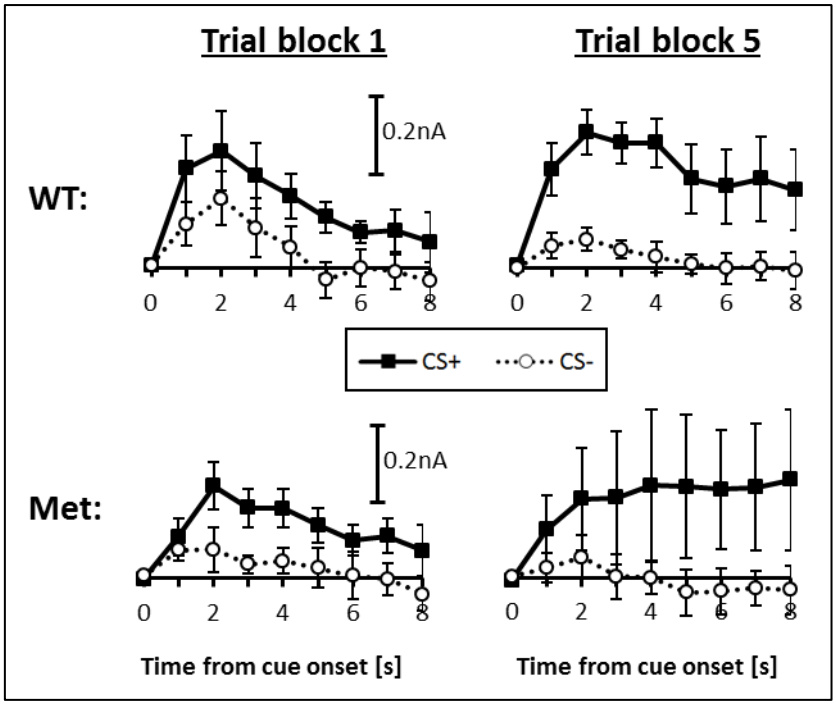
Supplementary Figure S6-1. Magazine head entry duration during cue and pre-cue baseline across 16 Pavlovian training sessions. A. *Average amount of time spent in the food magazine in the 8 seconds preceding auditory cue onset ('baseline', collapsed across all trials).* ***B****. Average amount of time (baseline-subtracted) spent in the food magazine during CS+ and CS- presentations. Data points represent means of blocks of two sessions. Data are mean ± SEM.*



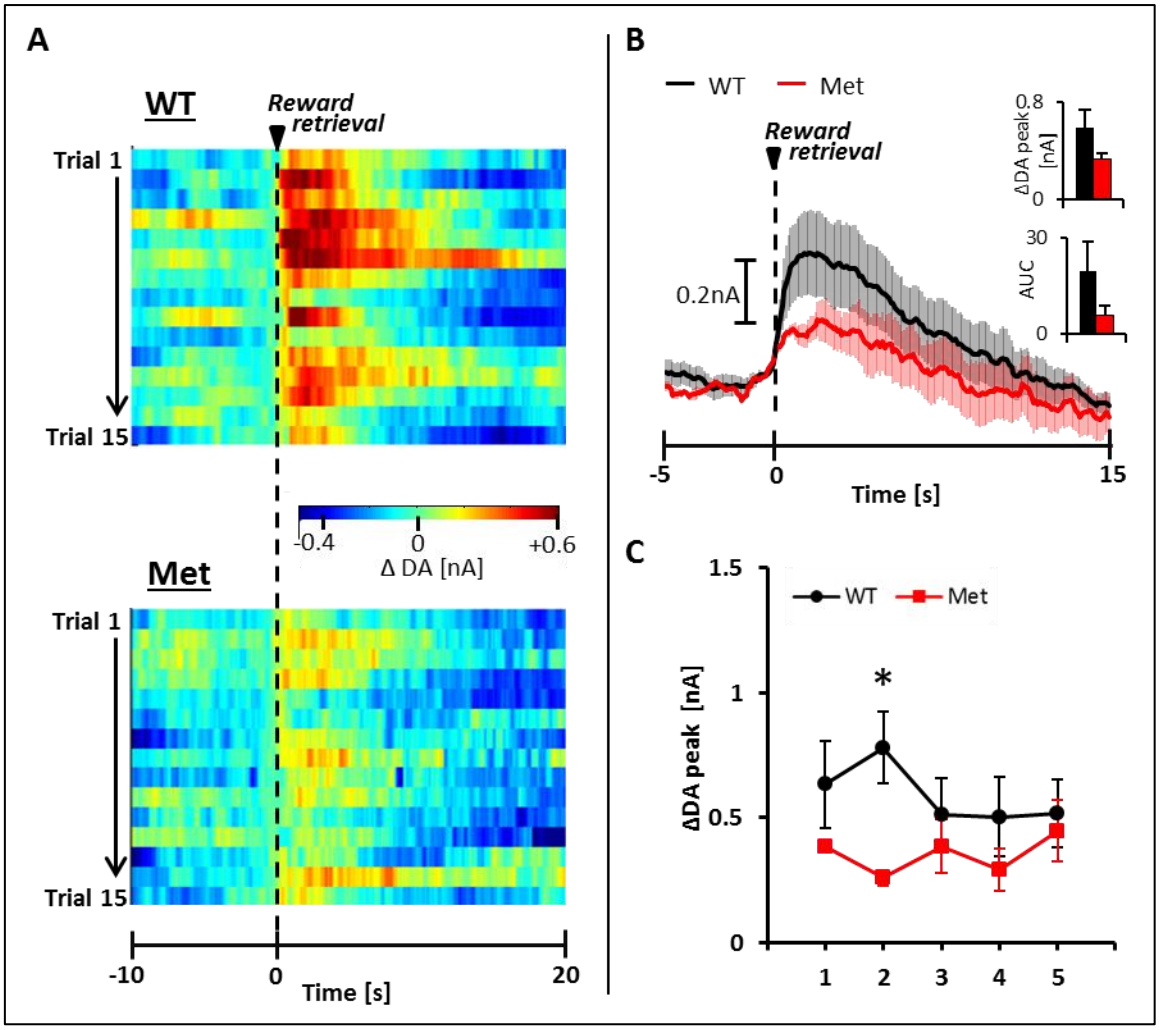
Supplementary Figure S6-2. FSCV recording locations used in Pavlovian conditioning experiment (Chapter 6). *Outlines depict coronal brain sections at 0.98mm (top), 1.18mm (middle), and 1.42mm (bottom) distance from bregma. Placements of recording electrodes are indicated by black (WT) and red (COMT-Met) circles.*



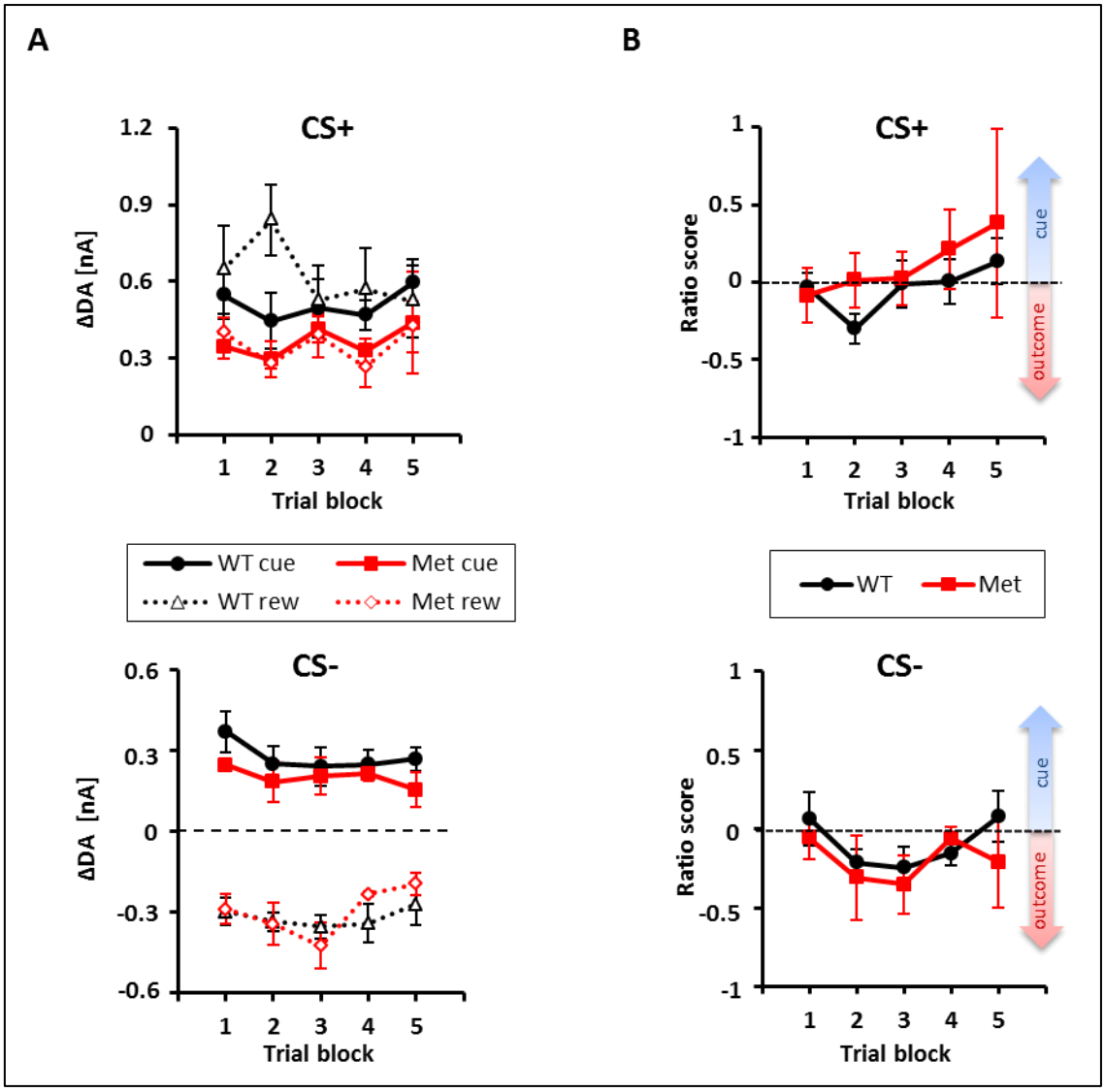
Supplementary Figure S6-3. Overview of behavioural performance of mice contributing to the dopamine data. A. *Normalised magazine responding to CS+ and CS- across the eight 2-session blocks of Pavlovian discrimination learning. Data are mean ± SEM.*



Supplementary Figure S6-4. *Change in dopamine recorded in the course of CS+ and CS- cue presentation from the start of the session (trial block 1, left) to the end of the session (trial block 5, right), in WT (top panel) and COMT-Met (bottom panel) mice. There were no significant effects of or interactions with genotype when these data were analysed using RM ANOVA (all p > 0.48).*



Supplementary Figure S6-5. Dopamine release time-locked and re-baselined at retrieval of reward from magazine. A. *Pseudocolour plots show change in dopamine signal (nA, colour coded) over the course of a trial (x-axis), relative to a 0.5s baseline before the time of reward retrieval on CS+ trials. Each horizontal line depicts one trial, averaged across all animals in the WT (top) and COMT-Met (bottom) groups.* ***B.*** *Dopamine current change in WT (black) and COMT-Met (red) mice time-locked to retrieval of reward from food magazine, averaged across all 15 CS+ trials of the first discrimination learning session. Insets depict mean dopamine peak (top) and area under a 8s window from reward retrieval (bottom).There were no genotype differences (*all $t_9 < 1.4$, all $p > 0.2$*).* ***C.*** *Mean reward-evoked peak dopamine currents [nA] across five blocks of the first discrimination learning session (Met > WT on block 2, RM ANOVA, genotype × block interaction and pairwise comparisons, * $p < 0.05$*). Error bars are ± SEM.*



Supplementary Figure S6-6. Relationship between cue- and outcome-evoked peak dopamine across the five three-trial blocks of the first Pavlovian training session. A. *Peak dopamine signal change evoked by cue (peak within 8s cue, solid lines) and reward outcome (dashed lines) on CS+ (top) and CS- (bottom) trials. In the case of CS+ trials, "reward" refers to peak dopamine change within 8s time-locked to reward retrieval. In the case of CS- trials, "reward" refers to the maximum dopamine suppression from pre-cue baseline in the 8s after CS- termination.* ***B.*** *Within-subject ratio scores showing progression of the relationship between cue- and outcome-evoked dopamine signal change. Scores of > 0 indicate greater cue-evoked signal change while scores of < 0 indicate greater outcome-evoked signal change. In the case of CS- trials, absolute values of ΔDA were used in all analyses and to calculate the ratio score. There were no significant effects or interactions with genotype in any of the measures presented here.*

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