The Role of Serotonin in the Development of Psychiatric Disorders: Studies on Drug Dependence and Anxiety-like Behaviour Following the Genetic Reduction of the Serotonin Transporter

by

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List of Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
5-HTP	5-hydroxy-l-tryptophan
5-HTTLPR	Serotonin transporter polymorphic region
ACTB	Beta-actin
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
cDNA	Complementary deoxyribose nucleic acid
CPP	Conditioned place preference
Cq	Quantification cycle
DA	Dopamine
DAT	Dopamine reuptake transporter
DOPAC	3,4-dihydroxyphenylacetic acid
ENU	N-ethyl-n-nitrosourea
EPM	Elevated plus maze
FST	Forced swim test
GAPDH	Glyceraldehyde 3-phospate dehydrogenase
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
i.p.	Intraperitoneal injection
i.v.	Intravenous injection
l-allele	Long allele of the 5-HTTLPR
MDMA	(±) 3, 4-methylenedioxymethamphetamine
MNE	Mean normalised gene expression
mRNA	Messenger ribonucleic acid
NE	Norepinephrine
NET	Norepinephrine reuptake transporter
NSF	Novelty supressed feeding task
OCD	Obsessive compulsive disorder
pCPA	Para-chlorophenylalanie

PFC	Pre-frontal cortex
PPI	Pre-pulse inhibition
qPCR	Quantitative real time polymerase chain reaction
REM	Rapid eye movement
RT-PCR	Reverse transcription polymerase chain reaction
s-allele	Short allele of the 5-HTTLPR
s.c.	Subcutaneous injection
SEM	Standard error of the mean
$SERT^{-/-}$	Homozygous SERT knock-out
$SERT^{+/-}$	Heterozygous SERT
$SERT^{+/+}$	Homozygous SERT wild-type
SERT	Serotonin reuptake transporter
SLC6A4	Solute carrier 6 member 4
SNP	Single nucleotide polymorphism
SSRI	Serotonin selective reuptake inhibitor
STin2	Tandem repeat in the second intron of the SLC6A4 gene
TAE	Tris(hydroxymethyl)aminomethane-acetate-ethylenediaminetetraacetic acid
TRP	L-tryptophan

Abstract

Rationale: Given the high prevalence and large burden of psychiatric disorders it is imperative to determine the underling etiology in order for better understanding and treatment. The neurotransmitter serotonin (5-HT) has been associated with mental disorders in humans both pharmacologically and genetically. Individuals with the short-allele of a prominent polymorphism within the 5-HT transporter (SERT) show increased incidence of mood disorders and drug dependence. However, whether or not dysregulation in the 5-HT system causes, or is just associated with, psychiatric disorders is impossible to determine from human studies alone. Consequently, it is imperative to employ an animal model of down-regulated SERT function. To better understand the role of 5-HT in drug dependence, the rat's behavioural response to the psychostimulant (±) 3, 4-methylenedioxymethamphetamine (MDMA), a preferentially serotonergically mediated drug, was assessed. Finally, the ability to rescue the anxiety-like phenotype in the SERT$^{-/-}$ rat by altering extracellular 5-HT during early development was also evaluated.

Objective: The primary objective of the current thesis was to determine whether dysregulation of 5-HT is directly linked to the occurrence of psychiatric disorders, particularly drug dependence and anxiety.

Methods: A model of down-regulated SERT function, the SERT knock-out (SERT$^{-/-}$) rat, was used for all experiments in order to determine a causal relationship between 5-HT dysregulation and psychiatric disorders. In Chapter 2, the response of the SERT$^{-/-}$ rats to various tasks usually disrupted by MDMA was assessed. In Chapter 3, the sensitivity of the SERT$^{-/-}$ rats to the reinforcing effects of MDMA was determined using the self-administration paradigm. Finally, in Chapter 4, whether the anxiety-like behaviour of the SERT$^{-/-}$ rat could be rescued through normalising excessive extracellular 5-HT neonatally was assessed. An attempt was also made to determine a mechanism by which 5-HT dysregulation could alter behaviour. To this end, gene expression previously found to be up- or down-regulated in the SERT$^{-/-}$ rat was assessed in the neonatally treated rats.

Results: The results of Chapter 2 indicated the SERT is necessary for MDMA's disruption of startle habituation but not its psychomotor effects. Moreover, for those rats that could discriminate low dose MDMA from saline, genetic removal

of the SERT resulted in the inability to discriminate MDMA from amphetamine, implying that, in these rats, MDMA was now subjectively indistinguishable from amphetamine. Indeed, this alteration also resulted in enhanced sensitivity to the reinforcing properties of MDMA, giving MDMA the qualities of a traditional psychostimulant in SERT$^{-/-}$ rats (Chapter 3). Finally, lowering the excessive 5-HT during neonatal development in SERT$^{-/-}$ rats led to a rescue of mild, but not high, anxiety-like behaviour in males. However, mRNA levels of long 3'NTR BDNF and 5-HT1a, genes associated with neurodevelopment, remained unchanged across genotypes and treatment groups (Chapter 4).

Conclusions: Genetic removal of the 5-HT transporter results in an altered behavioural response to MDMA, in particular an increased sensitivity to its reinforcing properties. However, while the genetic removal of the SERT results in enhanced extracellular 5-HT, the pathological phenotypes present in this rat are likely due to this increase occurring in early development, not its continued presence in adulthood. Overall, these findings contribute to the growing body of literature indicating that enhanced brain 5-HT during early development can lead to pathological behaviour in adulthood.

Chapter 1: General Introduction

Approximately 1 in 3 New Zealanders has experienced some form of mental illness. Of those individuals, approximately half are diagnosed with depression, anxiety, or bipolar disorder (Ministry of Health, 2013; Oakley Browne, Wells, & Scott, 2006). Astonishingly, 47% of the population are predicted to meet the criteria for some form of psychiatric disorder within their lifetime (Oakley Browne et al., 2006). In addition to the emotional burden, the OECD states the direct and indirect costs associated with mental illness, such as treatment costs and loss of productivity, can exceed 4% of Gross Domestic Product (OECD, 2014). Consequently, it is imperative research focuses on reducing this burden. It is important then to understand the underlying etiology of mental illness in order to both allow the creation of more effective targeted treatments and to gain better insight into risk factors.

The neurotransmitter serotonin (5-hydroxytryptamine or 5-HT) has been associated with a wide range of psychiatric disorders. For instance, selective 5-HT reuptake inhibitors (SSRIs, such as fluoxetine, trade name Prozac), which increase synaptic 5-HT through preventing its reuptake back into the presynaptic neuron, are commonly used to treat a myriad of disorders, including major depressive disorder, anxiety disorder, obsessive-compulsive disorder (OCD), bulimia nervosa, and panic disorder. Moreover, SSRI use during pregnancy, particularly during the first trimester, has been associated with autism in boys (Harrington, Lee, Crum, Zimmerman, & Hertz-Picciotto, 2014). 5-HT is also postulated to play a role in drug dependence, with SSRIs found to decrease the reinforcing efficacy of both cocaine and amphetamine in rodents (Carroll, Lac, Asencio, & Kragh, 1990a; Leccese & Lyness, 1984).

5-HT was first isolated and named in 1948 following its recognition as a vasoconstrictor (Rapport, Green, & Page, 1948). Since then, its wide ranging role in the body has been recognised, most notably in neuronal development and the regulation of reward related processes such as emotion, food intake, and sexual desire (Cools, Roberts, & Robbins, 2008; Gaspar, Cases, & Maroteaux, 2003; Pfaus, 2009; Vitalis & Parnavelas, 2003; Wirtshafter, 2001). 5-HT is synthesised in two steps: 1) the essential amino acid l-tryptophan is metabolized into 5-hydroxy-l-tryptophan (5-HTP) by the rate-limiting enzyme tryptophan

hydroxylase; and 2) 5-HTP is converted into 5-HT by the enzyme L-amino acid decarboxylase. Being an essential amino acid, L-tryptophan must be obtained from the diet, with deficiencies leading to depletions of 5-HT in the brain. Following this pathway, 5-HT is then metabolised into 5-Hydroxyindoleacetic acid (5-HIAA). 5-HT is widely expressed throughout the brain, with serotonergic neurons centred in the raphe nuclei projecting to many brain regions, including the cerebral cortex, hippocampus, basal ganglia, and cerebellum. 5-HT neurotransmission is regulated in a number of ways; at least 15 different 5-HT receptors have been discovered that mediate the serotonergic signal. Of these receptors, the most widely spread throughout the brain is the G-protein coupled 5-HT1a receptor. In the raphe nuclei 5-HT1a receptors are mostly autoreceptors located on 5-HT cell bodies: synaptic 5-HT interacts with 5-HT1a autoreceptors, the activation of which leads to hyperpolarisation of the pre-synaptic neuron, resulting in decreased 5-HT release. Extracellular 5-HT is also regulated by the 5-HT reuptake transporter (SERT), which acts by taking 5-HT back up into the presynaptic neuron where it is repackaged into vesicles and recycled, with remaining synaptic 5-HT metabolized by monoamine oxidase A.

Genetic epidemiological studies further support a role for 5-HT in psychiatric illness, demonstrating that genetic disruptions in the serotonergic system can lead to a wide range of psychiatric disorders. The most studied of these genetic disruptions is the serotonin transporter gene-linked polymorphic region (5-HTTLPR). The SERT protein is coded by a single gene, solute carrier 6 number 4 or SLC6A4, located on chromosome 17 (Kenna et al., 2012). Humans exhibit a genetic polymorphism in this gene that results in either a 43 base pair deletion (short-, or s-allele) or insertion (long-, or l-allele) in the promoter region (Heils et al., 1996; Wendland, Martin, Kruse, Lesch, & Murphy, 2006). The presence of the l-allele leads to increased transcriptional efficiency, resulting in increased production of SERT messenger ribonucleic acid (mRNA) and subsequent reuptake of 5-HT from the synaptic cleft (Heils et al., 1996; Lesch et al., 1996). Indeed, cultured human lymphoblast cells transfected with the s-allele exhibit a 50% reduction in [^{3}H]5-HT uptake compared with those homozygous for the l-allele (l/l) (Lesch et al., 1996). Furthermore, post-mortem brain sample analyses indicate that SERT mRNA is lower in s-allele carriers

(Little et al., 1998), while *in vivo* imaging has demonstrated decreased SERT availability in these individuals (Heinz et al., 2000).

The s-allele, carried by approximately 40% of individuals in Caucasian populations, was first associated with neuroticism and has since been associated with related affective disorders, including anxiety, bipolar disorder, and major depression following stressful life events (Caspi et al., 2003; Heils et al., 1996; Lesch et al., 1996; Sen, Burmeister, & Ghosh, 2004) for review see Kenna et al. (2012)). The homozygous s-allele (s/s) genotype has also been associated with resistance to SSRI treatment in depression (Serretti, Kato, De Ronchi, & Kinoshita, 2006). Moreover, the presence of the s-allele has been associated with the abuse of cocaine, methamphetamine and heroin (Cao, Hudziak, & Li, 2013; Enoch, Gorodetsky, Hodgkinson, Roy, & Goldman, 2011; Gerra et al., 2007). While there has been some inconsistency in the replication of the above findings (e.g. Jorm et al. (1998)), it is possible these discrepancies are due to methodological differences, particularly in regard to participant populations and measurements used. For instance, differences between ethnicities can dramatically alter findings. While the s-allele is more prominent in Asian populations than Caucasian, with 70% of Asian individuals carrying the s-allele, Asian individuals are not more likely to suffer from affective disorders (Weissman et al., 1996). Moreover, studies using African American populations demonstrate the l-allele as the risk allele, as opposed to the s-allele (Cao et al., 2013). Additionally, the subjective and indefinite nature of retrospective self-report used to measure variables such as anxiety calls into question the validity of currently described genotype differences. However, when more robust endophenotypes are considered, a genotype effect is still present. For example, s-allele carriers display increased baseline amygdala reactivity that correlates positively with life stress, potentially indicative of heightened rumination (Canli et al., 2006).

Since the discovery of the 5-HTTLPR, other SLC6A4 variants have been discovered and linked to psychiatric disorders, with some revealing previously unknown associations between the 5-HTTLPR and mental illness (Murphy & Moya, 2011). Notably, recent evidence suggests that the bi-allelic distinction typically used when investigating the 5-HTTLPR may underestimate the

associated psychopathology. Although individuals are typically parsed into s-allele carrying versus l/l groups for comparison, an adenosine/guanine single nucleotide polymorphism (SNP), rs25531, predominately found within the l-allele repeat, has been described which separates the l-allele into L_A (high SERT expressing) and L_G (low SERT expressing and comparable to the s-allele (Hu et al., 2005). While the bi-allelic comparison yields no clear association between OCD and either the s- or l-alleles, the L_A/L_A genotype occurs twice as often in individuals with OCD (Hu et al., 2005). While this finding has not been consistent (Wendland, Kruse, Cromer, & Murphy, 2007), when the SNP rs25532 (also found within the 5-HTTLPR) is included in analyses, the allele associated with increased SERT activity is more common in individuals with OCD when occurring alongside the L_A allele (Wendland et al., 2008). Consequently, it is likely that pooling the two l-alleles may underestimate true associations.

Other polymorphisms found within the SLC6A4 gene have also been associated with psychiatric disorders. One more commonly studied is the variable number tandem repeat identified in the second intron (STin2) of the SLC6A4, which contains 7, 9, 10 or 12 repeats of a 17 base pair sequence (Fan & Sklar, 2005). The 12 base pair repeat has been associated with schizophrenia and bipolar disorder, while the 9 base pair repeat has been associated with major depression (Fan & Sklar, 2005; Kunugi et al., 1996; Ogilvie et al., 1996). Additionally, an uncommon polymorphism in the coding region of the SLC6A4, the SNP I425V, has been linked with OCD, Asperger's syndrome, autism spectrum disorder, and anorexia nervosa (Ozaki et al., 2003). Moreover, polymorphisms occurring in the genes of some 5-HT receptors have also been linked to psychiatric disorders e.g. (Seneviratne et al., 2013; Unschuld et al., 2007).

It is important to note, that while many of the polymorphisms occurring in 5-HT associated genes have been linked to particular disorders, the functional consequence of the polymorphism is not always known. For instance, although the STin2 12 base pair repeat is suspected to act as a transcriptional regulator (MacKenzie & Quinn, 1999), its exact biological function remains unknown (Fan & Sklar, 2005). Conveniently, genetically engineered rodent models have been created for which the exact transporter function is known.

Both SERT knock-out ($SERT^{-/-}$) mice and rats, which have no SERT function whatsoever, exhibit similar pathological phenotypes to humans carrying low functioning SERT polymorphisms, as discussed in greater detail below (Holmes, Yang, Lesch, Crawley, & Murphy, 2003; Olivier et al., 2008).

Overall, there is considerable genetic evidence that disruptions in the serotonergic system lead to a wide range of psychiatric impairments. However, the exact mechanism underlying this relationship is unclear. Given the pathological phenotypes found in both s-allele carriers and $SERT^{-/-}$ rodents, one might conclude that, despite the aforementioned association between increased serotonergic function and OCD/alcoholism, down-regulation of the SERT, and the resulting increase in extracellular 5-HT, plays a causative role in the accompanying psychiatric disorders. Paradoxically however, SSRIs, which also lead to an increase in extracellular 5-HT, lead to a *decrease* in anxiety, depression, and the reinforcing efficacy of drugs of abuse (Carroll et al., 1990a; Leccese & Lyness, 1984; Magni et al., 2013). Given that these genetic alterations occur from very early on in development, it is possible that these long lasting functional consequences are in fact adaptations from variations in brain development (Alexandre et al., 2006; Olivier et al., 2008).

Indeed, alterations in 5-HT during development have previously been shown to lead to pathological behaviours in offspring. For instance, neonatal SSRI use has been associated with a 3-fold increase in autism spectrum disorder in boys (Harrington et al., 2014), and increased behavioural problems in 3 year olds (Oberlander et al., 2010). However, no studies have yet tracked offspring into adulthood, so it is unknown whether further problematic phenotypes develop. More importantly, due to the comorbidity of affective disorders such as depression and anxiety in these mothers, it is difficult to ascribe any increased risk in negative outcomes in offspring to prenatal SSRI use directly. However, animal studies, in which it is much easier to both track offspring and control extraneous variables, have also linked early life 5-HT alterations to pathological phenotypes in adulthood (Ansorge, Morelli, & Gingrich, 2008; Ansorge, Zhou, Lira, Hen, & Gingrich, 2004; Forcelli & Heinrichs, 2008; Olivier et al., 2011; Zhang et al., 2006). For instance, postnatally decreasing 5-HT through depletion of the 5-HT precursor L-tryptophan in rats leads to an increase in anxiety- and

depression-like phenotypes in adulthood, as indicated by performance in the forced swim test (FST), and the elevated plus maze (EPM) (Zhang et al., 2006). Moreover, increases in 5-HT following administration of fluoxetine during early development (both pre- and post-natally) produces an anxiety-like phenotype in the novelty suppressed feeding (NSF), open field, and EPM tasks (Ansorge et al., 2008; Ansorge et al., 2004; Forcelli & Heinrichs, 2008; Olivier et al., 2011). Moreover, these offspring show increased sensitivity to cocaine, exhibiting increased cocaine-induced conditioned place preference (CPP) and increased resistance to extinction following cocaine self-administration (Forcelli & Heinrichs, 2008). At the present time there is uncertainty regarding why these disruptions can be associated with very different psychiatric illnesses in humans. It is, for instance, unclear why some s-allele carriers become depressed, while others become addicted to drugs of abuse. It is likely the interaction between these genetic disruptions and the environment plays a role (Caspi et al., 2003).

To better understand why disruptions in the serotonergic system lead to psychiatric illness, genetic disruptions in the SERT must be thoroughly investigated. While it is important to consider data from humans, the necessary correlational nature of the data limits what can be concluded about the causal role of any polymorphisms. Moreover, in addition to the subjective nature of mental illness measures, the multitude of various SNPs within the SLC6A4, as well as further distinct genetic factors, means there is a low level of control over the experimental group. Consequently, it is important to use animal models to complement evidence from human studies. Animal models for the SERT polymorphism include $SERT^{-/-}$ mice (Bengel et al., 1998). $SERT^{-/-}$ mice show a 60-80% reduction in tissue 5-HT, and increased extracellular 5-HT (Bengel et al., 1998; Fabre et al., 2000). Moreover, as with s-allele carriers, they display anxiety-like behaviour, as indicated by their performance in the EPM, open field, and home cage emergence tasks compared to wild type ($SERT^{+/+}$) littermates (Holmes et al., 2003). While results are mixed in tests of behavioural despair (FST, tail suspension test) (Holmes, Yang, Murphy, & Crawley, 2002; Lira et al., 2003), the more robust sucrose preference test, a measure of anhedonia, reveals no depression-like phenotype in $SERT^{-/-}$ mice (Kalueff, Gallagher, & Murphy,

2006). Both cocaine- and (+)-amphetamine-induced locomotor activity is unaltered in SERT$^{-/-}$ mice (Bengel et al., 1998; Sora et al., 2001). Moreover, while SERT$^{-/-}$ mice show enhanced cocaine-induced CPP (Sora et al., 1998), (+)-Methylenedioxymethamphetamine- (MDMA) induced locomotor activity and racemic MDMA self-administration are extinguished (Bengel et al., 1998; Trigo et al., 2007).

Although these mice show high phenotypic similarities with s-allele carriers, there are differences between mice and other species, which leaves some reservations as to what can be concluded. For instance, rats and mice exhibit differential physiological responses to the 5-HT1a antagonist 8-OH-DPAT. While the 5-HT1a antagonist 8-OH-DPAT induces hypothermia in both mice and rats, this is mediated by the presynaptic 5-HT1a auto receptor in the mice, but the postsynaptic 5-HT1a receptor in rats (Bill, Knight, Forster, & Fletcher, 1991). Furthermore, MDMA produces differential long term effects in mice and rats. For instance, racemic MDMA acts as a selective DA neurotoxin in mice, leading to a decrease in striatal DA and its metabolites, as well as significantly reducing DAT, with minimal effects on 5-HT (Colado, O'Shea, & Green, 2004; Kindlundh-Högberg, Schiöth, & Svenningsson, 2007; O'Callaghan & Miller, 1994; O'Shea, Esteban, Camarero, Green, & Colado, 2001). Whereas in humans, non-human primates, and rats, there is evidence that long-term use of MDMA leads to selective 5-HT depletion and a decrease in SERT expression, increasing the addictive potential of MDMA (Buchert et al., 2004; Easton & Marsden, 2006; Kindlundh-Högberg et al., 2007; O'Shea, Granados, Esteban, Colado, & Green, 1998). Thus, the rat model is more consistent with the effects of MDMA in humans. Consequently, it is useful to also consider the behavioural effects of MDMA in a rat model. Such a model was created in 2006 using the mutagen N-ethyl-N-nitrosourea or ENU (Smits et al., 2006). ENU was intraperitoneally injected into male rats resulting in random point mutations, primarily in spermatogonial stem cells. Following breeding with healthy, untreated, females, the first generation was screened for genes of interest, which resulted in the discovery of a female with a point mutation in the SLC6A4 gene. This mutation produced a premature stop codon, resulting in a complete eradication of SERT mRNA from these animals due to nonsense mediated RNA

decay. SERT$^{-/-}$ rats have no SERT protein as indicated by radioactively labelled citalopram binding, and consequently 5-HT uptake is reduced by 72% in hippocampal synaptosomes (increasing to 100% with the addition of a norepinephrine transporter (NET) blocker), and extracellular 5-HT in the hippocampus is increased 9-fold as determined by microdialysis (Homberg et al., 2007). There are no compensatory changes in monoamine oxidase A activity in either the cortex or Caudate Putamen following this increased extracellular 5-HT. Moreover, tissue 5-HT levels are reduced to ~50-75% and 5-HIAA levels are reduced to ~45-55% in the hippocampus, caudate putamen, cortex, and amygdala (Homberg et al., 2007). However, 5-HT neurons in the dorsal raphe nuclei remain unchanged (Olivier et al., 2008). The heterozygous variant (SERT$^{+/-}$) displays a 50% reduction in SLC6A4 resulting in a 40% reduction in radioactively labelled citalopram binding, with 5-HT uptake reduced by 13.4% (Homberg et al., 2007). Both DA transporter (DAT) and NET concentrations, as well as uptake and concentrations of DA, norepinephrine (NE), and their metabolites (aside from NE levels in the amygdala), remain unchanged in SERT$^{-/-}$ *ex vivo* (Homberg et al., 2007). However, *in vivo* the response of SERT$^{-/-}$ rats to NET and DAT blockers is altered compared to SERT$^{+/+}$ rats: In SERT$^{-/-}$ rats, but not SERT$^{+/+}$ rats, the DAT blocker GBR12909 induces prolonged hyperthermia, and low doses of the NET blocker atomoxetine attenuate stress-induced hyperthermia (Olivier, Cools, Deen, Olivier, & Ellenbroek, 2010). Similar to mice, SERT$^{-/-}$ rats display an anxiety-like phenotype in the open field, EPM, home cage emergence, and NSF (males only) tasks (Olivier et al., 2008). A depression-like phenotype is also apparent in the FST and sucrose preference task (Olivier et al., 2008). Moreover, SERT$^{-/-}$ rats are more sensitive to the reinforcing properties of cocaine, as demonstrated by increased self-administration of cocaine at low doses, increased breakpoint for progressive ratio at high doses, and increased cocaine-induced CPP compared with SERT$^{+/+}$ littermates (Homberg et al., 2008). Unlike SERT$^{-/-}$ mice, SERT$^{-/-}$ rats also displayed increased cocaine-induced locomotor activity, which at low doses was potentiated by the 5-HT1a agonist 8-OH-DPAT (Homberg et al., 2008; Sora et al., 2001). It is likely that compensatory changes in the 5-HT1a receptor, likely the desensitisation of the 5-HT1a receptor (Homberg et al., 2008), facilitate the reinforcing effects of cocaine at

low doses. Given their functional and behavioural similarities to humans with the s-allele, SERT$^{-/-}$ rats are a good candidate to model this variant. While it is important to note the heterozygous animals are more representative of the s/s genotype, it is crucial to initially perform tests with the full knockout model to validate any initial hypotheses before moving onto the less extreme version of altered SERT function.

While these findings support the notion that the serotonergic system plays a role in the reinforcing efficacy of cocaine, it is important to note that cocaine is particularly reinforcing. Consequently, increases in the reinforcing efficacy from SERT$^{+/+}$ to SERT$^{-/-}$ are small. In order to better tease apart the underlying consequences of the lack of SERT on drug addiction, it is pertinent that the reinforcing effects of different, less rewarding, drugs of abuse are assessed. A promising candidate is MDMA, the main psychoactive component of the street drug ecstasy. MDMA, commonly used for its ability to produce euphoria, enhanced sensory perception, as well as feelings of well-being and self-confidence, has long been thought of as a safe, non-addictive drug. However, recent studies have shown prevalence rates are increasing, with New Zealanders amongst the highest consumers of MDMA in the world (UNODC, 2012). Moreover, MDMA has been found to be self-administered in rats, mice and monkeys, highlighting its abuse potential (Schenk, 2009). Indeed, while the addictive potential of MDMA is now well recognised, it is lower than that of most other drugs of abuse. While virtually all animals will readily learn to self-administer cocaine, only ~50% will learn to self-administer MDMA (Fantegrossi, Ullrich, Rice, Woods, & Winger, 2002; Schenk et al., 2007). This is likely due to MDMA's larger influence on the serotonergic system compared with other psychostimulants (Cole & Sumnall, 2003; Crespi, Mennini, & Gobbi, 1997; Han & Gu, 2006). While MDMA exerts its effects by blocking and inducing reverse transport of SERT, DAT, and NET, it has a much higher affinity for SERT relative to DAT, in contrast with cocaine, amphetamine, and methamphetamine, which all have a higher affinity for DAT (Han & Gu, 2006). It has repeatedly been shown that increasing 5-HT leads to a reduction in the rewarding properties of drugs of abuse (Baumann et al., 2011; Carroll et al., 1990a), while decreasing 5-HT leads to an increase in such properties (Carroll, Lac, Asencio, & Kragh,

1990b; Leccese & Lyness, 1984; Pelloux, Dilleen, Economidou, Theobald, & Everitt, 2012). Indeed, previous studies indicate 5-HT inhibits DA-mediated effects (Baumann et al., 2011; Rothman & Baumann, 2006). However, the exact role of 5-HT in regulating drug intake is still unknown.

Taking into consideration MDMA's increased affinity for the 5-HT system, and 5-HT's dampening on the dopaminergic effects of drugs of abuse, it would be interesting to see whether SERT$^{-/-}$ rats display an increased propensity to self-administer MDMA. While it is curious that SERT$^{-/-}$ mice failed to self-administer MDMA, this bears repeating in a rat model given the aforementioned differences between mice and rats in the response to MDMA (Kindlundh-Högberg et al., 2007; Koch & Galloway, 1997; O'Shea et al., 2001). While abuse of MDMA has not been considered in individuals with low activity 5-HTTLPR genotypes, use of MDMA is not significantly higher in individuals with the s-allele (Martín-Santos et al., 2010; Roiser, Cook, Cooper, Rubinsztein, & Sahakian, 2005), although there was a trend for increased use in s/s genotypes in Martín-Santos et al. (2010). Given that these studies used the biallelic distinction of the 5-HTTLPR, which fails to account for additional l-allele polymorphisms that also lead to a reduction in SERT activity, any existing differences may be underestimated. Moreover, there is some evidence these individuals may be more sensitive to the cognitive effects of ecstasy use, with s-allele carrying ecstasy users displaying deficits in attention and decision making (Cuyàs et al., 2011; Roiser et al., 2005; Roiser, Rogers, Cook, & Sahakian, 2006). Additionally, ecstasy using s/s individuals report increased sedation compared to l-allele carriers following ecstasy, and are more likely to suffer from non-substance-induced mood disorders such as depression (Martín-Santos et al., 2010; Pardo-Lozano et al., 2012; Roiser et al., 2005). Given the small number of studies and their correlational nature, it is difficult to draw a firm conclusion from these data, other than to say there are indications that s-allele carriers react differently to ecstasy than l/l controls.

It is possible that decreased SERT activity influences some of the behavioural effects of MDMA while leaving others uninterrupted. Indeed, MDMA studies in rodents have indicated that different monoamines govern different behavioural effects. For instance, MDMA disrupts attentional processes such as

habituation to a loud stimulus, which appears to be mediated by 5-HT (Kehne et al., 1992). However, increases in locomotor activity appear to be governed by both DA and 5-HT mechanisms (Daniela, Brennan, Gittings, Hely, & Schenk, 2004; Kehne et al., 1996). Consequently, it is important to determine which of the behavioural effects of MDMA are altered in the SERT$^{-/-}$ rat.

While confirming and extending the phenotype of SERT$^{-/-}$ rats is important, it is also crucial to determine the mechanisms underlying this alteration. As mentioned above, it is believed that the increased 5-HT during development is responsible for the pathological phenotype that characterises humans and rats with low SERT expression. Consequently, it is important to establish whether early intervention can prevent the pathological phenotype associated with the genetic reduction of SERT. An elegant study in SERT$^{-/-}$ mice has yielded promising results: The administration the 5-HT neurotoxin para-chlorophenylalanine (pCPA) during early development was found to attenuate the effects of reduced SERT function on rapid eye movement (REM) sleep in adulthood in SERT$^{-/-}$ mice (Alexandre et al., 2006)**.** This finding indicates that 'normalising' extracellular 5-HT levels during development may rescue the pathological phenotype. While the effect of normalising 5-HT levels during development on anxiety- and depression-like behaviours in SERT$^{-/-}$ rodents has not yet been attempted, the same study found that when SERT$^{-/-}$ mice were treated with the 5-HT1a receptor antagonist WAY 100635 during early development their depression-like behaviour was normalised in the tail suspension test, indicating that prevention of 5-HT1a stimulation can reverse the depression-like phenotype (Alexandre et al., 2006). Given 5-HT's wide ranging role during development, including neuronal migration, division, and differentiation (Gaspar et al., 2003), it is likely there could be many mechanisms underlying this change. However, no one has yet attempted to rescue the phenotype for SERT$^{-/-}$ rats, or has attempted to elucidate precisely why increased extracellular 5-HT during development causes this adult phenotype.

Current thesis

Given the state of the literature, the current project has three parts, which will be addressed consecutively in the following three chapters:

1) Although MDMA increases 5-HT, DA, and NE, it is unclear in which way the relative contributions play a role in the behavioural efficacy of MDMA in the brain. This will be investigated in Chapter 2 by looking at the effect of MDMA in the $SERT^{-/-}$ rat on various behavioural tasks with a predominant serotonergic or dopaminergic mechanism of action.
2) As $SERT^{-/-}$ rats are more sensitive to the reinforcing effects of cocaine, but $SERT^{-/-}$ mice fail to self-administer MDMA, Chapter 3 will seek to determine whether $SERT^{-/-}$ rats are more likely to self-administer MDMA than $SERT^{+/+}$ rats, and are therefore more sensitive to the reinforcing effects of MDMA.
3) To further elucidate the theory that increased 5-HT during development plays a role in adult phenotype, experiments presented in Chapter 4 will determine whether decreasing 5-HT in $SERT^{-/-}$ rats during development will rescue their anxious phenotype. Further to this, the thesis will attempt to elucidate the mechanism of a possible change by establishing if increased early life 5-HT leads to alterations in the mRNA of chosen candidate genes.

Chapter 2: The role of serotonin in the behavioural effects of MDMA

The subjective effects of (±) 3,4-methylenedioxymethamphetamine (MDMA), namely feelings of wellbeing, closeness, and empathy, differ from those of classical psychostimulants such as cocaine and amphetamine. This dissociation is likely due to the distinct pharmacology of MDMA. While most drugs of abuse primarily exert their actions through an increase in dopamine (DA), MDMA preferentially increases serotonin (5-HT) compared with DA and norepinephrine (NE) (Han & Gu, 2006). This increased potency for the 5-HTsystem likely underlies the decreased abuse potential of MDMA compared to cocaine and amphetamine. While close to 100% of animals will learn to press a lever for an intravenous infusion of either cocaine or amphetamine, only around 50% will learn this for MDMA (Schenk, Gittings, Johnstone, & Daniela, 2003). In addition to its propensity to be self-administered by rats, MDMA has a complex behavioural profile. For instance, it modifies attention and movement (Baumann, Clark, & Rothman, 2008; Kehne et al., 1992; Padich, McCloskey, & Kehne, 1996). However, while MDMA increases release of 5-HT, DA, and NE, it is unclear what the relative contribution of each of these neurotransmitters is in regulating behaviour. So far, this has almost exclusively been studied using pharmacological tools, i.e. by more or less selectively blocking specific receptors.

First, MDMA disrupts attentional processes (Kehne et al., 1992; Padich et al., 1996). For instance, acute MDMA interrupts sensorimotor gating, the regulation of sensory information to the motor cortex (Padich et al., 1996). Sensorimotor gating is commonly measured through pre-pulse inhibition (PPI), which refers to the ability of a weak stimulus immediately preceding a startle stimulus to inhibit the usual response to that startle stimulus (Graham, 1975). Interruption of auditory PPI by MDMA appears to be governed by 5-HT, with blockade of the 5-HT2a receptor, but not the DA D2 receptor, attenuating MDMA-induced disruption of auditory PPI (Padich et al., 1996). Also interrupted by MDMA is startle habituation, the eventual habituation to a startle stimulus following repeated exposure (Moyer, 1963). MDMA dose dependently decreases habituation to both acoustic and tactile startle stimuli, an effect prevented by both serotonergic neurotoxicity as well as the SSRI fluoxetine, but not by NE

transporter or DA D2 receptor blockade (Kehne et al., 1992). Overall, these particular attentional processes appear to be serotonergically mediated. However, these conclusions are tentative given the limited number of studies performed.

Second, MDMA has been demonstrated to alter locomotor activity (Baumann et al., 2008; Colussi-Mas & Schenk, 2008; Daniela et al., 2004; Gold, Koob, & Geyer, 1988; Kehne et al., 1996; Spanos & Yamamoto, 1989). For instance, acute MDMA dose dependently increases forward locomotion as well as stereotyped behaviours characteristic of 5-HT syndrome, such as forepaw treading, head weaving and low body posture (Gold et al., 1988; Spanos & Yamamoto, 1989). It is unclear however, whether this alteration of movement is mediated by serotonergic or dopaminergic mechanisms. For instance, increases in both 5-HT and DA have been correlated with MDMA-induced increases in stereotypy and ambulation (Baumann et al., 2008). Moreover, blockade of the either the DA D1 or the 5-HT2a receptor abolishes MDMA-induced locomotor activity in rats (Daniela et al., 2004; Kehne et al., 1996). Repeated exposure to MDMA produces a sensitised locomotor response (Colussi-Mas & Schenk, 2008; Ramos, Goñi-Allo, & Aguirre, 2004; 2005 2005; Spanos & Yamamoto, 1989), which also appears to involve a complex interaction between 5-HT and DA. For instance, while blockade of the DA D1 receptor by SCH23390 prevented the expression of MDMA-induced sensitisation, follow up studies have indicated this effect is due to the action of this antagonist on 5-HT2A receptors, not the D1 receptor (Ramos et al., 2004, 2005). Thus these findings demonstrate a complex behavioural profile for the involvement of MDMA in movement yet to be fully elucidated.

Last, as mentioned, the subjective effects of MDMA differ from those of classical psychostimulants. Indeed, in the drug discrimination paradigm, in which rats are taught to associate an operant response with a particular substance, rats can learn to differentiate MDMA and *d*-amphetamine (Goodwin & Baker, 2000; Harper, Langen, & Schenk, 2014), indicating the subjective properties of MDMA and amphetamine dissociate, at least at low doses of MDMA. While this makes sense given the preferential serotonergic affinity of MDMA, when rats are trained to distinguish MDMA from saline, *d*-amphetamine

partially generalises to MDMA (Oberlender & Nichols, 1988; Schechter, 1988), although MDMA does not generalise to *d*-amphetamine in *d*-amphetamine trained rats (Oberlender & Nichols, 1988). More recent investigations have revealed that, while doses of MDMA equal to and lower than the MDMA training dose generalise to MDMA, higher doses generalise to amphetamine (Harper et al., 2014), suggesting a complex interaction between 5-HT and DA in the subjective profile of MDMA. Indeed, both DA and 5-HT antagonists have been found to decrease the discriminative stimulus properties of MDMA in MDMA trained rats (Schechter, 1988). However, the relative contribution of DA and 5-HT in the subjective effects of MDMA is still far from clear.

Pharmacological studies are widely criticised for their non-specificity. For instance the D1 receptor antagonist SCH23390 also binds to the D5, 5-HT1a, and 5-HT2c receptors (Millan, Newman-Tancredi, Quentric, & Cussac, 2001; Ramos et al., 2005). In addition, there are 5 dopaminergic receptors and at least 15 5-HT receptors. Since MDMA is an indirect agonist (i.e. it enhances the release of 5-HT and DA), it could, in theory, act on all these receptors. Moreover, in the locomotor activity paradigm, it is possible that some antagonists have locomotor depressant effects when administered alone, such as DA D2 receptor antagonists (Millan, Seguin, Gobert, Cussac, & Brocco, 2004), an effect difficult to ascertain due to the low baseline locomotion in saline comparison groups. It is desirable then to use a method known not to have locomotor depressant effects. Therefore, in order to further discern the relative contributions of 5-HT, DA, and NE in the behavioural effects of MDMA, we decided to make use of a genetic model, the 5-HT reuptake transporter knock-out ($SERT^{-/-}$) rat. It has been shown previously that this rat lacks a functional SERT and as a result is insensitive to the effects of the selective 5-HT releaser fenfluramine (Homberg et al., 2008). Consequently, it is anticipated that behaviours in which the disruption by MDMA has been previously proposed to be serotonergically mediated (PPI, startle habituation, possibly stereotypy) will be abolished in these rats. Given the apparent role of both 5-HT and DA in forward locomotion following MDMA, the $SERT^{-/-}$ rat will be useful in determining whether 5-HT is necessary for the locomotor activating and/or sensitising effects of MDMA. Finally, given that MDMA is unable to release 5-HT in these animals whatsoever,

it is anticipated that the subjective effects of MDMA would resemble those induced by amphetamine and thus in the drug discrimination paradigm, we predict that amphetamine be indistinguishable from MDMA in the $SERT^{-/-}$ rats.

Method

Subjects

Serotonin transporter knockout rats (SLC6A4^{1Hubr}, SERT$^{-/-}$) were originally created via N-ethyl-N-nitrosourea (ENU)-induced mutagenesis in a commercial (Harlan, Ter Horst, The Netherlands) wild-type Wistar rat (Smits et al., 2006). Experimental animals were generated by breeding pairs of SERT$^{-/-}$ or wild-type (SERT$^{+/+}$) animals who had been bred by crossing heterozygous animals (SERT$^{+/-}$) that had been outcrossed for at least 10 generations. Parent animals had been previously genotyped at 3 weeks of age. Ear samples were taken following the administration of anaesthesia produced by isoflurane and used for genotyping (See Smits et al. (2006) for a more detailed description). Experimentally naïve adult male rats were used for consecutive experiments. Female rats were not used in the current set of experiments due to unpublished findings from our lab that indicate self-administered MDMA significantly increased mortality in female rats. Animals were bred and reared at the vivarium of the School of Psychology at Victoria University of Wellington. Animals were housed in groups of 3 to 6 until they reached 200 - 250 g, at which point they were housed in pairs in hanging polycarbonate cages in a temperature (19 - 21 ^{o}C) and humidity (55%) controlled room. For PPI and locomotor activity testing, animals were kept on a normal 12 hr light/dark cycle (lights on from 07.00 – 19.00 h), with testing being conducted during the light phase. For drug discrimination testing, animals were kept on a reverse 12 hr light/dark cycle (lights on from 19.00 – 07.00h), with testing being conducted during the dark phase. Aside from testing hours, water and food (aside from the duration of the drug discrimination paradigm) was available *ad libitum*. All testing environments were temperature (19 - 21 ^{o}C) and humidity (55%) controlled. All protocols were approved by the Victoria University of Wellington Animal Ethics Committee (AEC-2012R2).

Apparatus and procedure

Pre-pulse inhibition and startle habituation. Twenty-nine experimentally naïve adult male rats (14 SERT$^{-/-}$, 15 SERT$^{+/+}$) weighing 200 - 250 g were used (7 - 8 per group). Tests were conducted in standard startle

equipment (San Diego Instruments, San Diego). These consisted of a clear Perspex cylinder, 6" (L) x 2 1/4" (ID), mounted on a platform with a piezo electrical element, housed in a sound-attenuated chamber. Trial delivery and data acquisition were controlled by an interfaced microcomputer using a commercially available software package (SR-LAB™ Startle Response System, San Diego Instruments, San Diego).

Sessions were conducted between 11:00 and 15:00 hours. Fifteen minutes prior to the session start, rats were injected with 5 mg / kg intraperitoneal (i.p.) MDMA or vehicle, and then placed back in their home cage. They were then placed in the cylinder for the duration of the sessions. Each session was of 20-minute duration, and began with 5 minutes of habituation (white noise 70 dB) followed by 71 trials. The session both began and finished with 5 startle trials (120dB, 20ms). In between these blocks were pre-pulse inhibition trials, consisting of 72, 74, 78, and 86 dB stimuli (20 ms) preceding a startle stimulus by 100 ms, as well as further startle stimuli, and 'no stimulus' trials. A startle response in response to the startle stimuli was measured. Given the results of the first set of trials, the experiment was repeated using 10 mg / kg i.p. following a 1 week wash-out period. In order to minimise any sustained effect of the acute dose of MDMA, those animals who had previously received saline received MDMA, and vice versa.

Locomotor Activity. Adult male rats that had been used previously for PPI were given 2 weeks off, and then used for locomotor activity. In order to minimise drug effects, those animals that had been administered saline in the last 10 mg / kg MDMA in the PPI trial were allocated to the MDMA group, and vice versa. All apparatus and procedures used were identical to those used previously in our lab (Colussi-Mas & Schenk, 2008). Locomotor activity was assessed in open field activity systems consisting of eight clear Plexiglas activity chambers (42 x 42 x 30 cm) set in sound-attenuated chambers (ENV-515-16, Med Associates Inc., Vermont). Location was tracked using 2 pairs of 8 evenly spaced infrared sources and sensors positioned perpendicular to one another around the chambers, and recorded by a commercially available software package (Med-PC IV, Med Associates Inc., Vermont). The infrared beams divided the chamber into squares, with a region set to the approximate dimensions of a

rat (3 x 3 squares) generated around the rat. Any beam breaks within this region were counted as stereotypy. These movements have previously been shown to predominantly comprise of forepaw treading and head weaving (Baumann et al., 2005). One ambulatory count was registered if 3 beams were successively broken within 1000ms outside the region. A white noise generator masked extraneous auditory disturbance during testing, and the room was illuminated with red light. Prior to and after each behavioural test session, the chamber interiors were cleaned and wiped with Virkon 'S' disinfectant (Southern Veterinary Supplies, Palmerston North, New Zealand).

On each day of testing, animals were first habituated to the activity chambers for 30 min. They were then injected with 2.5 mg / kg i.p. MDMA or saline (n = 7 - 8 per group) once daily for 5 consecutive days, and then placed into the activity chamber for 2 hours. This dose was chosen as it was both relatively low while still being behaviourally active within this paradigm (Spanos & Yamamoto, 1989). Following a two-day withdrawal period, animals were given a challenge injection of saline or MDMA (2.5 mg / kg, i.p.).

Drug discrimination. Fourteen adult male rats (7 $SERT^{-/-}$, 7 $SERT^{+/+}$) that had been used previously for PPI and locomotor activity testing were given 3 weeks off, and then used for drug discrimination. Water was available *ad libitum* while commercial rat chow was rationed to maintain 85 - 90% free feeding weight. All training and testing took place in standard operant conditioning chambers measuring 28 x 21 x 21 cm equipped with three retractable levers (ENV-008, Med Associates Inc., Vermont). A sucrose pellet delivery apparatus was located in the centre front panel, while one lever was situated to the right and one to the left. A third lever was located at the centre of the back panel but was not used for the present experiment. Standard 100 mA white lights were situated above every lever. Sucrose pellet delivery and data acquisition were controlled by an interfaced microcomputer using a commercially available software package (Med-PC IV, Med Associates Inc., Vermont). Sucrose pellets (45 mg Dustless Precision Pellets, F0042) were obtained from Bio Serv (Frenchtown, NJ).

Training began with 14 days of 30-minute autoshaping sessions which train the rat to associate the pressing of a lever with a sucrose reward. During

autoshaping sessions one of the two levers (randomly determined) was inserted into the chamber and the light above was illuminated for 12 seconds. If subjects responded, or 15 seconds had elapsed (whichever occurred first), they were rewarded with a sucrose pellet. Thirty seconds later a new trial was initiated. Subjects could receive a maximum of 60 pellets in each session. Following 10 days, the interval for which a free pellet was dispensed following a failure to respond was increased to 60 seconds for four sessions in order to increase responding in two subjects. Finally, the fixed ratio, or the number of responses required to obtain a reward, was increased to 3, 5 and then 10 over three additional sessions. All subjects reliably responded following autoshaping.

Discrimination training then commenced. Rats were administered MDMA (1.5 mg / kg) or saline i.p. at the start of each session. Training doses were selected on the basis of previous research (Goodwin & Baker, 2000; Harper et al., 2014; Schechter, 1988). MDMA and saline were given in pseudorandom order, with each condition administered on no more than two consecutive days. Sessions began with 15 minutes of no trials in order to allow the drug to take effect, and continued until either 60 reinforcers were gained or 45 minutes had elapsed. For the first 15 sessions training was errorless, with only the drug appropriate lever presented. Following this, both levers were presented simultaneously, though only responses on the drug appropriate lever led to reward. Responses made on the alternate lever reset the response counter.

All rats were run between 13:00 and 15:00 hours Monday - Friday, with drug-lever selection differing in half of the subjects. Subjects were required to meet a criterion of 85% drug appropriate lever responses, prior to the delivery of the first reinforcer, over 3 consecutive sessions in order to move onto the testing procedure. If animals had failed to meet discrimination criterion following 55 training sessions they were deemed not to have met criterion.

After subjects met criterion, generalization tests with 0.5 mg / kg amphetamine i.p. were conducted. Sessions were identical to those performed in training, however sessions ceased following 10 responses and no reinforcers were given. Probe sessions were run 2 - 3 times with at least two standard training days in between. If the subject did not maintain 85% accuracy

generalisation tests were not recommenced until at least 2 consecutive discrimination days, including both MDMA and saline, met the 85% criterion. Data were collapsed across different sessions for the same probe condition.

Drugs

MDMA HCl and *d*-amphetamine were both obtained from Environmental Science and Research (Porirua, New Zealand). All drugs were dissolved in a sterile solution of 0.9% physiological saline and administered at a volume of 1 ml / kg body weight. All weights refer to the anhydrous salt and not the acid.

Data Analysis

All statistical analyses were performed using IBM SPSS Statistics version 22 for windows. The alpha level for statistical significance was set at $p < .05$.

Pre-pulse inhibition and startle habituation. To determine there was no interaction between PPI intensity and either genotype or treatment, a repeated measures analysis of variance (ANOVA) was initially run. Upon confirming there were no interactions present, PPI intensities were pooled and further ANOVA's run. ANOVA tests were used for all analyses with genotype and treatment as fixed factors. Following this, univariate ANOVA tests were used to ascertain treatment effects within each genotype, with treatment entered as the between subjects factor. PPI scores were calculated by dividing the mean of all the PPI scores (2, 4, 8, and 16 dB) by the mean of the startle trials within the middle block and deriving a percentage change in response. Mean basal startle response was calculated using the average startle response following the first block of 120 dB stimuli. The startle habituation score was measured as the percentage change in basal habituation between the first and last blocks of startle stimuli. Post hoc tests, where appropriate, employed Tukey's Honestly Significant Difference test. The Greenhouse-Geisser correction was employed in all cases where sphericity was violated

Locomotor activity. To determine whether groups differed in their baseline locomotor activity, activity during the first 30 minutes (prior to drug administration) was analysed using a univariate ANOVA, with Tukey's Honestly Significant Difference test used during post hoc analyses. To determine whether

genotypes differed overall, genotype groups were pooled (regardless of yet to be experienced drug condition), and another univariate ANOVA was run. A multivariate ANOVA was employed to determine any post treatment differences following both acute and repeated MDMA, with genotype and treatment entered as between subjects' factors. Follow up *t*-tests were employed where appropriate. To determine whether repeated MDMA resulted in locomotor sensitisation, paired *t*-tests comparing the ambulatory counts and stereotypic counts following acute treatment and challenge after successive treatment of each genotype were undertaken. Extreme outliers were identified using SPSS's outlier test and removed.

Drug discrimination. Genotype differences in the proportion of rats who met the discrimination criterion were analysed using a Kaplan-Meyer survival curve analysis with genotype entered as the between subjects variable. Response rate was also assessed. A measure of average responses per second was obtained by averaging the time each subject took to complete the first fixed ratio 10 trial during their final three discrimination sessions. An independent samples *t*-test was used, with genotype entered as the between subject variable. The data from one $SERT^{-/-}$ animal was excluded due to technical difficulties in the recording of latency data. Further to this, proportion of responses made on each lever was assessed by determining the percentage of responses made on the saline and MDMA appropriate levers during the 10 responses in the amphetamine probe session. A mixed repeated ANOVA was used, with lever as the within-subject variable and genotype as the between subject variable. In the case of a significant main effect or interaction, post hoc *t*-tests were used to determine the source of the significance, with equal variances not assumed if Levene's test for equality of variances was significant.

Results

Pre-pulse inhibition and startle habituation

As animals were exposed to different treatments following the first set of trials, it was first ascertained whether or not the repeated exposure to stimuli affected either PPI or startle habituation in the saline group. For PPI scores there were no significant differences between genotype $F_{1,25} = .00$, $p = .98$, day of trial, $F_{1,25} = 3.56$, $p = .07$, or interaction, $F_{1,25} = 1.25$, $p = .27$. Additionally for startle habituation scores there were no significant differences for genotype, $F_{1,25} = .17$, $p = .68$, day of trial $F_{1,25} = .93$, $p = .34$, and no interaction, $F_{1,25} = .23$, $p = .63$. Consequently, the data for saline treatments between experiments were amalgamated.

Table 2.1 displays mean basal startle responses of each genotype following pre-treatment with either saline, 5, or 10 mg / kg MDMA. No main effect of genotype, $F_{1,52} = 3.06$, $p = .09$, or treatment, $F_{1,52} = .18$, $p = .84$, was found, as well as no interaction, $F_{1,52} = .54$, $p = .59$, suggesting there are no differences between groups in terms of basal startle response.

Table 2.1.

Basal startle response in SERT $^{+/+}$ and SERT $^{-/-}$ animals following pre-treatment with either saline (14 - 15 per group), 5 or 10 mg / kg MDMA i.p (7 - 8 per group).

Treatment	SERT $^{+/+}$	SERT $^{-/-}$
saline	344 ± 95.1	548 ± 124.6
5 mg / kg MDMA	285 ± 92.8	605 ± 127.2
10 mg / kg MDMA	362 ± 115.4	392 ± 135.1

While a significant effect of PPI intensity was observed, $F_{2.38,123.98} = 52.96$, $p = .00$, there were no significant interactions present (Intensity by genotype: $F_{2.38,1\ 23.98} = 1.09$, $p = .35$; intensity by treatment: $F_{4.77,1\ 23.98} = .86$, $p = .51$; intensity by genotype by treatment $F_{4.77,1\ 23.98} = .52$, $p = .75$). Consequently, PPI intensity was pooled into total PPI scores. Figure 2.1 displays the total PPI scores. MDMA failed to significantly disrupt PPI $F_{2,52} = .48$, $p = .62$, and this

pattern was consistent across genotype, $F_{1,52} = .00$, $p = .99$. Additionally, no interaction was found $F_{2,52} = 1.36$, $p = .27$.

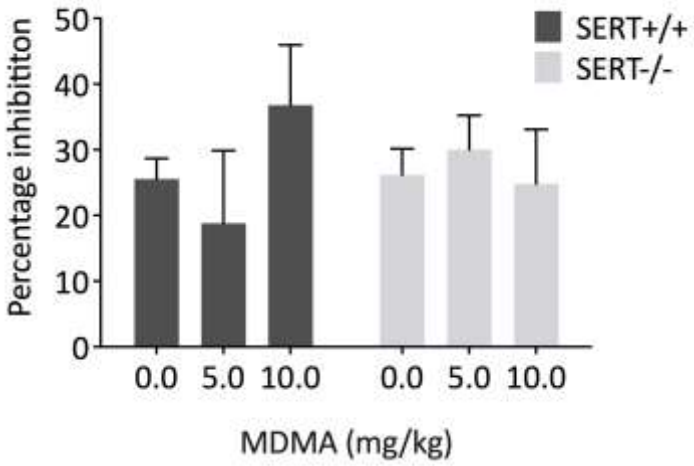

Figure 2.1. Percentage inhibition for SERT$^{+/+}$ and SERT$^{-/-}$ animals injected with saline (0 mg/kg), 5 mg / kg or 10 mg / kg MDMA i.p. Values were obtained by dividing pre-pulse inhibition scores by the mean of the startle trials. n = 7 – 8 per MDMA group, n = 14 -16 per saline group. Bars represent the mean (+SEM).

Figure 2.2 displays the percentage change in basal habituation between the first and the second block of startle stimuli. A significant effect of genotype, $F_{1,52} = 10.42$, $p = .002$, and dose, $F_{2,52} = 4.12$, $p = .02$, was found, as well as a significant interaction, $F_{2,52} = 5.12$, $p = .01$. This effect was driven by a significant difference in SERT$^{+/+}$ rats, $F_{2,25} = 4.75$, $p = .02$. Follow up tests indicate that for SERT$^{+/+}$ animals there was a significant decrease in startle habituation from the first to the second startle stimulus blocks following 5 mg / kg MDMA, but not 10 mg / kg, compared to saline ($p = .02$), suggesting MDMA disrupts normal startle habituation. For SERT$^{-/-}$ animals however, there was no significant effect of MDMA dose on habituation to the startle stimuli, $F_{2,27} = .19$, $p = .83$, indicating that MDMA does not affect startle habituation in animals lacking the SERT.

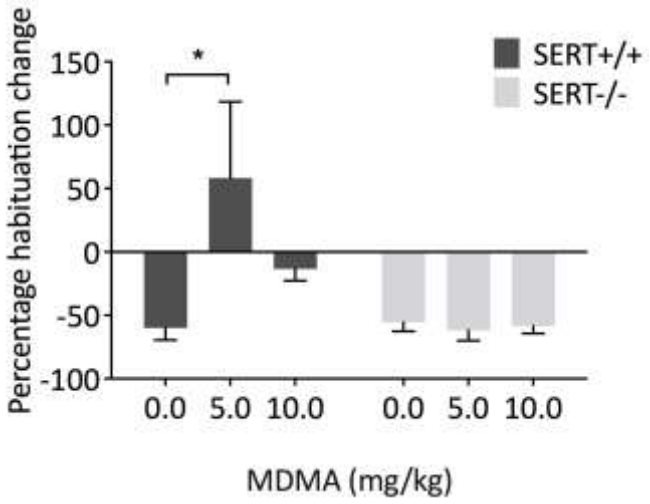

Figure 2.2. Percentage change in habituation between the first and second blocks of startle stimuli for SERT $^{-/-}$ and SERT $^{+/+}$ animals injected with saline (0 mg / kg), 5 mg / kg or 10 mg / kg MDMA i.p. Negative values denote decreased mean startle response to startle stimuli from the first to second blocks. n = 7 – 8 per MDMA group, n = 14 -16 per saline group. Bars represent the mean (+SEM). * p < .05.

Locomotor Activity

Baseline activity. As expected, baseline ambulatory counts between animals that would be receiving saline or drug within each genotype did not differ (SERT$^{+/+}$: t_{12} = .1, p = .92; SERT$^{-/-}$: t_{12} = -2.03, p = .07). Consequently, genotypes were pooled for analysis (Figure 2.3a). Pooled analysis revealed a significant difference between genotype, t_{26} = -3.15, p = .01, with an increase in baseline ambulation for SERT$^{-/-}$ rats.

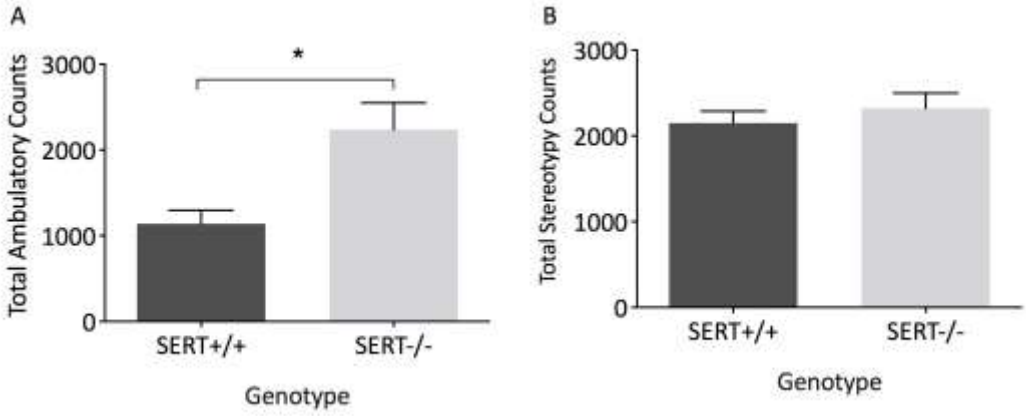

Figure 2.3. A) Baseline ambulatory counts in the open field for both SERT $^{+/+}$ and SERT $^{-/-}$ animals; and B) Baseline stereotyped behaviour in the open field for both SERT $^{+/+}$ and SERT $^{-/-}$ animals. N = 7 – 8 per group. Bars represent the mean (+SEM). *p < .05

Additionally, baseline stereotypy counts between animals that would be receiving saline or drug within each genotype did not differ either ($SERT^{+/+}$: t_{12} = -.03, p = .98; $SERT^{-/-}$: t_{13} = -1.22, p = .25), and so genotypes were also pooled for analysis (Figure 2.3b). These analyses indicated no genotype differences were present, t_{27} = -.76, p = .46.

Acute MDMA. Figure 2.4a shows the locomotor response to acute 2.5 mg / kg MDMA or saline (0.0 mg / kg MDMA). MDMA increased locomotor activity in both genotypes, $F_{1,25}$ = 8.06, p = .01, however, although this effect appears to be diminished in $SERT^{-/-}$ rats, no effect of genotype was present, $F_{1,25}$ = .16, p = .69, and no significant interaction was observed, $F_{1,25}$ = .70, p = .41.

Acute MDMA also increased stereotyped behaviour, Figure 2.4c, $F_{1,25}$ = 13.60, p < .001, and a difference between genotypes was also observed, $F_{1,25}$ = 5.86, p = .02, though no interaction was found $F_{1,25}$ = 1.26, p = .27 (Figure 2.4b). Follow-up tests indicate there was a tendency for the $SERT^{+/+}$ animals to show more stereotypy following acute MDMA compared to $SERT^{-/-}$ rats, $t_{8.34}$, = 2.04, p = .07.

Repeated MDMA. Figure 2.4b shows the number of ambulatory counts following repeated exposure to MDMA. MDMA (2.5 mg / kg) increased locomotor activity in both genotypes, $F_{1,25}$ = 10.38, p < .001, however the amount of locomotor activity did not differ between genotypes, $F_{1,25}$ = .00, p = .95, and no significant interaction was observed, $F_{1,25}$ = .09, p = .76. Neither genotype exhibited sensitisation to MDMA following repeated exposure to MDMA, if anything, ambulation was decreased following repeated MDMA, although this was not significant ($SERT^{+/+}$ animals, t_6 =1.53, p = .18; $SERT^{-/-}$ animals t_6 = 1.33, p = .23).

Figure 2.4d shows stereotypy following repeated MDMA. An effect of treatment was found, $F_{1,25}$ = 13.06, p < .001, however, the amount of stereotypy did not differ between genotypes, $F_{1,25}$ = 1.15, p = .29. No significant interaction was observed, $F_{1,25}$ = .38, p = .54. Neither genotype exhibited sensitisation of stereotyped behaviour to MDMA following repeated exposure to MDMA ($SERT^{+/+}$, t_6 = 1.5, p = .18; $SERT^{-/-}$, t_6 = 1.33, p = .23).

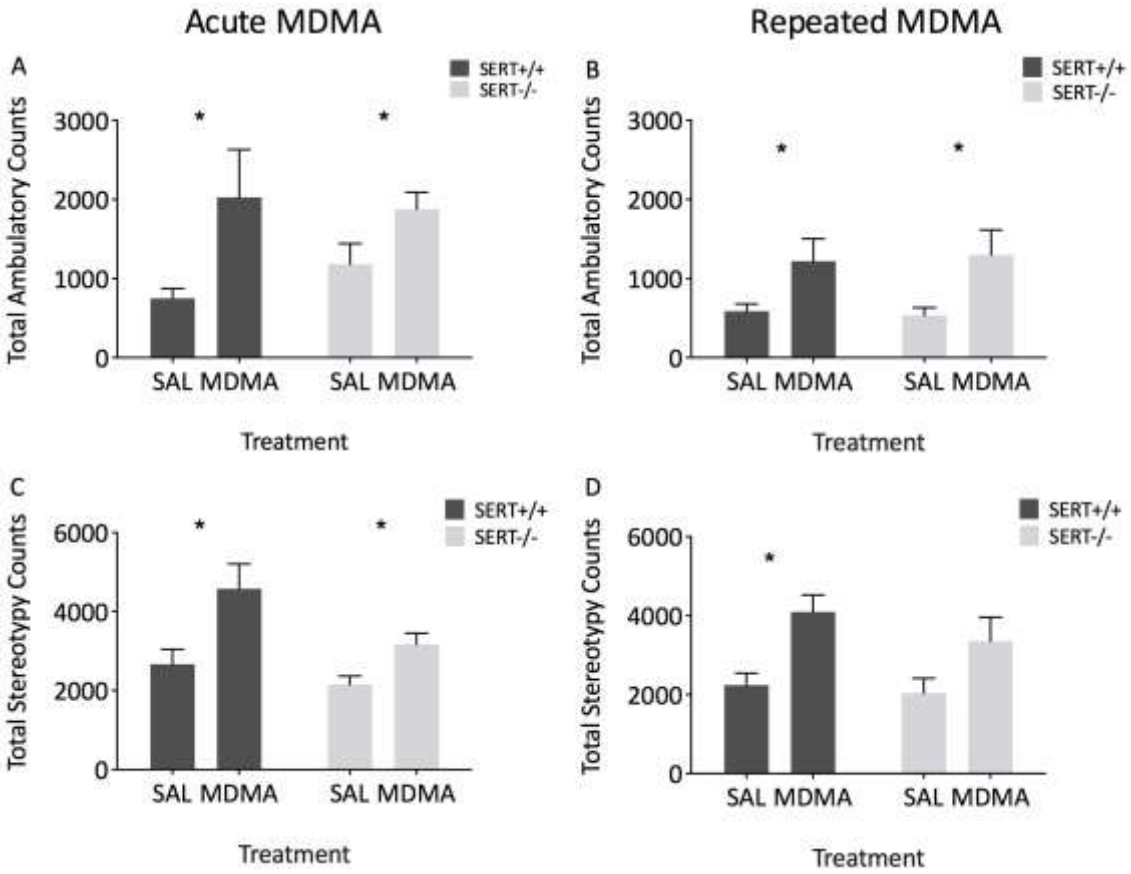

Figure 2.4. A) Ambulatory counts for SERT $^{+/+}$ and SERT $^{-/-}$ animals following an acute injection of 2.5 mg / kg MDMA or saline (0.0 mg / kg MDMA) i.p. within the open field; C) Stereotypy counts for SERT $^{+/+}$ and SERT $^{-/-}$ animals following an acute injection of 2.5 mg / kg MDMA or saline (0.0 mg / kg MDMA) i.p. within the open field. *N* = 7 - 8 per group; B) Ambulatory counts for SERT $^{+/+}$ and SERT $^{-/-}$ animals following an acute injection of 2.5 mg / kg MDMA or saline (0.0 mg / kg MDMA) i.p. following exposure to MDMA each day or 5 days followed by a 2 day withdrawal; C) Stereotypic counts for SERT $^{+/+}$ and SERT $^{-/-}$ animals following an acute injection of 2.5 mg / kg MDMA or saline (0.0 mg / kg MDM A) i.p. following repeated exposure to MDMA. *N* = 7 - 8 per group. Bars represent the mean (+SEM). *p < .05.

Drug Discrimination

Figure 2.5a shows the cumulative percentage of animals that met the discrimination criterion. Although the majority of SERT$^{+/+}$ rats (70%) learnt to discriminate saline and MDMA, only around 30% of SERT$^{-/-}$ did. A Kaplan–Meier survival analysis showed a significant leftward and upward shift in the curve of the SERT$^{+/+}$ group compared with the SERT$^{-/-}$ group, $\chi^2_1 = 9.41$, $p = .02$. These results indicate that the discriminative stimulus properties of MDMA were

significantly lowered in SERT$^{-/-}$ compared with SERT$^{+/+}$ rats. As displayed in figure 2.5b, there were no differences between genotypes for response rate, $t_{7.15}$, .54, p = .60.

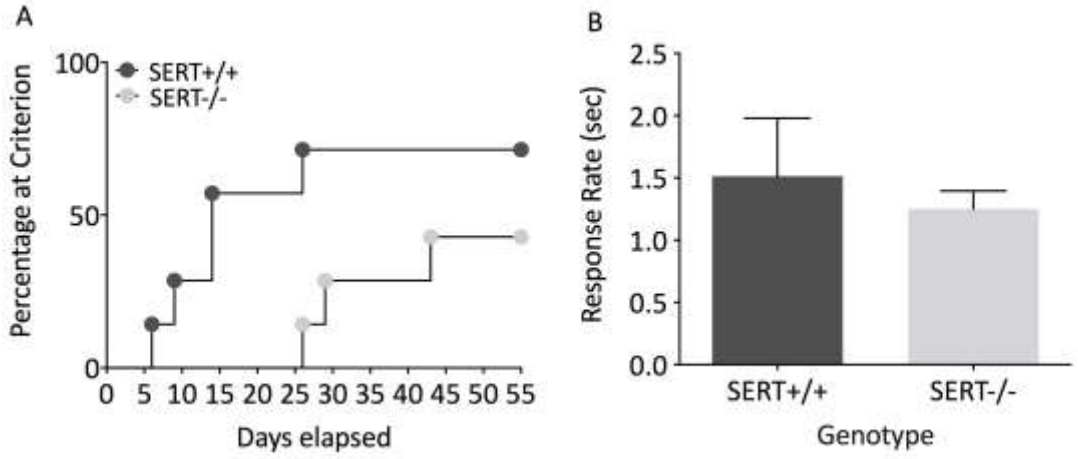

Figure 2.5. A) Cumulative percentage of SERT$^{-/-}$ (n = 7) and SERT$^{+/+}$ (n = 7) rats that met discrimination criterion as a function of test session. B) Overall mean response rate (responses per second) to complete the first fixed ratio 10 during the final ten discrimination sessions, for both SERT$^{-/-}$ (n = 6) and SERT$^{+/+}$ (n = 7) rats. Bars represent the mean (+SEM). *p < .05.

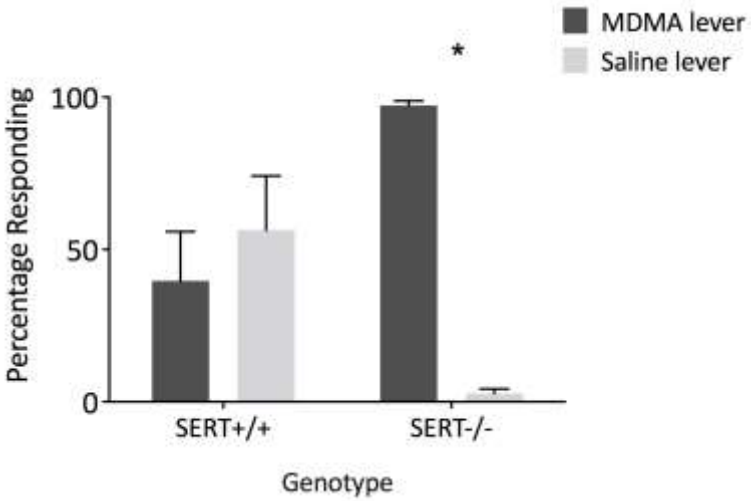

Figure 2.6. Mean number of responses of SERT$^{-/-}$ (n = 5) and SERT$^{+/+}$ (n = 3) rats on either the saline- or MDMA-appropriate lever following a 0.5 mg / kg amphetamine i.p probe. Bars represent the mean (+SEM). *p < .05.

Only five SERT$^{+/+}$ rats, and three SERT$^{-/-}$ rats met the discrimination criterion and moved on to probe generalisation with 0.5 mg / kg amphetamine

(Figure 2.6). SERT$^{+/+}$ animals showed partial stimulus generalisation to MDMA, with an average of 40% responses on the MDMA-appropriate lever, while SERT$^{-/-}$ animals responded almost exclusively on the amphetamine-appropriate lever. This resulted in a significant lever by genotype interaction, $F_{1,6} = 6.09$, $p = .049$, but no effect of lever, $F_{1,6} = 2.98$, $p = .14$, or genotype, $F_{1,6} = .56$, $p = .48$. Follow up *t*-tests indicated that no differences existed between lever choice for SERT$^{+/+}$ animals, $t_4 = -.50$, $p = .65$, while a significant difference was found for SERT$^{-/-}$ rats, $t_2 = 32.13$, $p < .01$.

Discussion

Pre-pulse inhibition and startle habituation

MDMA-induced disruption of startle habituation was abolished in SERT$^{-/-}$ rats, indicating that the SERT is necessary for the disruption of MDMA to startle habituation. These results are in line with previous pharmacological studies (Kehne et al., 1992). While no difference was observed between genotypes in MDMA-induced disruption of PPI, given that MDMA failed to disrupt PPI in the control SERT$^{+/+}$ group, it is impossible to establish whether genetic abolition of the SERT affects MDMA's effect on sensorimotor gating. A possible explanation for the lack of disruption in control rats is that a relatively modest range of MDMA was used (5 and 10 mg / kg MDMA). This range was deliberately selected to prevent any possible ceiling or floor effects (upper or lower limits for which any differences between groups for a dependant variable can no longer be discerned). However, previous studies used the higher dose of 20 mg / kg MDMA (Padich et al., 1996), indicating that very high doses of MDMA may be required to disrupt PPI. Nevertheless, pilot testing of this dose led to mortality in SERT$^{-/-}$, rats, and so this dose was not continued for animal welfare reasons.

Although the doses selected may have been too low to affect PPI, 5 mg / kg MDMA did abolish habituation to a startle stimulus in the SERT$^{+/+}$ rats, but not SERT$^{-/-}$ rats. Given that previous studies have demonstrated disruption of startle habituation with 20 mg / kg MDMA (Kehne et al., 1992), it is strange that disruption was not observed in control rats following 10 mg / kg MDMA. This is likely due to the effects of sustained habituation between sessions. Ideally, the two MDMA doses would have been counter balanced in order to prevent order effects. However, 5 mg / kg MDMA was initially the sole dose employed. Given its lack of efficacy in significantly disrupting PPI in SERT$^{+/+}$ rats, the experiment was repeated using 10 mg / kg MDMA. In order to minimise drug effects, 10 mg / kg MDMA was given to those rats which had previously been administered saline (and vice versa). While a 1 week washout period was given between sessions, the disruption of startle habituation by MDMA was diminished in the 10mg / kg group. Habituation to a startle stimulus has previously been shown to be sustained for up to 7 days (Moyer, 1963), so it is possible habituation was

sustained in these animals. However, there was no significant difference in habituation across the two saline groups, one of which was not naive to the paradigm so this conclusion is tentative. As noted above, pilot testing with 20 mg / kg MDMA was unsuccessful; however 10 mg / kg MDMA should be repeated in paradigm naïve animals in order to determine whether the effect of 10mg / kg MDMA on startle habituation is really diminished in these animals. Indeed, subsequent data collected in our laboratory group has shown the same effect of MDMA-induced disruption of startle habituation in experimentally naïve $SERT^{+/+}$ rats with 10 mg / kg MDMA.

It should be noted that, unlike previous studies that employed a discrete startle trial, the startle trials in the current experiment were given within a session that included PPI trials (Kehne et al., 1992; Stevenson & Gratton, 2004). Consequently, instead of measuring habituation across the whole session, habituation was measured as the percentage change in basal habituation between two blocks of 5 startle stimuli. While this paradigm fails to account for possible sensitisation within the first block (Halberstadt & Geyer, 2009) this is unlikely to be of great concern given the significant results found between genotypes. If anything, it is possible the effect was underestimated. Retesting the effects of MDMA in a pure startle habituation session (i.e. a session where only startle stimuli are presented) would help elucidate this.

Locomotor activity

Disruption of forward locomotion and stereotypy by MDMA was found to be largely unaltered in $SERT^{-/-}$ rats, although the effects of MDMA did appear to be slightly diminished in $SERT^{-/-}$ rats. These findings indicate the SERT may not be necessary for the locomotor activating effects of MDMA, and are in spite of previous pharmacological studies indicating that MDMA-induced locomotor activity is mediated by 5-HT, as well as DA (Daniela et al., 2004; Kehne et al., 1996). However, $SERT^{-/-}$ rats did display an increase in baseline forward locomotion, indicating that 5-HT may mediate locomotion in response to novelty. Finally, sensitisation to the locomotor effects of MDMA following repeated exposure was not observed in either genotype, and so it is impossible to determine whether abolition of the SERT alters MDMA sensitisation.

It is possible the different conclusions regarding the role of 5-HT in the locomotor activating effects of acute MDMA between the current study and previous pharmacological studies are mediated by the lack of specificity within these pharmacological agents. More importantly, findings published following the completion of the current study indicate that MDMA-induced increases in velocity were diminished in SERT$^{-/-}$ rats (Lizarraga et al., 2014). It is possible that MDMA-induced decreases in hyperactivity in SERT$^{-/-}$ rats are only present at higher doses of MDMA given that Lizarraga et al. (2014) used 10mg / kg MDMA (compared with 2.5 mg / kg in the current study). However, there are methodological issues that limit any such conclusions. First, while locomotor activity is usually measured as ambulatory counts or distance travelled, Lizarraga et al. (2014) used the less often used velocity (cm per minute) measurement, and so it is unclear what a decrease in MDMA-induced speed implies. Second, Lizarraga et al. (2014) treated all groups as one variable (treatment group) as opposed to considering genotype and drug treatment separately, which violates the assumption of an ANOVA that groups are independent. Consequently, future studies ought to replicate the current experiment using higher doses of MDMA to indicate whether the SERT is necessary for the locomotor activating effects of MDMA.

Stereotypy following acute MDMA was similarly increased in both genotypes, although there was a trend for this increase to be greater in SERT$^{+/+}$ compared with SERT$^{-/-}$. While the increase in stereotypy in SERT$^{+/+}$ rats is in keeping with previous findings that MDMA leads to 5-HT syndrome (Baumann et al., 2005; Spanos & Yamamoto, 1989), it is surprising there is an increase in stereotypic counts in SERT$^{-/-}$ rats, given that MDMA cannot increase 5-HT in these rats. It is possible that the method of measuring stereotypy in the present study was not ideal. Although stereotypy counts within the open field paradigm have previously been found to comprise head weaving and forepaw treading, markers of 5-HT syndrome (Baumann et al., 2005), these behaviours were not measured directly in the currently study. Moreover, increases in DA have been previously correlated with increases in stereotypy within the open field (Baumann et al., 2008), and cocaine and amphetamine also increase stereotypy counts without inducing clear head weaving or forepaw treading (Kuczenski,

Segal, & Aizenstein, 1991). Consequently, it is likely the stereotypy measured in this particular paradigm may have been influenced by increases in DA.

SERT$^{-/-}$ rats did demonstrate increased baseline forward locomotion, but not stereotypy, compared with SERT$^{+/+}$ rats, indicating SERT$^{-/-}$ show enhanced activity in their response to novelty. While previous studies with the SERT$^{-/-}$ rats have not found any increase in forward locomotion following exposure to novel environments (Homberg et al., 2008; Olivier et al., 2008), unpublished results from our lab have replicated this effect, indicating this finding warrants further investigation. It is surprising that baseline stereotypic behaviour failed to differ across genotypes, given that SERT$^{-/-}$ rats have 9-fold increased 5-HT, and MDMA-induced stereotypy is thought to be 5-HT mediated. However, as mentioned above, it is possible the particular paradigm used to measure stereotypy is sensitive to increases in DA. Moreover there is compensatory down-regulation in 5-HT receptors that may prevent changes in baseline behaviour (Baumann et al., 2008; Homberg et al., 2008).

Following repeated MDMA, the psychomotor response to MDMA was unaltered in both SERT$^{+/+}$ and SERT$^{-/-}$ rats, indicating that sensitisation to the locomotor activating effects of MDMA was not observed in either genotype. Consequently, it is impossible to determine whether any differences exist in MDMA sensitisation following the ablation of the SERT. It is likely the dose used in the current study was too low to produce sensitisation. Indeed, previous studies that have demonstrated sensitisation following repeated MDMA have used much higher doses than those used in the current study (5 and 10 mg / kg (Colussi-Mas & Schenk, 2008; Ramos et al., 2004)). The dose of 2.5 mg / kg was chosen for the current study as it is both behaviourally active but not so high so as to produce a ceiling effect whereby no effect of genotype could be elucidated. However, as no effect of sensitisation was observed in the control SERT$^{+/+}$ rats, it would be useful to assess multiple doses of MDMA within this paradigm.

Drug discrimination

While only a small number of SERT$^{-/-}$ rats managed to discriminate low dose MDMA from saline in the learning phase of the task, those that could were unable to differentiate amphetamine from low dose MDMA. MDMA preferentially increases extracellular 5-HT and has a relatively diminished effect

on extracellular DA in normal animals (Baumann et al., 2008; Han & Gu, 2006). Thus, whereas, in line with previous studies, amphetamine only partially generalised to MDMA in animals with a functional SERT (Kueh & Baker, 2007; Oberlender & Nichols, 1988), it fully substituted for MDMA in SERT$^{-/-}$ rats. These findings support previous assertions that 5-HT plays a crucial role in the discriminative stimulus effects of low dose MDMA (Harper et al., 2014). Moreover, it is well established that the discriminative stimulus properties of amphetamine are almost exclusively mediated via DA (Callahan, Appel, & Cunningham, 1991). The data therefore suggest that in the SERT$^{-/-}$ rats the discriminative stimulus properties of MDMA are also mediated via DA. Consequently, it is possible that when administered to SERT$^{-/-}$ rats, MDMA shares the well-established greater abuse potential seen with self-administration of amphetamine. Indeed, this hypothesis is tested in Chapter 3.

The differential effect on the SERT and the DAT likely explains the low number of SERT$^{-/-}$ rats that learned to discriminate low dose MDMA from saline. As MDMA cannot act upon the SERT in the SERT$^{-/-}$ rats, it is likely that the very small dopaminergic effect seen with this dose of MDMA was not strong enough to allow the SERT$^{-/-}$ rats to reliably differentiate MDMA from saline. While it is likely learning would increase if training occurred with a high dose of MDMA, such as 3 mg / kg, this dose would produce an increase in the DA effect of MDMA, and may therefore mask any existing differences between genotypes.

Although genetic reductions in SERT function found in humans do not lead to a complete ablation of the SERT, as seen in the SERT$^{-/-}$ rats, in humans, polymorphisms have been reported in the promoter region of the SERT. Some of these polymorphisms (most notably the short (s-) allele of the SERT linked polymorphic region or 5-HTTLPR) lead to a significant reduction in SERT function (Lesch et al., 1996). The findings of the current study back up evidence in humans that genetically reduced SERT function leads to alterations in their response to MDMA. Although this has not been studied in great detail, there are indications that s-allele carriers are more sensitive to some of the behavioural effects of ecstasy, such as deficits in attention and decision making (Cuyàs et al., 2011; Roiser et al., 2005; Roiser et al., 2006), as well as increased occurrences of

mood disorders (Martín-Santos et al., 2010). Furthermore, the results of the drug discrimination study indicate these individuals may subjectively experience MDMA as being more amphetamine-like than individuals who do not have this genetic variation.

Conclusion

The current results indicate that genetic removal of the SERT results in an alteration in the behavioural effects of MDMA. While this genetic removal resulted in an abolishment of MDMA-induced disruption to startle habituation, it failed to affect MDMA-induced increases in locomotor activity, implying that the SERT is necessary for MDMA's disruption of startle habituation but not its psychomotor effects. Moreover, for those rats that could discriminate low dose MDMA from saline, genetic removal of the SERT resulted in a removal of the ability to partially discriminate MDMA from amphetamine, supporting the notion that 5-HT plays a crucial role in the discriminative stimulus effects of a low dose of MDMA. Moreover, these findings imply that, in these rats, MDMA was now subjectively indistinguishable from amphetamine. Overall, the current findings support studies in humans that indicate individuals with genetically reduced SERT functions display an altered response to MDMA under certain conditions.

Chapter 3: A genetic deletion of the serotonin transporter greatly enhances the reinforcing properties of MDMA in rats

This chapter was adapted from Oakly, Brox, Schenk, and Ellenbroek (2014).

Many drugs of abuse preferentially increase synaptic dopamine (DA) but also exert effects on other brain systems that might either enhance or limit subsequent rewarding effects and abuse liability. For example, serotonin (5-HT) is inhibitory to DA signalling (Baumann et al., 2011). Therefore, the ability of many drugs of abuse to also enhance 5-HT might limit reward-mediated self-administration (Hayes & Greenshaw, 2011). Accordingly, variations in drug-produced DA and/or 5-HT release might explain between-subject variability in the propensity to self-administer drugs of abuse. (±) 3, 4-methylenedioxymethamphetamine (MDMA) is an excellent candidate to test this hypothesis because, while many other drugs of abuse, such as cocaine and amphetamine, preferentially enhance extracellular levels of DA (by blocking or reversing the DA transporter or DAT), MDMA preferentially binds to the serotonin transporter (SERT) thereby enhancing extracellular 5-HT levels to a greater extent than DA (Cole & Sumnall, 2003; Han & Gu, 2006). This pharmacological effect would be expected to limit the rewarding effects of MDMA because manipulations that increase extracellular 5-HT, such as selective 5-HT reuptake inhibitors (SSRI), are not positively reinforcing and have been found to reduce the rewarding properties of other psychostimulant drugs (Baumann et al., 2011; Carroll et al., 1990a). Indeed, although the abuse liability of MDMA is now well established, it is lower than that of most other drugs of abuse. This is evident both in humans and rats with only a relatively small proportion of MDMA users progressing to dependence (Degenhardt, Bruno, & Topp, 2010) and only ~50% of Sprague-Dawley rats developing reliable MDMA self-administration (Schenk, Colussi-Mas, Do, & Bird, 2012).

In the present study, we tested the hypothesis that MDMA's acute effects on the SERT underlie its reduced abuse liability by measuring MDMA self-administration in rats with a genetic deletion of the SERT. Although these animals have a 9-fold higher tonic synaptic (extracellular) level of 5-HT, drug effects mediated by the SERT are absent (Homberg et al., 2007). This animal

therefore provides a unique opportunity to establish whether the binding of MDMA to the SERT and the subsequent change in extracellular 5-HT is an important factor in the development and maintenance of MDMA self-administration.

Method

Subjects

Serotonin transporter knockout rats (SLC6A4^{1Hubr}, SERT$^{-/-}$) were originally created via N-ethyl-N-nitrosourea (ENU)-induced mutagenesis in a commercial (Harlan, Ter Horst, The Netherlands) wild-type Wistar rat (Smits et al., 2006). Experimental animals were generated by breeding pairs of SERT$^{-/-}$ or wild-type (SERT$^{+/+}$) animals who had been bred by crossing heterozygous animals (SERT$^{+/-}$) that had been outcrossed for at least 6 generations. Parent animals had been previously genotyped at 3 weeks of age. Ear samples were taken and used for genotyping (performed at Transnetyx, Cordova, USA). Twenty four experimentally naïve adult male rats (13 SERT$^{-/-}$, 11 SERT$^{+/+}$) weighing 300 - 400 g were used for self-administration. Female rats were not used in the current set of experiments due to unpublished findings from our lab that indicate self-administered MDMA significantly increased mortality in female rats. Animals were bred and reared at the vivarium of the School of Psychology at Victoria University of Wellington. Animals were housed in groups of three to six until they reached 300 - 400 g (approximately 90 days), at which point they were housed individually in hanging polycarbonate cages in a temperature (19 - 21 ^{o}C) and humidity (55%) controlled room. Animals were kept on a normal 12 hr light/dark cycle (lights on from 07.00 – 19.00 h), with testing being conducted during the light phase. Aside from testing hours, water and food were available *ad libitum*. All protocols were approved by the Victoria University of Wellington Animal Ethics Committee (AEC-2012R2).

Apparatus and procedure

Surgery. Surgery protocols were identical to those used in Schenk, Harper, and Do (2011). One week prior to self-administration, rats were surgically implanted with a chronic indwelling catheter, while under deep anaesthesia produced by intraperitoneal (i.p.) injections of a ketamine (90.0 mg / kg; Phoenix Pharm Distributors LTD, New Zealand) and xylazine (9.0 mg / kg; Phoenix Pharm Distributors LTD, New Zealand) mix. Post anaesthesia, lubricant eye ointment was applied across the cornea of the eye to prevent excessive dryness and Carprofen (5.0 mg / kg, subcutaneous (s.c.); Norbrook NZ LTD, New

Zealand) was administered for pain. Fur on the skull and chest was shaved, and an iodine-ethanol mix was applied. A small incision was made in the chest and the right external jugular vein was isolated and tied off with an ethanol soaked polyamide thread. A silastic catheter (cured tubing coated in silicone, fitted to a 2 cm length of 22 gauge stainless steel cannula) was passed subcutaneously from an exposed portion of the skull to the chest. A small cut was then made into the jugular vein into which the catheter was inserted and tied off. The incision was closed with super glue, and topical antibiotic (Terramycin powder; Pfizer Animal Health, Australia) was applied. The catheter was secured to the skull using dental acrylic adhered to 4 small stainless steel jeweller's screws (Centrostyle, Italy, Ref.00395) embedded in the skull. Additionally, the head of a 6-32 x 3/8 counter sunk stainless steel screw was adhered to the dental acrylic for the purpose of attaching the rat to the spring encasement in the operant chamber. Topical antibiotic was also applied to the skull incision. To prevent dehydration and aid recovery, 5mL of a compound sodium lactate solution (Hartmann's solution) was injected subcutaneously on each side of the rat (10 mL total). Testing began following a 7 day recovery period. For 2 days following surgery, rats were given Carprofen (5.0 mg / kg, s.c.) for pain. Each day following surgery catheters were infused with 0.2 ml sterilised 0.9% saline solution, containing heparin (3.0 I U / mL) and penicillin G potassium (250 000 I U / mL), to maintain catheter patency, and to prevent infection and the formation of clots and fibroids. The day immediately prior to testing, and every seven days thereafter, catheter patency was confirmed: Catheters were infused with 0.1 mL of heparin-penicillin solution, and then 0.04 mL was drawn up. If blood was successfully drawn the catheter was deemed functional. If not, it was infused with 0.15 mL of sodium pentobarbital (5.0 mg / kg, intravenous (i.v.)). Loss of righting reflex confirmed catheter patency. Rats were maintained in their home cages in the animal facility until testing.

Apparatus. Self-administration tests were conducted in 28 x 21 x 21 cm operant conditioning chambers (ENV-001, Med Associates Inc., Vermont) equipped with two levers. Depression of one lever (the 'active' lever) resulted in a 12.0-s intravenous infusion (0.1 mL) of MDMA, as well as the illumination of a stimulus light above the lever. Depression of the other lever (the 'inactive' lever)

was without programmed consequence, but was recorded. Infusions were delivered via a mechanical pump (Razel, Model A with 1 rpm motor), which housed a 20 mL syringe, by which drug was delivered i.v. via silastic tubing. Tubing entered the chamber from above via a swivel (Harvard Apparatus, USA), which allowed for free movement by the animal in the chamber. Drug delivery and data acquisition were controlled by an interfaced microcomputer using a commercially available software package (Med-PC IV, Med Associates Inc., Vermont). The testing room was temperature (19 - 21 ^{o}C) and humidity (55%) controlled.

Procedure. Testing was conducted during daily 2-h sessions which took place between 12:00 and 17:00 from Monday–Saturday. Both immediately prior to, and following, each daily test session, the catheters were infused with 0.2 mL of the heparin-penicillin solution. Rats were then placed in the operant conditioning chambers, and the exposed stainless steel tubing was attached to a length of silastic tubing through which drug was delivered.

Acquisition of MDMA self-administration. 13 SERT$^{-/-}$ along with 11 SERT$^{+/+}$ animals were used. Every session began with an experimenter delivered infusion of drug. Thereafter, each depression of the active lever (fixed ratio 1 reinforcement schedule) resulted in an automatic infusion of MDMA (1.0 mg / kg per infusion) paired with the illumination of a stimulus light located directly above the active lever. Pressing the incorrect lever was recorded but had no consequences. The criterion for acquisition was the same as that used previously in our lab (Schenk et al., 2012), rats were considered to have acquired self-administration if they obtained 90 infusions of MDMA within 25 days.

Maintenance of MDMA responding. Upon acquisition, rats (13 SERT$^{-/-}$ and 6 SERT$^{+/+}$) received additional tests to examine further the dose dependent nature of responding maintained by MDMA. Once animals obtained 90 infusions, the dose was halved to 0.5 mg / kg per infusion. Following stable responding on this dose (approximately 4 days), the reinforcement schedule was increased to fixed ratio 2, and then fixed ratio 5.

Progressive ratio responding. Remaining rats (10 SERT$^{-/-}$ and 6 SERT$^{+/+}$ rats) were subsequently tested under conditions of a progressive ratio

of reinforcement. For these tests, the ratio requirements increased logarithmically during each daily test as described previously (Richardson & Roberts, 1996). The session was terminated when the rat failed to gain an infusion following a 60-min period. The breakpoint and number of infusions earned were determined under this schedule, with breakpoint defined as the last ratio successfully completed. The 3 doses of MDMA used (0.25, 0.5 and 1.0 mg / kg per infusion) were assigned in pseudorandom order and given with one standard fixed ratio 5 session in between each dose.

Extinction and reinstatement of MDMA seeking behaviour.

Following progressive ratio testing, one standard fixed ratio 5 session was completed, after which drug seeking following abstinence was investigated (10 $SERT^{-/-}$ and 4 $SERT^{+/+}$ rats). During extinction, when the active lever was depressed, the stimulus light remained off and rats received an infusion of vehicle on an fixed ratio 5 reinforcement schedule. Once rats had reached a criterion of extinction (fewer than 20% of their average responses per session on the active lever) they were then reinstated with an i.p. injection of 10 mg / kg MDMA 10 minutes prior to commencement of the session, with the stimulus light turned back on, as previously used in our lab (Schenk, Gittings, & Colussi-Mas, 2011).

Drugs

MDMA HCl was obtained from Environmental Science and Research (Porirua, New Zealand). MDMA was dissolved in a sterile solution of heparinised (3 I U / mL) physiological saline. All weights refer to the anhydrous salt and not the acid.

Data Analysis

Acquisition of self-administration was compared between genotypes using the log-rank test to compare Kaplan-Meier survival estimates. Right-censoring was applied to rats that did not acquire within 25 days. Time-course data were converted into the number of responses that occurred in the first 30 minutes of the 2-hour session as a proportion of the total number of responses. This was in order to determine whether subjects were loading their responses

at the beginning of the session, a pattern typically found in MDMA self-administration (Schenk et al., 2003). The data for the first three experiment days, time-course, maintenance and progressive ratio were analysed using a repeated measures analysis of variance (ANOVA), with either experiment day / fixed ratio / dose entered in as a within subjects factors, and genotype entered as a between subjects factor. The Greenhouse-Geisser correction was used whenever sphericity was violated. Post-hoc analyses, where appropriate, were conducted using independent samples *t*-tests, with equal variances not assumed if Levene's test for equality of variances was significant. Extinction and reinstatement were analysed using independent samples *t*-tests. Extreme outliers were identified using SPSS's outlier tests and removed. The alpha level for statistical significance was set at $p < .05$. All statistical analyses were performed using IBM SPSS Statistics version 22 for windows.

Results

Acquisition

Figure 3.1a displays the average lever responses for 1.0 mg / kg MDMA over the initial 3 days of the experiment. Only the first 3 days of the experiment were considered in the initial analysis as this was prior to any subject meeting the acquisition criterion, as this event resulted in a change in MDMA dose and subsequent change in responding. While no main effect of experimental session was found, $F_{1.22, 28.04} = 1.13$, $p = .31$, there was a moderately significant effect of genotype, $F_{1, 23} = 4.11$, $p = .05$, as well as a significant interaction, $F_{1.22, 28.04} = 4.1$, $p = .045$. Post-hoc comparisons revealed that, while there was no significant difference between groups on day 1, $t_{23} = .15$, $p = .88$, there was a trend towards a significant difference on day 2, $t_{14.48} = 2.04$, $p = .06$, and a significant difference on day 3, $t_{13.06} = 2.31$, $p = .04$, indicating that, while both genotypes performed similarly upon experiment commencement, SERT$^{-/-}$ animals progressively responded more as the experiment continued.

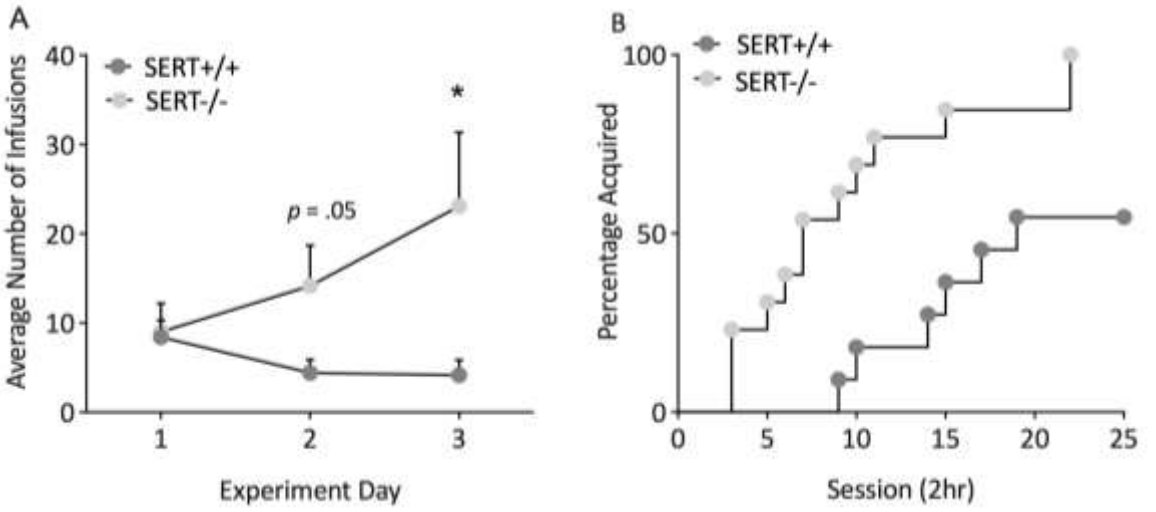

Figure 3.1. A) Average responses on the active lever (+SEM) for SERT $^{+/+}$ (n = 11) and SERT $^{-/-}$ (n = 13) during the first 3 days of self-administration of 1.0 mg / kg MDMA; and B) Survival curves for the acquisition of MDMA self-administration for SERT$^{-/-}$ and $^{+/+}$ rats. *p < .05.

In order to establish whether SERT$^{-/-}$ animals acquired self-administration behaviour significantly more than SERT$^{+/+}$ animals, an acquisition criterion previously established in our lab (more than 90 presses on the active lever within 25 days; (Schenk et al., 2012) was applied to all animals.

Within 25 days, all 13 SERT$^{-/-}$ rats (100%) acquired self-administration behaviour, compared with only 6 of 11 (55%) SERT$^{+/+}$ animals. A log rank test was run to determine if there were differences in the survival distribution between the SERT$^{-/-}$ and SERT$^{+/+}$ rats. Figure 3.1b shows the survival curves for the acquisition of self-administration, with SERT$^{-/-}$ rats displaying a significant increase in the probability of acquiring MDMA self-administration over the time period considered, $\chi^2_1 = 9.41$, $p < .001$. This suggests that the reinforcing properties of MDMA were stronger in the SERT$^{-/-}$ rats compared to the SERT$^{+/+}$ animals.

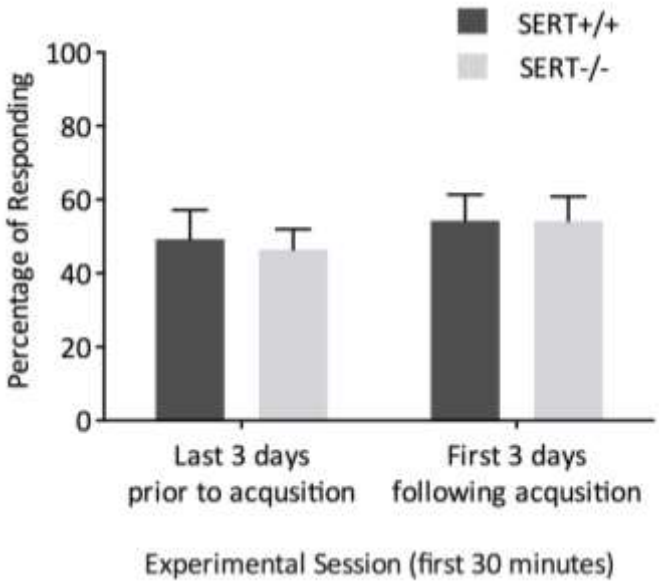

Figure 3.2. Average proportion of responding occurring in the first 30 minutes of the 2 hours session for both the last 3 days prior to acquisition (1.0 mg / kg MDMA), and the first 3 days after acquisition (0.5 mg / kg MDMA) for both SERT$^{-/-}$ (n = 13) and SERT$^{+/+}$ (n = 11) rats. Bars represent mean (+SEM). *$p < .05$.

Figure 3.2 displays the proportion of responding occurring in the first 30 minutes both prior to, and following acquisition. All subjects appear to perform a majority of their responses within the first 30 minutes, as has been found previously (Schenk et al., 2003). Statistical analyses found no effect of experimental day, $F_{1, 17} = 1.25$ $p = .28$, and no effect of genotype, $F_{1, 17} = 34.54$, $p = .87$. This indicates there are no differences between SERT$^{-/-}$ and SERT$^{+/+}$ rats in the pattern of responding.

Maintenance

An enhanced susceptibility of the SERT$^{-/-}$ rats to MDMA was also apparent during maintenance (Figure 3.3) with statistical analysis showing a significant main effect of fixed ratio , $F_{1.32, 22.38} = 34.54$, $p < .001$, and genotype, $F_{1, 17} = 5.73$, $p = .03$, but no interaction, $F_{1.32, 22.38} = 2.20$, $p = .15$. Follow up post-hoc tests revealed that SERT$^{-/-}$ animals infused significantly more MDMA than SERT$^{+/+}$ animals with a fixed ratio of 1, $t_{17} = 2.33$, $p = .03$, as well as when the fixed ratio was increased to 2, $t_{17} = 2.55$, $p = .02$. However, when the fixed ratio was increased to 5, the increase in responding by SERT$^{-/-}$ was only marginally significant, $t_{17} = 1.98$, $p = .05$.

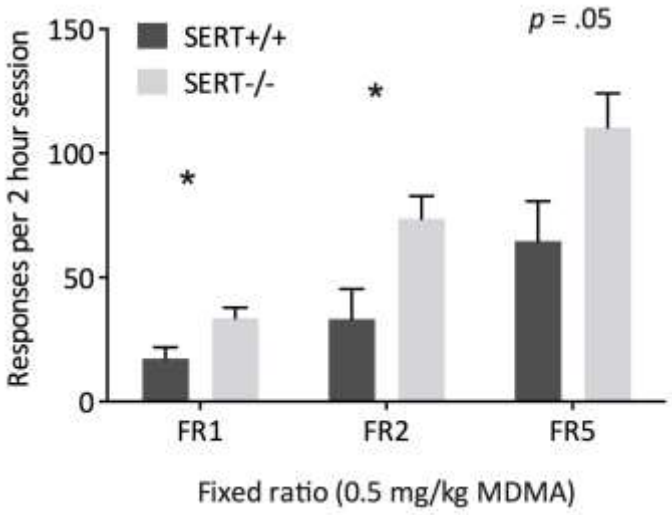

Figure 3.3. Responding maintained by a fixed ratio of 1, 2 or 5 with 0.5 mg / kg MDMA for SERT $^{-/-}$ ($n = 13$) and SERT $^{+/+}$ ($n = 6$) animals. Bars represent mean number of active lever responses (+SEM) during daily 2-hour sessions. *$p < .05$.

Progressive ratio

Means are displayed in Figure 3.4a. A dose by genotype interaction was found, $F_{2, 22} = 4.83$, $p = .02$. However no main effect of dose $F_{2, 22} = 2.17$, $p = .14$, or genotype, $F_{1, 11} = 3.38$, $p = .09$, was observed. While follow up post-hoc tests indicate that for 0.25 mg / kg there was no significant difference between SERT$^{+/+}$ and SERT$^{-/-}$ animals, $t_{914} = -.12$, $p = .91$, significant differences between groups were found for the higher doses: 0.5 mg / kg: $t_{9.03} = 2.87$, $p = .02$; and 1.0 mg / kg: $t_{11.18} = 2.61$, $p = .03$. This suggests that SERT$^{-/-}$ animals will work harder for higher doses of MDMA compared with SERT$^{+/+}$ animals.

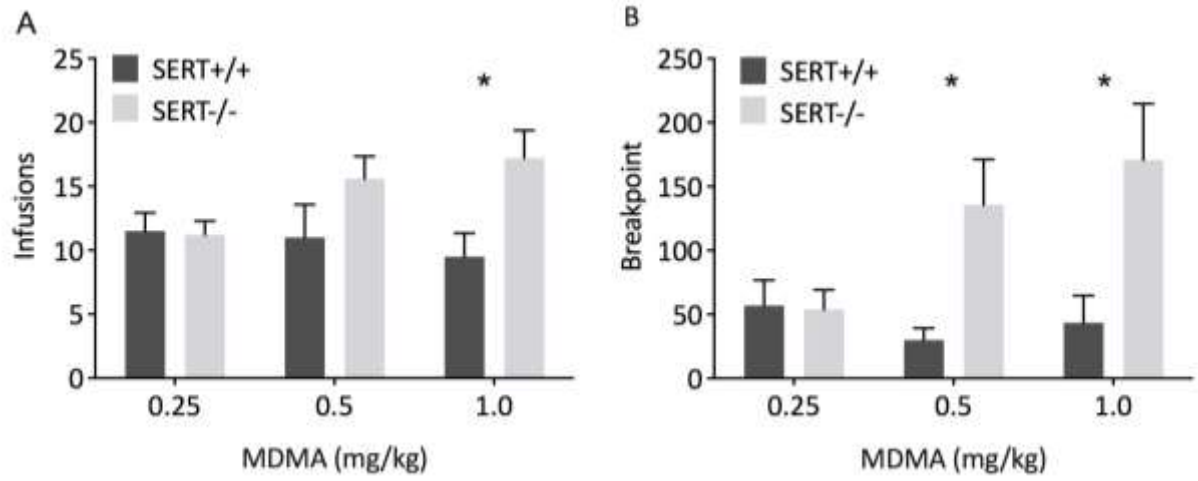

Figure 3.4. Mean number of infusions earned (A) or break point (B) as a function of MDMA dose for SERT $^{-/-}$ (n=10) and SERT $^{+/+}$ (n= 6) animals during progressive ratio tests of self-administration. Bars represent the mean (+SEM). *p < .05.

Extinction and reinstatement

There was no significant difference in the time to extinguish drug seeking behaviour between SERT$^{+/+}$, figure 3.5a, t_{13} = -.32, p = .75. Following extinction, animals were reinstated with a dose of 10mg / kg MDMA plus light cue. However, there was no effect of genotype on reinstatement, figure 3.5b, t_{12} = .43, p = .67.

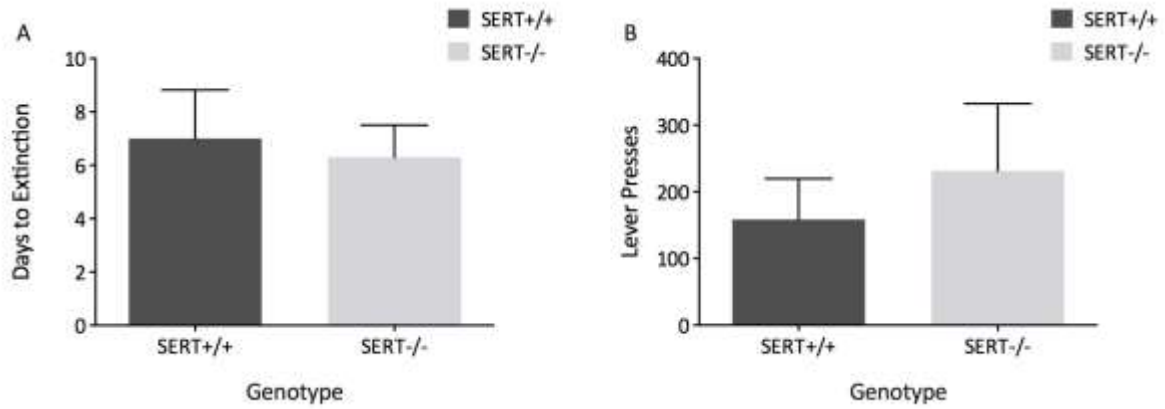

Figure 3.5. A) Mean number of days to extinguish lever pressing behaviour following MDMA self-administration paradigm for SERT$^{-/-}$ (n=10) and SERT $^{+/+}$ (n= 5) animals; B) Mean number of lever presses following reinstatement of MDMA self-administration with 10mg / kg MDMA plus light cue for SERT$^{-/-}$ (n=10) and SERT $^{+/+}$ (n= 4). Bars represent the mean (+SEM).

Discussion

These data show that SERT$^{-/-}$ rats are more sensitive to both the initial and subsequent reinforcing properties of MDMA. This is remarkably similar to previous findings from our lab that showed animals with reduced 5-HT neurotransmission following selective lesions of 5-HT neurons by the neurotoxin 5,7-dihydroxytryptamine display enhanced acquisition of MDMA self-administration (Bradbury et al., 2013). Moreover, this pattern is consistent with the findings of studies using other psychostimulants such as cocaine and amphetamine (Leccese & Lyness, 1984; Pelloux et al., 2012). Interestingly, while the lesioned animals and the SERT$^{-/-}$ rats share their lack of the SERT, SERT$^{-/-}$ rats have 9-fold *increased* basal level of extracellular 5-HT (Homberg et al., 2007), while the lesioned animals have significantly *reduced* basal 5-HT levels. This strongly suggests that, rather than the basal (tonic) level, it is the drug-induced phasic increase in extracellular 5-HT which limits the reinforcing properties of MDMA (and by analogy of other psychostimulants such as cocaine).

Although so far, the distinction between tonic and phasic 5-HT release has not received much attention, it has played a major role in our understanding of the functional role of DA (Grace, 1995, 2000). Given the structural similarities between 5-HT and DA implying comparable function, it is possible the serotonergic system is similarly regulated. DA regulation is proposed to occur in two ways: a fast acting, brief, phasic response; and a slow acting, longer lasting, tonic response. The phasic response refers to the high volumes of DA released from the pre-synaptic neuron in response to behaviourally relevant stimuli. This phasically released DA stimulates postsynaptic DA receptors but is rapidly taken back up into the presynaptic neuron by DAT. In the tonic response, DA is released by the pre-synaptic neuron in response to excitatory stimulation (e.g. by glutamate), at levels too low to be taken back up into the presynaptic neuron by DAT. This tonically released DA then diffuses out of the synapse, where it acts to homeostatically regulate the dopaminergic system by activating presynaptic autoreceptors, which in turn regulates the release of phasic DA though various processes including decreased DA cell firing and decreased DA synthesis.

MDMA primarily exerts its effects both by blocking the SERT and inducing reverse transport. This phasic release of 5-HT is then able to diffuse into the extrasynaptic space, thereby increasing tonic 5-HT levels. The increased tonic 5-HT will then stimulate 5-HT autoreceptors, thereby down regulating the serotonergic response, resulting in the dysphoric mood reported in the days following MDMA use (Parrott & Lasky, 1998). This process will differ in $SERT^{-/-}$ rats due to their complete lack of SERT. As with animals with lesions in the serotonergic system, when MDMA is administered to $SERT^{-/-}$ rats it is unable to phasically increase 5-HT. MDMA is still able to phasically increase DA in these animals however, and so the reinforcing effects of MDMA can be experienced without inhibition by 5-HT (Baumann et al., 2011; Rothman & Baumann, 2006), thereby increasing the reinforcing efficacy of MDMA in these animals. It is therefore likely that the inability of drugs of abuse to induce a phasic 5-HT release, rather than a sustained, tonic reduction in 5-HT levels, that enhances the reinforcing properties in animals with serotonergic lesions. This suggests that reducing the 5-HT levels in adulthood in $SERT^{-/-}$ rats is unlikely to reverse the enhanced sensitivity to MDMA.

The present study is the first to investigate the impact of the SERT on MDMA self-administration in genetically modified rats. The impact of this deletion is substantial and indicates a much greater role of drug produced 5-HT efflux in the acquisition and maintenance of self-administration than was previously shown when acquisition of cocaine self-administration was measured (Homberg et al., 2008). Accordingly, the data indicate a much more significant role of SERT in the reinforcing effects of MDMA than previously considered. Indeed, with repeated exposure, MDMA leads to a down-regulation of SERT (Schenk et al., 2007) which would explain the eventual acquisition of responding by some of the $SERT^{+/+}$ rats. Given the important role of DA D1 and D2 receptors in MDMA self-administration (Brennan, Carati, Lea, Fitzmaurice, & Schenk, 2009), this suggests that $SERT^{-/-}$ rats may have an increased sensitivity to dopaminergic stimulation. However, a recent microdialysis study has shown that, while cocaine-induced increases in extracellular 5-HT release in the nucleus accumbens and hippocampus are smaller in $SERT^{-/-}$ rats than $SERT^{+/+}$, no genotype differences were found for extracellular DA (or norepinephrine

(NE)) release (Verheij, Karel, Cools, & Homberg, 2014). Future experiments are required to determine whether this finding is consistent across brain structures and whether this also holds for MDMA, given its unique pharmacological properties.

Our present set of data from SERT$^{-/-}$ rats are diametrically opposite to results obtained from SERT$^{-/-}$ mice. While SERT$^{-/-}$ mice also display anxiety-like behaviour (Holmes et al., 2003), as well as increased cocaine-induced conditioned place preference (Sora et al., 1998), MDMA self-administration in these mice is abolished (Trigo et al., 2007). This difference reinforces the idea that the role of 5-HT in the behavioural response to MDMA differs between species. Significantly, MDMA produces differential long term effects in mice and rats. For instance, MDMA acts as a selective DA neurotoxin in mice, leading to a decrease in striatal DA and its metabolites, as well as significantly reducing DAT, with minimal effects on 5-HT (Colado et al., 2004; Kindlundh-Högberg et al., 2007; O'Callaghan & Miller, 1994; O'Shea et al., 2001). Whereas in humans, non-human primates, and rats there is evidence that long-term use of MDMA leads to selective 5-HT depletion and a decrease in SERT expression, increasing the addictive potential of MDMA (Buchert et al., 2004; Easton & Marsden, 2006; Kindlundh-Högberg et al., 2007; O'Shea et al., 1998). Thus, the rat model is consistent with effects that are produced in humans who consume MDMA and increase their intake over time. Consequently, it is important to consider the effects of a down-regulated SERT in a rat model.

Pharmacogenetic studies in humans have shown that carriers of the short (s-) allele of the SERT linked polymorphic region (5-HTTLPR) of the solute carrier 6 member 4 (SLC6A4) gene (leading to a 50% reduction in SERT activity (Lesch et al., 1996)) are more likely to abuse psychostimulants, such as cocaine and methamphetamine (Cao et al., 2013; Gerra et al., 2007). While abuse of MDMA has not been considered, use of MDMA is not significantly higher in these individuals (Martín-Santos et al., 2010; Roiser et al., 2005), although there was a trend for increased use in s/s genotypes in Martín-Santos et al. (2010). Given that these studies used the biallelic distinction of the 5-HTTLPR (i.e. only considered the s- and the l-allele, not the various versions of the l-allele), which fails to account for additional polymorphisms in the alternate long (l-) allele

that also lead to a reduction in SERT activity, these findings are likely to be underestimated. Importantly, s-allele carriers have been found to be more sensitive to some of the effects of MDMA on the central nervous system, including attention deficits, impaired emotional processing, risky choice making, increased mood disorders, and sedation (Cuyàs et al., 2011; Martín-Santos et al., 2010; Pardo-Lozano et al., 2012; Roiser et al., 2005; Roiser et al., 2006). While cognisant of the need for judicious translation from animal data to humans, the data suggest that individuals with a lower SERT activity may be more sensitive to the reinforcing effects of MDMA.

Chapter 4: Normalising early life serotonin levels rescues mild anxiety-like behaviour in SERT knock-out rats

Genetic reductions in the serotonin reuptake transporter (SERT) protein have frequently been associated with pathological phenotypes in adulthood. For instance, humans with the short (s-) allele of the SERT linked polymorphic region (5-HTTLPR) are more likely to be anxious, depressed, and abuse illicit drugs such as cocaine, methamphetamine, and heroin (Cao et al., 2013; Caspi et al., 2003; Enoch et al., 2011; Gerra et al., 2007; Heils et al., 1996; Lesch et al., 1996; Sen et al., 2004). Further, rodent models with genetically reduced SERT function ($SERT^{-/-}$) also show anxiety- and depression-like behaviour (Holmes et al., 2003; Olivier et al., 2008), and $SERT^{-/-}$ rats, but not mice (Bengel et al., 1998; Sora et al., 2001) show increased sensitivity to both cocaine (Homberg et al., 2008), and MDMA (Chapter 3), as well as altered behavioural response to MDMA (Chapter 2). However, the mechanisms underlying these altered characteristics are currently unknown.

Due to the reduced ability of the SERT protein to transport extracellular serotonin (5-HT) back up into the presynaptic neuron, lymphoblast cell lines transfected with the s-allele showed a 50% reduction in [^{3}H]5-HT uptake compared to l/l cells (Lesch et al., 1996). Similarly, $SERT^{-/-}$ rats exhibit a 9-fold increase in extracellular 5-HT (Homberg et al., 2007). Paradoxically however, 5-HT reuptake inhibitors, or SSRIs, which result in increased extracellular 5-HT through blockade of the SERT, are commonly prescribed for their ability to decrease anxiety and depression, and have even been shown to reduce the rewarding efficacy of cocaine in rats (Carroll et al., 1990a). Given that SSRIs are commonly prescribed in adulthood, while potential genetic reductions in the SERT will be present from very early on in development, it is possible that the negative effects of genetic reductions in the SERT are mainly due to excessive extracellular 5-HT during early development.

Indeed, 5-HT is known to play a wide role in neurodevelopment, acting as a developmental signal in neuronal processes such as neuronal migration, cell division and cell differentiation (For review see (Gaspar et al., 2003)). There is evidence that disruptions in 5-HT homeostasis during neural development can lead to psychiatric disorders. For instance, gestational use of SSRIs has been

associated with a 3-fold increase in autism in boys (Harrington et al., 2014), as well as increased internalising behaviours in 3 year olds (Oberlander et al., 2010). However, no studies have yet tracked offspring beyond childhood, so it remains unknown whether further difficulties develop, or existing abnormalities persist into adulthood. Moreover, it is difficult to tease apart the effects of maternal depression and antidepressant treatment on offspring.

Nonetheless, studies in animals, which can more easily overcome the aforementioned issues associated with human research, support the hypothesis that exposure to SSRIs during neonatal development leads to pathological phenotypes in adulthood. For example, postnatal SSRI exposure leads to increased anxiety- and depression-like behaviours, as well as increased sensitivity to the reinforcing properties of cocaine (Ansorge et al., 2008; Ansorge et al., 2004; Forcelli & Heinrichs, 2008; Olivier et al., 2011). Interestingly, decreasing postnatal 5-HT also leads to pathological outcomes: neonatal depletion of the 5-HT precursor L-tryptophan (TRP), resulting in decreased extracellular 5-HT, also leads to anxiety- and depression-like behaviours in rodents (Zhang et al., 2006; Zoratto, Fiore, Ali, Laviola, & Macrì, 2013), while mice with a conditional knock-out of the 5-HT1a receptor (5-HT1a$^{-/-}$), in which the activation of 5-HT1a receptors by 5-HT is prevented, display increased anxiety-like behaviour only when synthesis of the 5-HT1a receptor is turned off during postnatal development, but not during adulthood (Gross et al., 2002).

Given the important role 5-HT plays in the development of anxiety and depression, it is important to establish whether early intervention can prevent the pathological phenotype associated with the genetic reduction of SERT. In a previous study, neonatal exposure to the 5-HT neurotoxin para-chlorophenylalanine (pCPA) has been shown to rescue disrupted rapid eye movement (REM) sleep in SERT$^{-/-}$ mice (Alexandre et al., 2006). This effect appears to be driven by the inability of 5-HT to excessively activate the 5-HT1a receptor in pCPA treated SERT$^{-/-}$ mice: When neonatal 5-HT1a receptor activation was blocked by the 5-HT1a antagonist WAY100635 in SERT$^{-/-}$ mice their depression-like behaviour was rescued. However, while SERT$^{-/-}$ mice normally exhibit down-regulated 5-HT1a receptor binding, these levels were

mostly unchanged in the rescued SERT$^{-/-}$ mice. The aforementioned study in conditional 5-HT1a$^{-/-}$ mice also support the role of 5-HT1a receptor activation during early development in mediating normal anxiety-like behaviour (Gross et al., 2002). However, given the low number of studies, and that they have only been performed in mice, the mechanism by which the behaviour is rescued is still far from clear.

Importantly, no one has yet attempted to rescue the behaviour of SERT$^{-/-}$ rats. This is particularly crucial given species differences between mice and rats in the physiological response to 5-HT1a antagonists and to genetic alterations in the SERT. For instance, while the 5-HT1a antagonist 8-OH-DPAT induces hypothermia in both mice and rats, this is mediated by the presynaptic 5-HT1a autoreceptor in the mice, but the postsynaptic 5-HT1a receptor in rats (Bill et al., 1991). Likewise, as was discussed in Chapter 3, while SERT$^{-/-}$ rats show significantly increased MDMA self-administration, SERT$^{-/-}$ mice show abolished MDMA self-administration (Trigo et al., 2007). Therefore, the aim of the current study was to reduce the 5-HT levels of SERT$^{-/-}$ rats during early development, and determine whether this intervention rescues their anxiety-like phenotype as assessed by the novelty supressed feeding (NSF) and open field tasks. While Alexandre and colleagues (2006) injected pCPA into pups, drug exposure in young rats is extremely distressing, with injections during early life leading to increased anxiety-like behaviour in adulthood (Girardi, Zanta, & Suchecki, 2014). Moreover, given that SERT$^{-/-}$ rats exhibit increased sensitivity to anxiogenic situations (Olivier et al., 2008), the use of an alternative, less invasive, technique was desirable. Accordingly, dams were fed a TRP depleted diet immediately following birth. While this technique has been performed previously e.g. (Zhang et al., 2006; Zoratto et al., 2013), its use has been infrequent, and never been performed before in our lab. Therefore we first performed a pilot study to assess the optimal concentration of TRP before implementing the actual study with SERT$^{-/-}$ rats.

Additionally, in order to investigate a mechanism for any apparent behavioural changes, gene expression of both 5-HT1a receptor and long 3' untranslated region brain-derived neurotropic factor (long 3'UTR BDNF) were assessed. The 5-HT1a receptor was selected given its aforementioned

involvement in the anxiety- and depression-like behaviour of mice (Alexandre et al., 2006; Gross et al., 2002). Moreover, the 5-HT1a receptor is down-regulated in the SERT$^{-/-}$ rats (Homberg et al., 2008), giving some support for the hypothesis. Likewise, BDNF is decreased in the leukocytes of both s-allele and other low functioning SERT polymorphisms (Molteni et al., 2010). Furthermore, total BDNF, as well as the long 3'UTR BDNF and other BDNF transcripts, are down-regulated in SERT$^{-/-}$ rats in the hippocampus and the pre-frontal cortex (PFC) (Calabrese et al., 2015; Molteni et al., 2010). This decreased expression of both total BDNF and the long 3'UTR BDNF develops from around postnatal day (P) 0 - P7 (Calabrese et al., 2013), indicating that this disruption occurs very early on in brain development.

Accordingly, it is hypothesised that the anxiety-like phenotype normally observed in SERT$^{-/-}$ rats, as well as the normally down-regulated mRNA of both long 3'UTR BDNF and 5-HT1a, will be rescued following neonatal TRP depletion.

Experiment 1: Early life tryptophan depletion

As neonatal TRP depletion has not been widely used previously, a pilot study was run with standard Sprague Dawley rats to determine optimal TRP levels for the experimental group. Control groups were fed 0.2g TRP / 100g diet (based on (Benevenga, Gahl, Crenshaw, & Finke, 1994)) while experimental groups were fed either 0.1g TRP / 100g diet or 0.0g TRP / 100g diet. Anxiety-like behaviour was then assessed in adulthood, followed by sensitivity to the locomotor activating effects of amphetamine in order to determine dopamine (DA) sensitivity.

Method

Subjects and General Protocols

Sprague Dawley dams were fed an experimental diet containing varying amounts of TRP from P0 until P20. Five female offspring per group were sacrificed directly following diet completion, while the remaining offspring were weaned at P30 (0.0g TRP n = 26; 0.1g TRP n = 17; Control n = 19). Male pups were weighed at P21, P30 and P60 to determine any weight changes resulting from the experimental diet. Behavioural testing was performed from P60 onwards, with anxiety-like behaviours first assessed using the NSF task, followed 1 week later by amphetamine-induced locomotor activity in the open field. Open field data were extracted from the first 5 minutes of the habituation phase occurring at the beginning of amphetamine-induced locomotor activity testing.

All animals were bred and reared at the vivarium of the School of Psychology at Victoria University of Wellington and housed in groups of three to four in hanging polycarbonate cages in a temperature (19 - 21 ^{o}C) and humidity (55%) controlled room. Animals were kept on a normal 12 hr light/dark cycle (lights on from 07.00 – 19.00 h), with testing being conducted during the light phase. Aside from testing hours, water and food were available *ad libitum*. All protocols were approved by the Victoria University of Wellington Animal Ethics Committee (AEC-2013R6).

Diet

All groups were fed ad libitum with a cornflour based diet (Table 4.1) modelled on that used previously (González et al., 2008). Cornflour (Maseca, Azteca Milling Ltd, Texas) was selected due to its extremely low TRP content (0.045g / 100g)(González et al., 2008) to which TRP could be supplemented.

Table 4.1.

Experimental Diet Composition

	Portion (g)		
	TRP 0.2g/100g	TRP 0.1g/100g	TRP 0.0g/100g
Corn flour	91.2	91.3	91.4
Soya oil	4.5	4.5	4.5
Vitamin and mineral mix	4.1	4.1	4.1
L- tryptophan	0.2	0.1	0.0
Total	100	100	100

Diets contained varying amounts of TRP: Experimental groups were fed a diet containing either 0.0g TRP or 0.1g TRP per 100g diet, while control groups were fed a diet of 0.2g TRP per 100g diet in order to gain the required amount of TRP for normal development (Benevenga et al., 1994). A vitamin and mineral mix (Berocca® Performance, Bayer) was added to prevent malnutrition from B vitamin deficiency. The amount of vitamin tablets per 100g diet was determined by comparing the contents of the tablets to a standard commercial mix (Teklad mix, Category numbers 40060 and 170760, Harlan Laboratories) as displayed in Table 4.2. On the basis of these comparisons it was decided 3 pills would be incorporated per 100g mix. Soya oil was also added to the diet to help binding. Soya oil amounts were based on those used previously (González et al., 2008). Vitamin and mineral mix pills were soaked in 25 ml of distilled H_2O overnight, added to dry ingredients along with ~150 ml of distilled H_2O, and then mixed to form a dough. Pellets were formed from the dough and dehydrated at 30 ^{o}C overnight.

The experimental diet was available *ad libitum* from P0 until P20 (based on (González et al., 2008). As this was the first time this protocol was used in

our lab, all dams were given the control diet for 24 hours, 2 weeks post mating, to habituate dams to the food.

Table 4.2.

Comparison of standard Vitamin/Mineral Mix to Berocca® Perfomance tablets

	Substance	
	Berocca® (mg /tablet)	Teklad Mix (mg/kg)
Magnesium	100	100
Calcium	100	293
Zinc	10	n/a
Vitamin B12	0.01	3
Vitamin H	0.015	0.44
Folic acid	0.04	0.2
Vitamin B1 and B2	15	2.2

HPLC

Following diet completion, 5 females from each group were sacrificed for monoamine analysis using high performance liquid chromatography (HPLC) in order to ensure the manipulation was successful. On P20 rats were taken between 09:00 and 14:00 hours and sacrificed by CO_2 asphyxiation. Rats were decapitated and brains rapidly removed and dissected on ice. Brains were placed dorsally in a metal brain matrix and razor blades were places in parallel slice channels producing 1 or 2 mm slices based on the protocol outlined by Heffner, Hartman, and Seiden (1980). Sections were placed on a glass petri dish on ice, and hippocampus and caudate putamen were dissected and placed in 1.5 mL Eppendorf tubes that had been previously weighed. Brain regions were weighed and stored immediately at -80 ^{o}C. Concentrations (ng / mg) of 5-HT, DA, norephinephrine (NE), and the 5-HT metabolite 5-Hydroxyindoleacetic acid (5-HIAA), as well as the DA metabolites 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in both the hippocampus and the caudate putamen were determined via HPLC (100 series, Agilent). Samples were homogenised in 10 µL 0.1 N perchloric acid per mg of tissue and centrifuged at 13,000 rpm at 4 ^{o}C for 30 minutes. The supernatant was filtered into vials and

injected into the column in pseudorandom order (C18 reversed phase; Agilent Eclipse XDB-C18, 4.6 × 150 mm, 5 μm particle size) and the mobile phase consisted of 75 mmol/L NaH_2PO_4, 1.7 mmol / L octanesulfonic acid, 0.25 mmol / L EDTA, 100 μL / l triethylamine, 10% (v/v) acetonitrile, pH 3, delivered at a constant flow rate of 1.0 ml / min. The injection volume and sensitivity were altered based on brain region (hippocampus: injection volume: 20 μl, sensitivity 500nA; caudate putamen: injection volume: 20 μl, sensitivity: 2nA). Chromatograms were acquired with Agilent software and peak heights of samples were compared to peak heights of standards with known concentrations of 5-HT, DA, NE, 5-HIAA, DOPAC, and HVA. Regression analysis of the calibration curves was then used to calculate the concentration of the neurochemicals. Turnover rates for 5-HT and DA were calculated by dividing the metabolite concentration by the monoamine concentration.

Amphetamine Locomotor Activity and Open Field

Locomotor activity was assessed in open field activity systems consisting of eight clear Plexiglas activity chambers (42 x 42 x 30 cm) set in sound-attenuated chambers (ENV-515-16, Med Associates Inc., Vermont). Location was tracked using 2 pairs of 8 evenly spaced infrared sources and sensors positioned perpendicular to one another around the chambers, and recorded by a commercially available software package (Med-PC IV, Med Associates Inc., Vermont). The infrared beams divided the chamber into squares, with a region set to the approximate dimensions of a rat (3 x 3 squares) generated around the rat. Any beam breaks within this region were counted as stereotypy. These movements have previously been shown to predominantly comprise of forepaw treading and head weaving (Baumann et al., 2008). One ambulatory count was registered if 3 beams were successively broken within 1000ms outside the region. One rearing count was registered if a second set of infrared beams placed 15 cm above the ground was broken. A white noise generator masked extraneous auditory disturbance during testing, and the room was illuminated with red light. Prior to and following each behavioural test session, the chamber interiors were cleaned and wiped with Virkon 'S' disinfectant (Southern Veterinary Supplies, Palmerston North, New Zealand).

On the day of testing, animals were first habituated to the activity chambers for 30 min. Open field data were collected from the first five minutes the animals were in the chamber. Animals were injected following the 30-minute habituation phase with 0.5 mg/kg i.p. amphetamine and remained in the chamber for another 60 minutes. This dose was chosen as it was both relatively low, while still being behaviourally active within this paradigm (Joyce & Koob, 1981).

Novelty Supressed Feeding

Novelty supressed feeding testing was performed in a dimly lit room using a large circular arena (1 m diameter), the base of which was covered with a layer of bedding and contained a circular piece of paper, upon which was placed a single food pellet (approx. 2 g). Subjects were food deprived for 24 hours prior to the start of the experiment. Upon test commencement, the subject was placed in a designated section on the side of the chamber, and the timer was started. The experiment was terminated when the rat obtained the food pellet or following 10 minutes, whichever occurred first. The latency to initiate feeding was recorded, and the rat was immediately removed and fed. The chamber was cleaned between subjects using 70% ethanol, and fresh bedding, paper, and food applied to the chamber floor. Data were recorded using EthoVision software (Version 9.1).

Drugs

d-amphetamine was obtained from Environmental Science and Research (Porirua, New Zealand). Amphetamine was dissolved in a sterile solution of 0.9% physiological saline and administered at a volume of 1 ml / kg body weight. All weights refer to the anhydrous salt and not the acid.

Data analysis

Offspring weight percentage change, HPLC, open field and NSF data were analysed using a one way analysis of variance (ANOVA) with TRP group as a between subjects factor. Follow up Tukey Honestly Significant Difference post-hoc tests were employed where necessary. For amphetamine-induced

locomotor activity, a two-way ANOVA was run with TRP group and drug group as between subjects factors. Although no interactions were present, exploratory independent *t*-tests were used in order to elucidate potential patterns, with equal variances not assumed if Levene's test for equality of variances was significant. Due to technical difficulties, the data for 5 rats were lost for open field analysis. Extreme outliers were identified using SPSS's outlier test and removed. All tests were run using IBM SPSS Statistics version 22 for windows. Alpha level was set at $p < .05$ for all data.

Results

Offspring weights

There was no difference between treatment groups for percentage weight change between P20 to P 30, $F_{2,61} = .92$, $p = .4$, or from P 30 to P60, $F_{2,61} = .07$, $p = .94$, Figure 4.1.

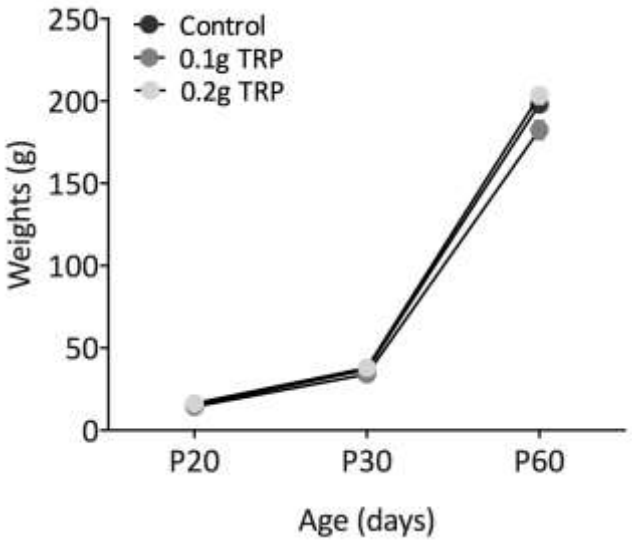

Figure 4.1. Offspring weights as a function of age (P20, P30 and P60) for all for litters neonatally TRP-deprived (0.0g or 0.1g TRP per 100g diet, $n = 26$ and 17 respectively) as well as controls (0.2g TRP per 100g diet, $n = 19$). Lines represent the mean (+SEM). * $p < .05$

HPLC

There was a tendency for decreased 5-HT levels after early life low TRP exposure in both the hippocampus, $F_{2,12} = 3.23$, $p = .08$ (Figure 4.2a), as well as the caudate putamen, $F_{2,12} = 3.27$, $p = .07$ (Figure 4.3a) at P20. Moreover, there was a significant dose dependent decrease in 5-HIAA in both the hippocampus, $F_{2,12} = 7.74$, $p = .01$ (Figure 4.2b), as well as the caudate putamen, $F_{2,12} = 8.78$, $p < .001$ (Figure 4.3b). Follow up post-hoc tests indicated this difference was between the 0.0g TRP and 0.2g TRP groups in the hippocampus ($p = .01$), and between the 0.0g TRP groups and both the other groups in the caudate putamen (0.1g TRP: $p = .01$; 0.2g TRP: $p = .01$).

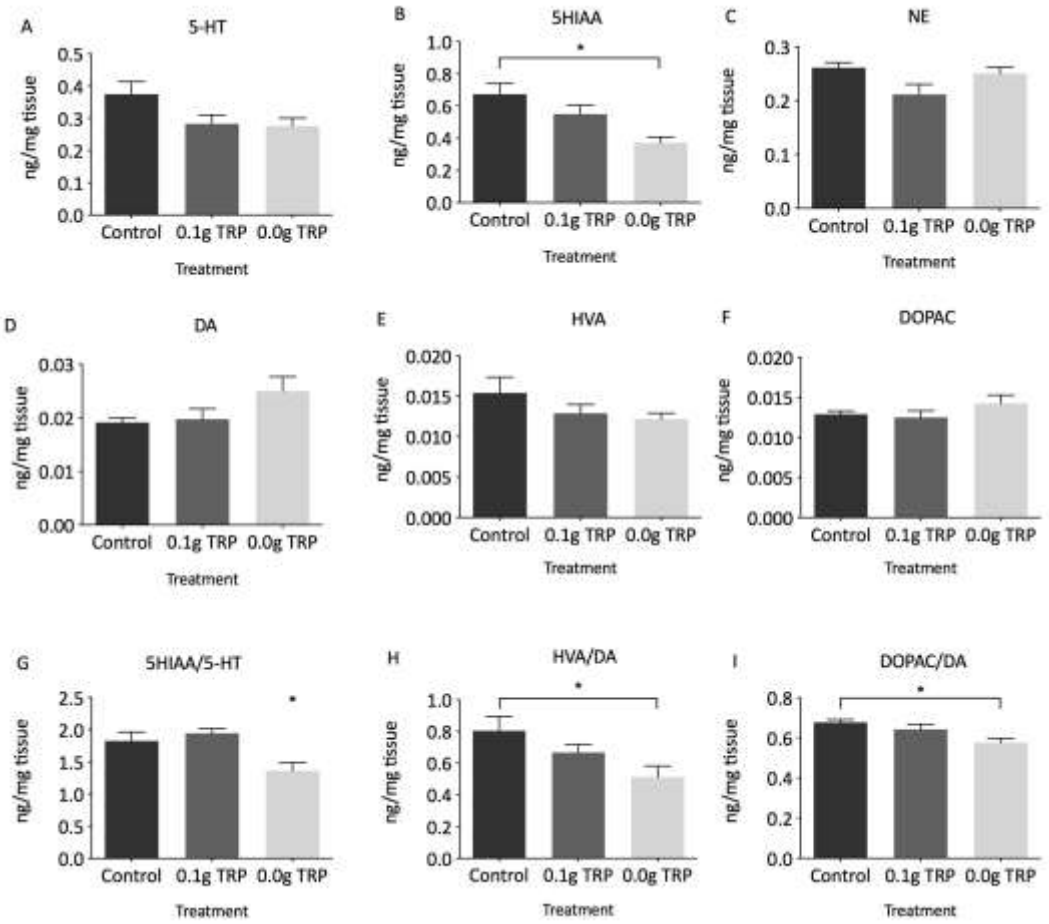

Figure 4.2. Hippocampus concentrations (ng/mg) of 5-HT, 5-HIAA, NE, DA, HVA, and DOPAC (A-F), plus 5-HT and DA turnover (G-I) for females neonatally TRP-deprived (0.0g or 0.1g TRP per 100g diet) as well as controls (0.2g TRP per 100g diet). n = 5 per group. Bars represent the mean (+SEM). * $p < .05$

There was also a significant effect on 5-HT turnover in the hippocampus, $F_{2,12} = 7.06$, $p = .01$ (Figure 4.2g), though this was not significant in the caudate putamen, $F_{2,12} = 1.61$, $p = .24$ (Figure 4.3g). Follow up tests indicated this difference in the hippocampus was between the 0.0g TRP group and both other groups (0.1g TRP: $p = .01$; 0.2g TRP group: $p = .04$).

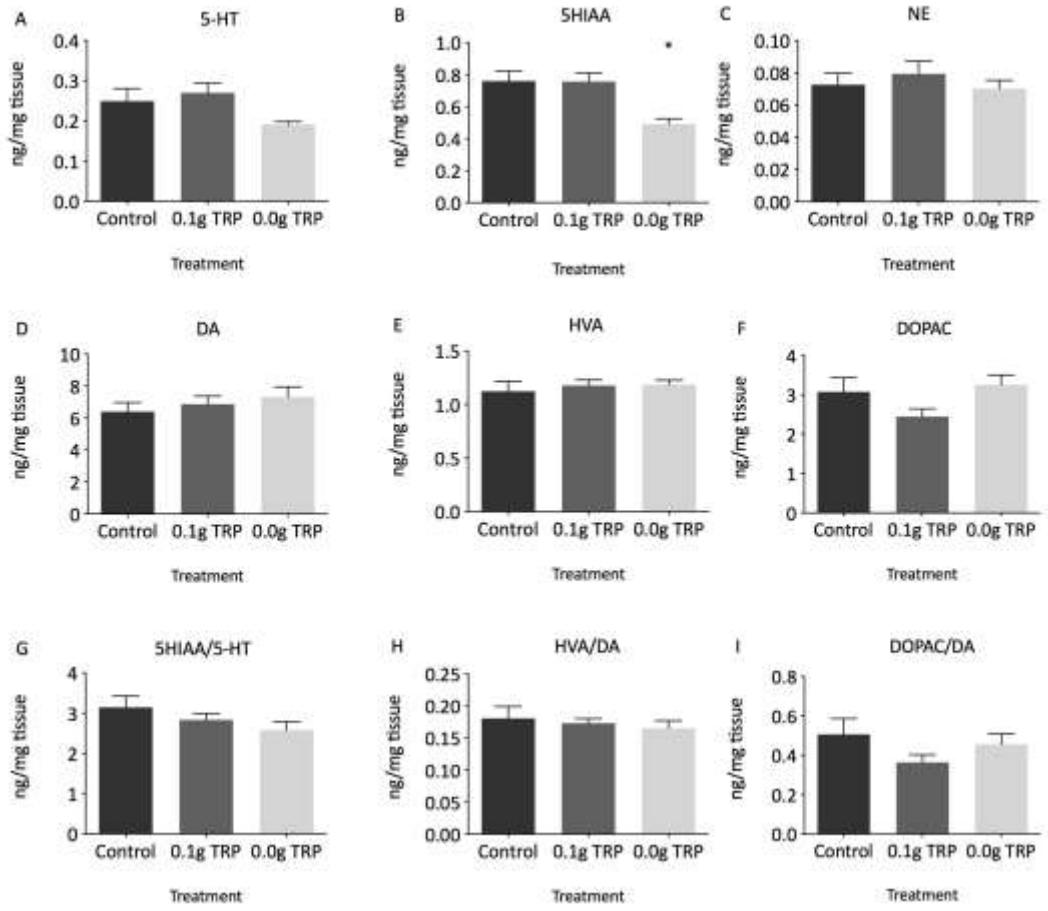

Figure 4.3. Caudate putamen concentrations (ng / mg) of 5-HT, 5-HIAA, NE, DA, HVA, and DOPAC (A-F), plus 5-HT and DA turnover (G-I) for females neonatally TRP-deprived (0.0g or 0.1g TRP per 100g diet) as well as controls (0.2g TRP per 100g diet). n = 5 per group. Bars represent the mean (+SEM). * $p < .05$**.**

Moreover, while there were no differences between groups for DA or its metabolites in either the hippocampus (DA: $F_{2,12} = 2.6$, $p = .12$, Figure 4.2d; HVA: $F_{2,12} = 1.2$, $p = .34$, Figure 4.2e; DOPAC: $F_{2,12} = 2.67$, $p = .11$, Figure 4.2f), or the caudate putamen (DA: $F_{2,12} = .66$, $p = .54$, Figure 4.3d; HVA: $F_{2,12} = .27$, $p = .77$, Figure 4.3e; DOPAC: $F_{2,12} = 2.28$, $p = .15$, Figure 4.3f), there was a significant treatment effect on DA turnover in the hippocampus, with decreased TRP dose-dependently decreasing DA turnover (HVA/DA: $F_{2,12} = 3.97$, $p = .05$, Figure 4.2h; DOPAC/DA: $F_{2,12} = 6.39$, $p = .01$, Figure 4.2i). Follow up tests indicated that 0.0g TRP differed significantly from 0.2g TRP (HVA/DA: $p = .04$; DOPAC/DA: $p = .01$). There was no effect on DA turnover in the caudate putamen (HVA/DA: $F_{2,12} = .26$, $p = .77$, Figure 4.3h; DOPAC/DA: $F_{2,12} = 1.49$, $p = .26$, Figure 4.2i). Finally, there

was no significant treatment effect on NE in either the hippocampus, $F_{2,12} = 3.30$, $p = .07$ (Figure 4.2c), or caudate putamen, $F_{2,12} = .99$, $p = .40$ (Figure 4.3c).

Anxiety Related Tests

Open Field. There was no significant effect of TRP group on time spent in the centre of the open field, $F_{2,53} = .58$, $p = .56$, or on distance travelled $F_{2,53} = 1.5$, $p = .23$, indicating that neonatal TRP depletion did not affect activity measured in the open field task (Figure 4.4).

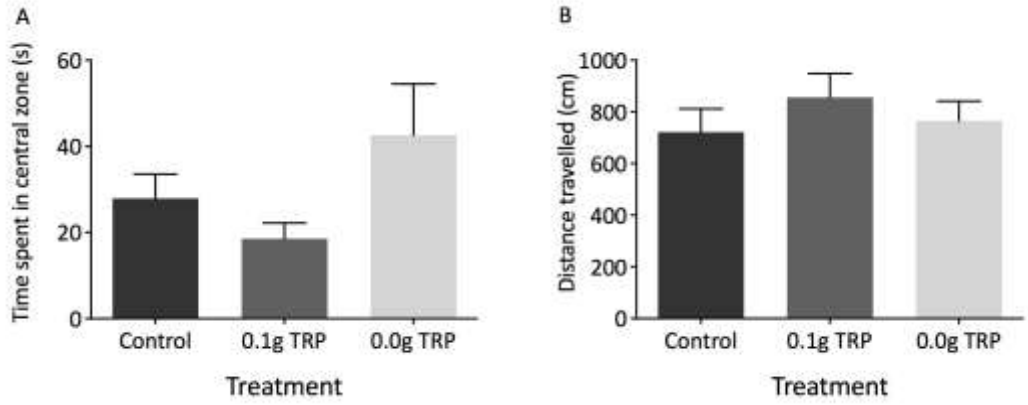

Figure 44. A) Time spent in the centre of the open field (s), and B) distance travelled in the open field (cm), during a 5 minutes novel exposure in rats neonatally TRP-deprived (0.0g or 0.1g TRP per 100g diet, $n = 23$ and 16 respectively) as well as controls (0.2g TRP per 100g diet, $n = 17$). Bars represent the mean (+SEM). * $p < .05$

Novelty Supressed Feeding. As indicated in Figure 4.5, a significant difference between groups was observed, $F_{2,56} = 4.21$, $p = .02$, with post-hoc tests indicating there was a significant difference between the 0.0g and 0.2 TRP groups ($p = .02$). This indicates that neonatal TRP depletion dose dependently increased latency to feed.

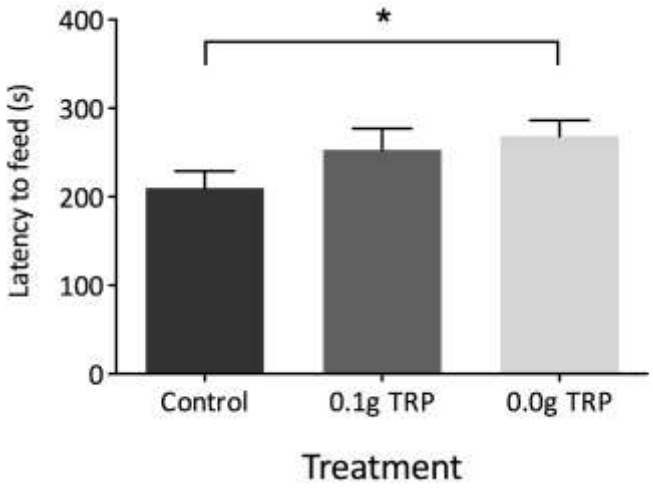

Figure 4.5. Latency to begin eating a food pellet in an open arena following 24 hours of food deprivation in animals neonatally TRP-deprived (0.0g or 0.1g TRP per 100g diet, n = 26 and 16 respectively) as well as controls (0.2g TRP per 100g diet, n = 19). Bars represent the mean (+SEM). * $p < .05$

Amphetamine locomotor activity

There was no effect of group for baseline ambulatory counts $F_{5,42} = .36$, $p = .87$, rearing $F_{5,42} = .24$, $p = .94$, or stereotypy, $F_{5,42} = .78$, $p = .57$. Amphetamine increased forward locomotion across all groups $F_{1,50} = 12.57$, $p < .001$, however there was no effect of TRP group $F_{2,50} = .82$, $p = .45$, and no interaction, $F_{2,50} = .79$, $p = .46$ (Figure 4.6a). Given the apparent trend of increased forward locomotion in the 0.0g TRP group compared to the other two groups, exploratory post-hoc *t*-tests were run. These indicated that, while there was a significant effect of amphetamine in the 0.0g TRP group $t_{12.57} = -2.9$, $p = .01$, there was no significant effect of amphetamine in the other TRP groups (0.1g TRP: $t_{14} = -1.59$, $p = .13$; 0.2 TRP: $t_{15} = -1.70$, $p = .11$).

While amphetamine increased rearing across all groups $F_{1,50} = 7.01$, $p = .01$, there was no effect of TRP group $F_{2,50} = .85$, $p = .43$, and there was no interaction, $F_{2,50} = .701$, $p = .50$ (Figure 4.6b). Again, given the apparent trend of decreased rearing in the 0.0g and 0.1g TRP groups following amphetamine compared to control, exploratory post-hoc *t*-tests were run. These indicated that there was a significant effect of amphetamine on rearing in the 0.2g TRP group $t_{9.93} = -2.65$, $p = .03$, but no significant effect of amphetamine on rearing

for the 0.1g TRP condition $t_{8.09}$ = -1.15, p = .28, or the 0.0g TRP group t_{15} = -.90, p = .38.

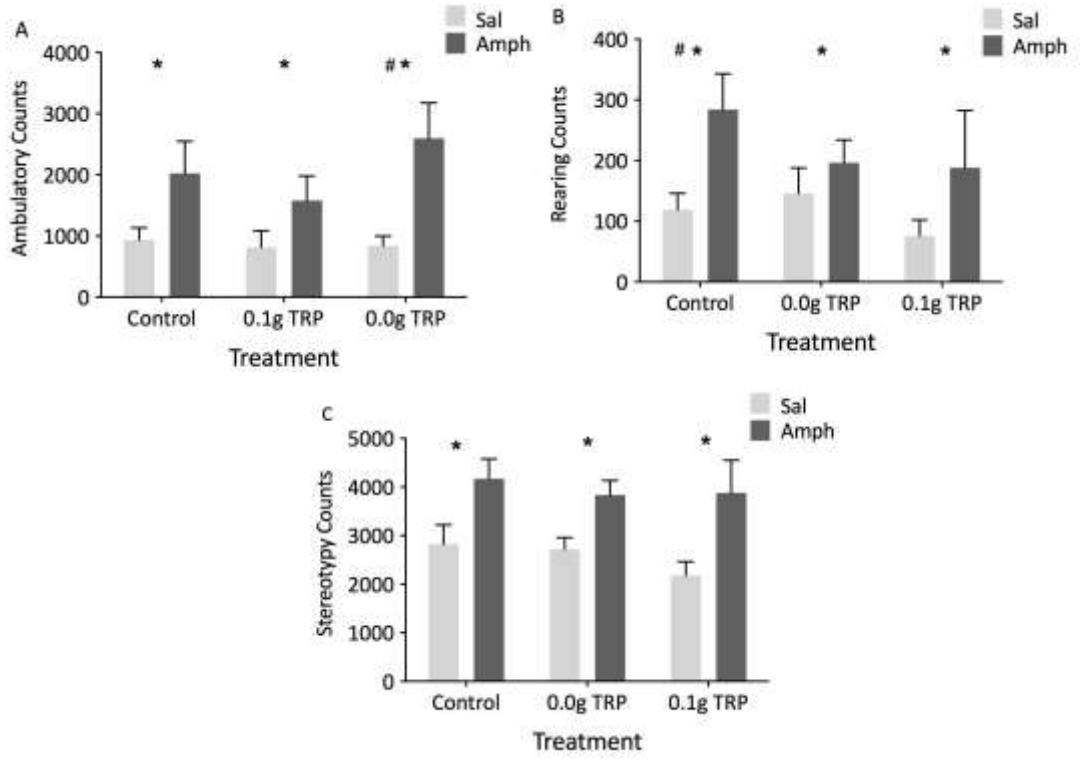

Figure 4.6. Psychomotor effects of either 0.5 mg / kg amphetamine or saline in neonatally TRP-deprived (0.0g or 0.1g TRP per 100g diet) as well as controls (0.2g TRP per 100g diet) animals. Graphs display A) ambulation, B) rearing, and C) stereotypy. 0.0g TRP: saline n = 11, amphetamine n = 12; 0.1g TRP: saline n = 8, amphetamine n = 8; 0.2g TRP: saline n = 9, amphetamine n = 8. Bars represent the mean (+SEM). * p < .05 between drug treatments, # p < .05 in exploratory post-hoc t-tests.

Last, while amphetamine treatment increased stereotypy across all groups $F_{1,50}$ = 19.13, p < .01, there was no effect of TRP group $F_{2,50}$ = .65, p = .52, and no interaction, $F_{2,50}$ = .29, p = .75 (Figure 4.6c). These findings indicate that amphetamine increased forward locomotion, rearing, and stereotypy, while there is some suggestion that acute amphetamine resulted in increased ambulation, but decreased rearing, in animals that had been neonatally TRP-deprived.

Comment

HPLC results confirmed neonatal TRP depletion led to a significant decrease in 5-HIAA in the hippocampus and the caudate putamen, as well as a trend for 5-HT decrease. Moreover, neonatal TRP depletion resulted in an increase in 5-HT turnover in the hippocampus, indicating the manipulation was successful, with the effect being much stronger in the group that received the 0.0g TRP per 100g diet. Moreover, while NE and DA metabolites were unaffected, there was a trend for increased DA levels in the hippocampus, which lead to a significant decrease in DA turnover in the hippocampus, indicating that reducing 5-HT influences DA expression. In adulthood, neonatally TRP-deprived rats showed an increase in anxiety-like behaviour as measured by the NSF task, but not the open field. Moreover, there was some support for an increase in sensitivity to the psychomotor effects of amphetamine in these animals. Taken together, the results from this pilot study indicate this manipulation is strong enough to affect anxiety-like, and possibly dopaminergically mediated, behaviours. Based on these findings, the 0.0g TRP / 100g diet was selected as the experimental dose given that it was both much more successful in decreasing 5-HT tone, as well as inducing behavioural changes, though not so strong as to induce severe physical deficits. However, it should be noted that, while Sprague-Dawley rats were used in the pilot (experiment 1), Wistar rats are used in the experiment proper. While this was necessary due to resource constraints, there exist considerable differences between strains. Consequently, few conclusions will be drawn from this pilot study.

Experiment 2: Early life tryptophan depletion in SERT knock-out rats

Method

Subjects and General Protocols

Serotonin transporter knockout rats (SLC6A4^{1Hubr}, SERT$^{-/-}$) were originally created via N-ethyl-N-nitrosourea (ENU)-induced mutagenesis in a commercial (Harlan, Ter Horst, The Netherlands) wild-type Wistar rat (Smits et al., 2006). Experimental animals were generated by breeding pairs of SERT$^{-/-}$, or SERT$^{+/+}$ fathers with SERT$^{+/-}$ mothers. Originally homozygous breeding has been attempted for SERT$^{-/-}$ animals, however poor maternal care exhibited by SERT$^{-/-}$ mothers resulted in low offspring survival. Offspring resulting from non-homozygous breeding were genotyped at the end of the experiment. Following brain extraction, tail cuts were taken for genotyping (performed at Transnetyx, Cordova, USA). Dams were given an experimental diet containing either a control amount of TRP (0.2g per 100g diet), or no TRP (0.0g per 100g diet), from P0 until P20. Five female and five male SERT$^{-/-}$ offspring per group were sacrificed directly following diet completion, while remaining animals were weaned at P30. Anxiety-like behaviour was assessed from P60 for remaining SERT$^{+/+}$ and SERT$^{-/-}$ animals (Male SERT$^{+/+}$ 0.0g TRP n = 26, 0.2g TRP n = 15, SERT$^{-/-}$ 0.0g TRP n = 9, 0.2g TRP n = 5; Female SERT$^{+/+}$ 0.0g TRP n = 11, 0.2g TRP n = 14, SERT$^{-/-}$ 0.0g TRP n = 9, 0.2g TRP n = 11), first using the open field, followed 1 week later by NSF. Two weeks following NSF, rats were sacrificed by CO_2 asphyxiation followed by decapitation. Brains were immediately frozen in an -80 ^{o}C freezer. Brains were later dissected and RNA was isolated for qPCR (N = 7-9 per group, aside from SERT$^{-/-}$ 0.2g TRP per 100g diet males, for which n = 4).

All animals were bred and reared at the vivarium of the School of Psychology at Victoria University of Wellington and housed in groups of three to four in hanging polycarbonate cages in a temperature (19 - 21 ^{o}C) and humidity (55%) controlled room. Animals were kept on a normal 12 hr light / dark cycle (lights on from 07.00 – 19.00 h), with testing being conducted during the light phase. Aside from testing hours, water and food were available *ad libitum*. All

protocols were approved by the Victoria University of Wellington Animal Ethics Committee (AEC-2013R6).

Diet

Based on the results from the pilot study, the 0.0g TRP / 100g diet was chosen for the experimental diet. Diet composition and schedules are identical to those described in Experiment 1.

HPLC

As with Experiment 1, following diet completion, 5 animals from each group (TRP and sex) were sacrificed for monoamine analysis using HPLC in order to ensure the manipulation was successful. Both male and female $SERT^{+/+}$ rats were used in order to discern whether any sex differences existed. Only $SERT^{+/+}$ rats were used due to the low number of $SERT^{-/-}$ rats available, with those $SERT^{-/-}$ born reserved for behavioural testing. Briefly, on P20 rats were taken between 09:00 and 14:00 hours and sacrificed by CO_2 asphyxiation. Structures were dissected as previously described and stored at -80 ^{o}C. Monoamine plus metabolite concentrations (5-HT, 5-HIAA, DA, DOPAC, HVA, NE) for the prefrontal cortex (PFC) were determined via HPLC (injection volume: 20µl, sensitivity: 500nA). All other methods are identical to those described in Experiment 1.

Anxiety Related Tests

In the open field task animals were removed from the activity chamber immediately once 5 minutes had elapsed. Apparatus and further procedures used, as well as all novelty supressed feeding methods, are identical to those described in Experiment 1.

RNA preparation and gene expression analysis by real time quantitative polymerase chain reaction (qPCR)

RNA extraction and purification. Brains were partially defrosted to -10 ^{o}C +/- 2 ^{o}C and the PFC was dissected using the method described previously (Experiment 1, current chapter). This region was selected based on previous

findings that the PFC is particularly sensitive to alterations in long 3'UTR BDNF in SERT$^{-/-}$ rats (Calabrese et al., 2015; Molteni et al., 2010). Tissue was placed in 1.5 mL Eppendorf tubes and immediately placed on dry ice, then stored at -80 ^{o}C. RNA was isolated from the PFC only. RNA was isolated using a guanidine isothiocyanate-phenol-chloroform extraction using TRIzol® reagent (Life Technologies) in accordance with the manufacturer's instructions. Tissue was homogenised in TRIzol® reagent (500 µL to max 50 g tissue) and incubated at room temperature for 5 minutes. To this, 100 µL of chloroform was added and tubes were inverted 10 times and incubated at room temperature for 2-3 minutes. Samples were centrifuged at 12,000 g for 15 minutes at 4 ^{o}C. The upper aqueous phase was removed and added to a new RNase free 1.5 mL Eppendorf tube. To this, 250 µL isopropanol was added and tubes were mixed by inverting, followed by incubation at room temperature for 10 minutes. Tubes were centrifuged at 12,000 g for 10 minutes at 4 ^{o}C. The supernatant was removed and 500 µL of 75% ethanol was added to the pellet. Tubes were vortexed briefly and then centrifuged at 7500 g for 5 minutes at 4 ^{o}C. The supernatant was removed and the pellet was air-dried either until the ethanol had evaporated or a maximum of 10 minutes. To this, 52 µL of RNase-free H_2O was added to rehydrate the pellet and tubes were incubated at 55 ^{o}C in a water bath for 15 minutes.

Quantification and integrity of RNA. The samples were quantified using a Nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific). A 2 µL aliquot of RNase-free H_2O was loaded onto the Nanodrop as a blank, followed by a 2 µL aliquot of sample. The Nanodrop provided measurements of RNA concentration (ng / ml), as well as measurements of purity ($A_{260/280}$ and $A_{260/230}$) for each RNA sample. Following this, RNA quality was determined using agarose gel electrophoresis. As different size particles migrate though the gel at different rates, agarose gel electrophoresis can determine whether the sample contains any DNA contamination, indicated by separated bands, or RNA degradation, indicated by visible smearing of bands. A 1% agarose gel was used consisting of 1g agarose, 100 mL distilled H_2O and 5 µL of SYBR® safe DNA gel stain (Life Technologies) and was manually cast. Gel was placed in the electrophoresis container (Bio-Rad) and filled with

tris(hydroxymethyl)aminomethane-acetate-ethylenediaminetetraacetic acid (TAE) running buffer (20 mL 50% TAE to 1 L deionised H_2O). A 1 µL aliquot of the RNA sample along with 9 µL RNase free H_2O and 2 µL of 6x loading dye (Thermo Scientific) were loaded into the gel wells. Five µL 1Kb DNA ladder (Thermo Scientific) was placed in the first and last wells. The gel was run at 90V for 30 minutes. Gels were then examined on an ultra violet transilluminator (ChemiDoc, Bio-Rad) using Bio-Rad software (Image Lab™ 5.0 Software).

Reverse Transcription Polymerase Chain Reaction (RT-PCR). RNA was then converted into complementary deoxyribonucleic acid (cDNA) in preparation for the qPCR. Up to 2.5 µg of RNA was added to 4 µL of reverse transcriptase premix (SuperScript® VILO™ MasterMix, Life Technologies) and up to 20 µL of RNase/DNase free H_2O. The mixture was gently mixed and incubated in a RT-PCR machine (Veriti Thermal Cycler ®, ThermoFischer Scientific) for the following cycles: 25 ^oC for 10 minutes; 42 ^oC for 60 minutes; 85 ^oC for 5 minutes. Cycles were then terminated. The undiluted cDNA was then stored at -20 ^oC until the qPCR.

Real Time Polymerase Chain Reaction (qPCR). Samples were processed for qPCR to assess long 3'UTR BDNF, and 5-HT1a (HTR1a) mRNAs. The housekeeping genes used were Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Beta-actin (ACTB). All primers were TaqMan® primer/probe based and ordered at Life Technologies (see Table 3). The qPCR reaction mix consisted of 40 µL RNase/DNase free H_2O, 50 µL TaqMan® gene expression master mix (Applied Biosystems®, Life Technologies), 5 µL primer (TaqMan® Gene Expression Assays (20x), Life Technologies), and 1 µL aliquot of cDNA sample (20 ng). Samples were analysed in 96 well plates (Bio-Rad) that were covered and briefly centrifuged. Samples were run in triplicate with 'no sample' controls. Thermal cycling (c1000™ thermal cycler, Bio-Rad), was initiated with the incubation of 50 ^oC for 2 minutes (RNA retro transcription) followed by incubation at 95 ^oC for 10 minutes (TaqMan® polymerase activation). Following this, 40 - 50 PCR cycles were performed, consisting of 15 seconds at 95 ^oC, to enable melting, followed by 1 minute of 60 ^oC, to enable annealing and extension reactions.

Table 4.3.

Forward and reverse primers and probes purchased from Life Technologies.

Gene	Assay ID
GAPDH	Rn01775763_g1
ACTB (Beta-actin)	Rn00667869_m1
HTR1a	Rn00561409_s1
long 3'UTR BDNF	Rn02531967_s1

Data Analysis

For HPLC data, a two-way ANOVA was used with TRP group and sex entered as between subjects factors. Given that previous experiments were all performed using males alone, as well as variance in anxiety-like behaviour observed in females during different oestrous cycles (Frye, Petralia, & Rhodes, 2000), sex was analysed separately for all tests. A two-way ANOVA was used with genotype and TRP group entered as between subjects factors. Follow up test following significant interactions were performed using independent *t*-tests.

The cycle at which the gene reached a detection threshold, or the quantification cycle (Cq) as well as the efficiency of the PCR plate were averaged and used to calculate the mean normalised gene expression (MNE) per sample. The Cq values of the target (5-HT1a and Long 3' UTR BDNF) and reference genes (GAPDH and BACT) as well as the efficiency of the PCR reactions per sample were used to determine the MNE for the target genes. Values were then log transformed. Following this, considering sex separately, a two-way ANOVA with genotype and TRP group entered as between subject factors was run to determine differences in log MNE between samples.

Extreme outliers were detected using SPSS's outlier test and removed. All tests were run using the IBM SPSS Statistics version 22 for windows. Alpha level was set at $p < .05$ for all data.

Results

HPLC

Monoamine concentrations following neonatal TRP depletion in SERT$^{+/+}$ rats in the PFC are presented in Figure 4.7, with ANOVAs in Table 4.4. Overall, a TRP-free diet led to significant decrease in 5-HIAA and a significant increase in DOPAC in SERT$^{+/+}$ rats in the PFC, and this did not differ between sexes.

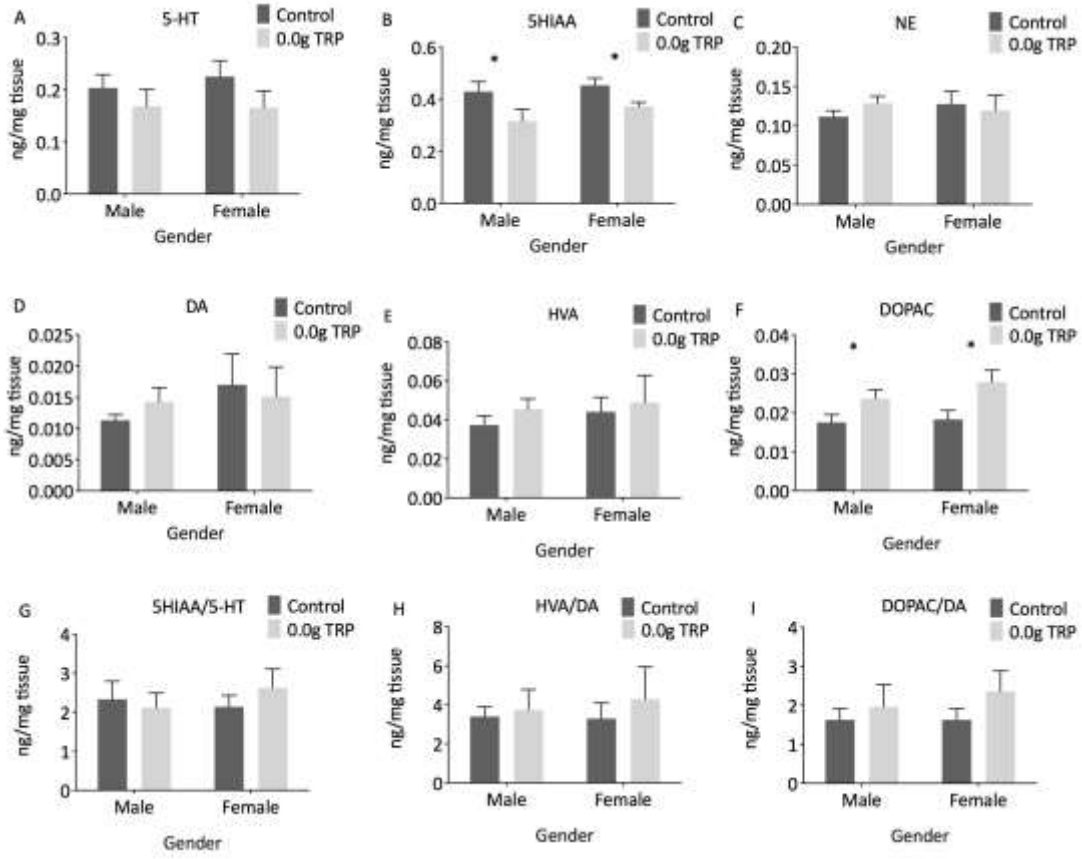

Figure 4.7. PFC concentrations (ng/mg) of 5-HT, 5-HIAA, NE, DA, HVA, and DOPAC (A-F), plus 5-HT and DA turnover (G-I) for SERT$^{+/+}$ males and females neonatally TRP-deprived (0.0g TRP per 100g diet) as well as controls (0.2g TRP per 100g diet). n = 5 per group. Bars represent the mean (+SEM). * $p < .05$.

Table 4.4.

Two-way ANOVA results of TRP depletion on various monoamines and their metabolites in the PFC across sex within SERT$^{+/+}$ rats.

Monoamines	Gender	TRP Group	Interaction
5-HT	$F_{1,16} = .10$, $p = .76$	$F_{1,16} = 2.47$, $p = .14$	$F_{1,16} = .17$, $p = .69$
5-HIAA	$F_{1,16} = 1.43$, $p = .25$	**$F_{1,16} = 8.28$, $p = .01$ ***	$F_{1,16} = .23$, $p = .64$
5-HIAA/5-HT	$F_{1,16} = .14$, $p = .71$	$F_{1,16} = .11$, $p = .75$	$F_{1,16} = .72$, $p = .41$
DA	$F_{1,16} = .80$, $p = .38$	$F_{1,16} = .02$, $p = .88$	$F_{1,16} = .44$, $p = .52$
HVA	$F_{1,16} = .35$, $p = .56$	$F_{1,16} = .55$, $p = .47$	$F_{1,16} = .04$, $p = .85$
DOPAC	$F_{1,16} = 1.06$, $p = .31$	**$F_{1,16} = 10.53$, $p = .01$ ***	$F_{1,16} = .50$, $p = .48$
HVA/DA	$F_{1,16} = .04$, $p = .84$	$F_{1,16} = .09$, $p = .76$	$F_{1,16} = .09$, $p = .76$
DOPAC/DA	$F_{1,16} = .02$, $p = .90$	$F_{1,16} = .62$, $p = .44$	$F_{1,16} = .62$, $p = .44$
NE	$F_{1,16} = .06$, $p = .81$	$F_{1,16} = .82$, $p = .38$	$F_{1,16} = .82$, $p = .38$

*$p < .05$

Anxiety Related Tests

Open Field. For male subjects, a significant genotype by TRP group interaction for time spent in the centre of the open field was observed, $F_{1,50} = 6.18$, $p = .02$ (Figure 4.8a). A main effect of genotype was also found, $F_{1,50} = 15.84$, $p < .001$, although no main effect of TRP group was observed, $F_{1,50} = 3.86$, $p = .06$. Follow up *t*-tests indicated the interaction was driven by a significant difference between TRP-deprived SERT$^{-/-}$ and SERT$^{+/+}$ subjects, $t_{8.5} = 3.41$, $p = .01$ in time spent in the centre compared to SERT$^{+/+}$ rats, $t_{43} = 1.64$, $p = .11$. This indicates that male SERT$^{-/-}$ rats that were deprived of TRP spent significantly more time in the centre of the open field than TRP-deprived SERT$^{+/+}$. For female subjects, no interaction between genotype and TRP group was observed, $F_{1,41} = 1.28$, $p = .27$. However, female SERT$^{-/-}$ rats did spend more time in the centre of the open field compare with SERT$^{+/+}$ rats, $F_{1,41} = 15.01$, $p < .001$ (Figure 4.8b), although no difference was observed between those deprived of TRP in early life and those not, $F_{1,41} = .24$, $p = .62$.

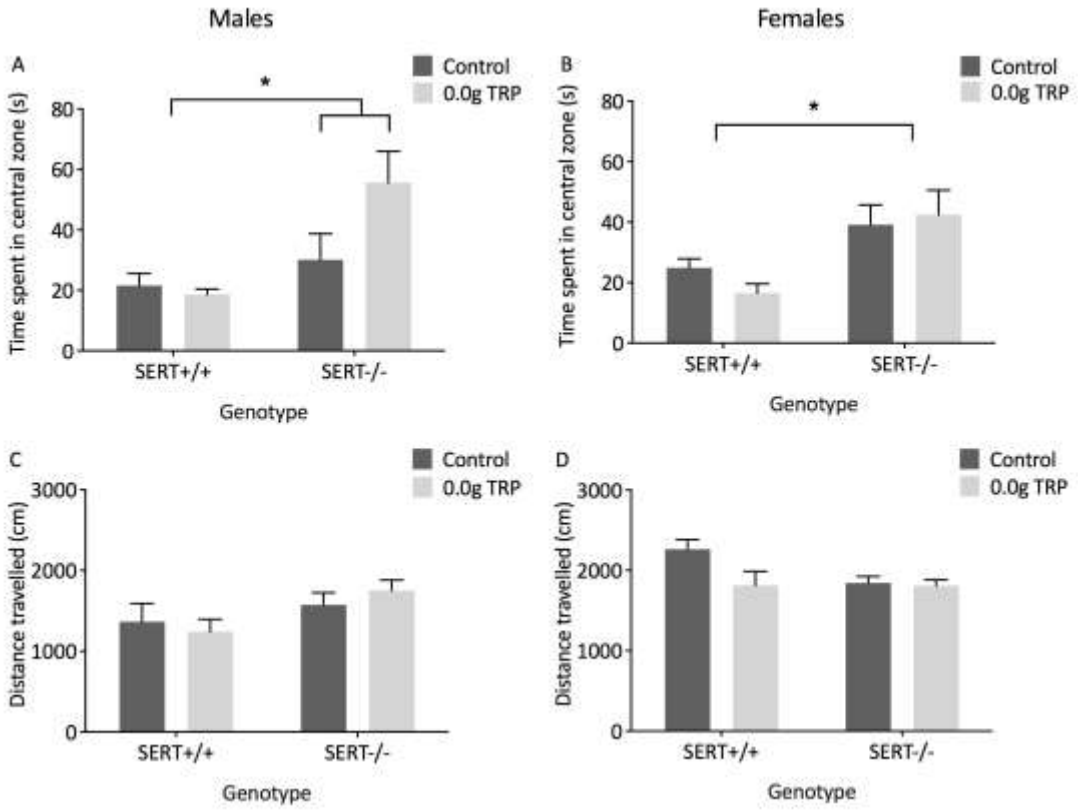

Figure 4.8. Time spent in the centre of the open field (s) for male (a) and female (b), and distance travelled in the open field (cm) for male (c) and female (d) SERT$^{-/-}$ and $^{+/+}$ rats neonatally TRP-deprived (0.0g TRP per 100g diet) as well as controls (0.2g TRP per 100g diet) during a 5 minutes novel exposure. Male SERT$^{+/+}$ 0.0g TRP n = 26, 0.2g TRP n = 15, SERT$^{-/-}$ 0.0g TRP n = 9, 0.2g TRP n = 5; Female SERT$^{+/+}$ 0.0g TRP n = 11, 0.2g TRP n = 14, SERT$^{-/-}$ 0.0g TRP n = 9, 0.2g TRP n = 11. Bars represent the mean (+SEM). * $p < .05$

Neither males (Genotype: $F_{1,41} = 2.88$ $p = .10$; TRP group: $F_{1,41} = 3.74$, $p = .06$; Genotype x TRP group: $F_{1,41} = 2.79$, $p = .10$), nor females (Genotype: $F_{1,50} = 2.02$, $p = .16$; TRP group: $F_{1,50} = .012$, $p = .91$; Genotype x TRP group: $F_{1,50} = .35$, $p = .55$) demonstrated any differences in distance travelled in the open field (Figure 4.8c-d). This indicates that the increased time spent in the centre of the open field demonstrated by TRP depleted SERT$^{-/-}$ rats is not driven by an increase in exploratory behaviour. However, pooled analysis indicated a significant effect of sex, with females moving a greater distance compared with males (Figure 4.9), $t_{123.53} = 5.15$, $p < .001$

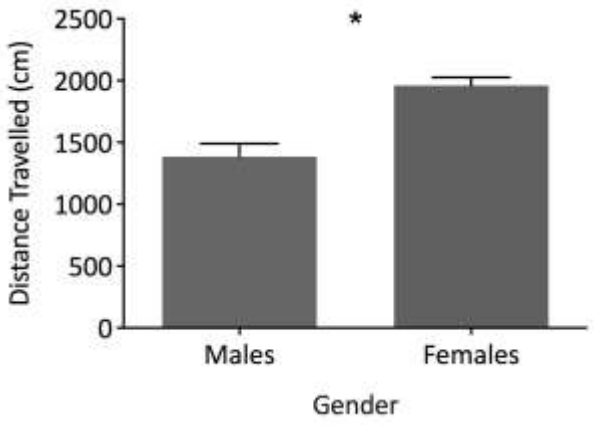

Figure 4.9. Pooled data showing distance travelled in the open field (cm) during a 5 minutes novel exposure for male (n = 54) vs. female (n = 45) subjects. Bars represent the mean (+SEM). * p < .05.

Novelty Supressed Feeding. As displayed in Figure 4.10a, male SERT$^{-/-}$ rats had a longer latency to feed than SERT$^{+/+}$ $F_{1,50}$ = 19.31 p < .001, regardless of TRP group, $F_{1,50}$ = .65, p = .42. There were no interactions between genotype and TRP group observed, $F_{1,50}$ = .01, p = .93. In females however (Figure 4.10b), while SERT$^{-/-}$ rats also had a longer latency to feed compared with SERT$^{+/+}$ rats, $F_{1,41}$ = 9.69, p < .001, neonatally TRP-deprived animals also showed increased latency to feed, $F_{1,41}$ = 4.38, p = .04. No interaction between the two variables was observed, $F_{1,41}$ = .05 p = .83.

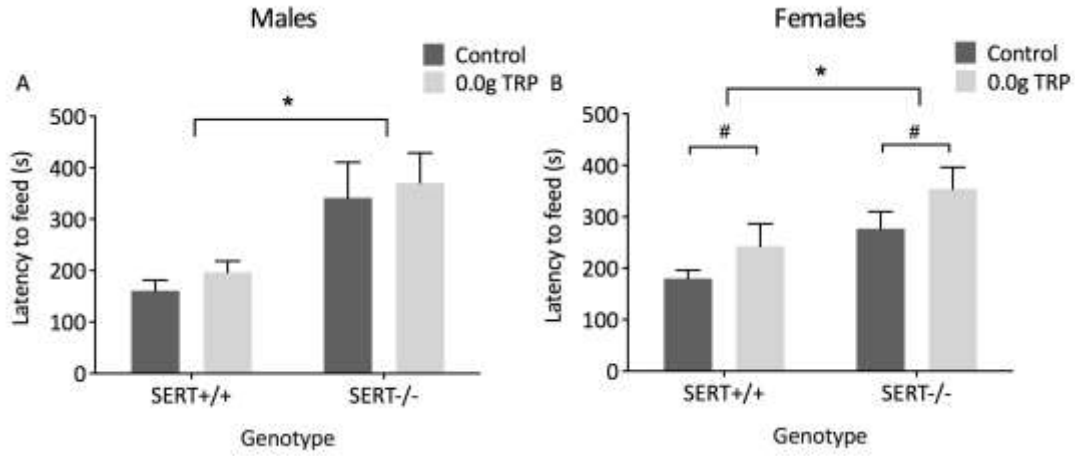

Figure 4.10. Latency to eat in food-deprived animals in the novelty supressed feeding task for male (a) and female (b) SERT$^{-/-}$ and $^{+/+}$ rats neonatally TRP-deprived (0.0g TRP per 100g diet) as well as controls (0.2g TRP per 100g diet). Male SERT$^{+/+}$ 0.0g TRP n = 26, 0.2g TRP n = 15, SERT$^{-/-}$ 0.0g TRP n = 9, 0.2g TRP n = 5; Female SERT$^{+/+}$ 0.0g TRP n = 11, 0.2g TRP n = 14, SERT$^{-/-}$ 0.0g TRP n = 9, 0.2g TRP n = 11. Bars represent the mean (+SEM). * p < .05 between genotypes, # p < .05 between TRP groups.

qPCR

Following RNA extraction, all samples were analysed for purity and quantity using a Nanodrop spectrophometer, as well as agarose gel electrophoresis to ensure quality. On the basis of these analyses, two samples were removed from further analyses as they did not contain enough RNA for qPCR testing.

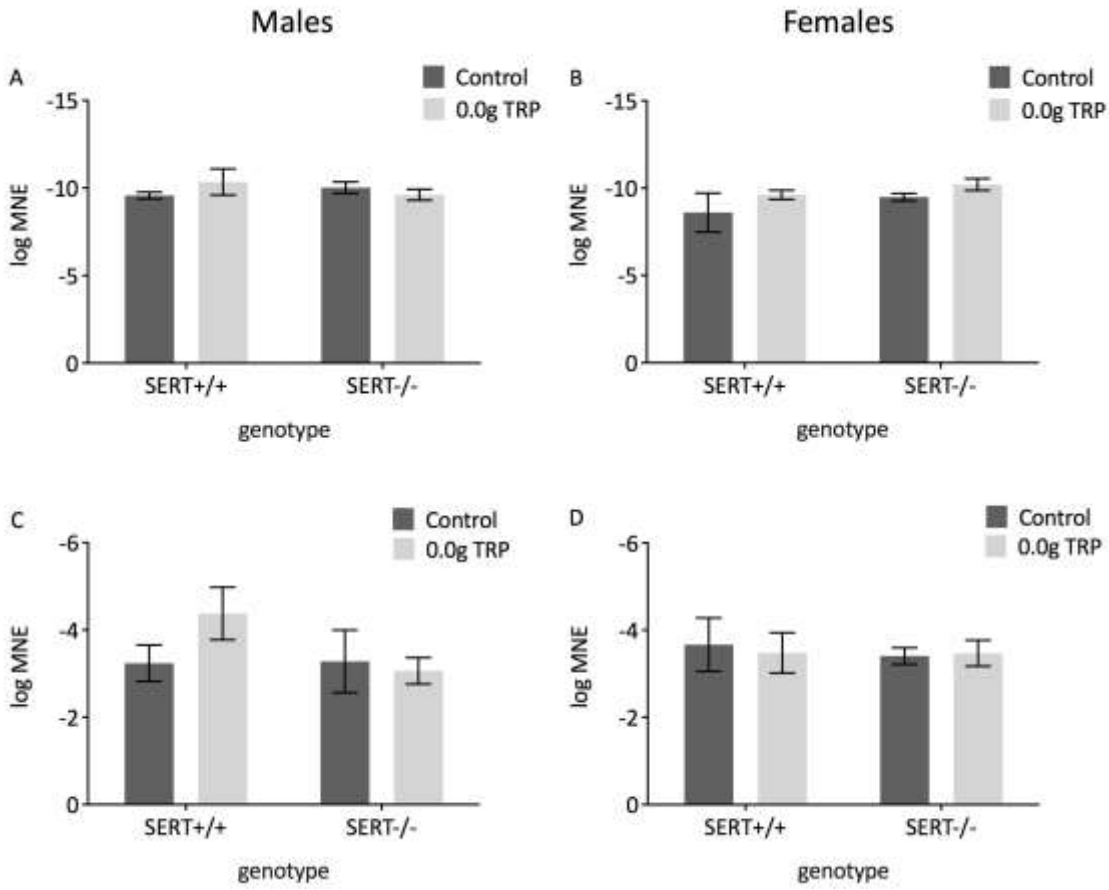

Figure 4.11. Log mean normalised gene expression (MNE) of BDNF (A,B) and 5-HT1a (C,D) for male and female SERT$^{-/-}$ and $^{+/+}$ rats neonatally TRP-deprived (0.0g TRP per 100g diet) as well as controls (0.2g TRP per 100g diet). *N* = 7-9 per group, aside from SERT$^{-/-}$ 0.2g TRP per 100g diet males, for which *n* = 4. Bars represent the mean (+SEM).

As indicated in Figure 4.11a, expression of long '3 UTR BDNF in male subjects did not differ between genotypes, $F_{1,25} = .07$, $p = .80$, or TRP group $F_{1,25} = .11$, $p = .74$, and no interaction was present, $F_{1,25} = 1.17$, $p = .29$. Similarly, expression of 5-HT1a in males (Figure 4.11c) did not differ between genotypes, $F_{1,25} = 1.4$, $p = .25$, or TRP group $F_{1,25} = .74$, $p = .40$, and no interaction was found, $F_{1,25} = 1.60$, $p = .22$. Expression of BDNF in females (Figure 4.11b) also failed to

differ between both genotype, $F_{1,27}$ = 1.40, p = .25, as well as TRP group $F_{1,27}$ = 1.93, p = .18, and no interaction was found, $F_{1,27}$ = .06, p = .81. Finally, expression of 5-HT1a in females demonstrated no difference between genotypes, $F_{1,27}$ = .10, p = .76, TRP group $F_{1,27}$ = .02, p = .89, and no interaction was found, $F_{1,27}$ = .09, p = .77 (Figure 4.11d).

Discussion

The aim of the current study was to determine whether neonatal TRP depletion would rescue the anxiety-like behaviour normally found in SERT$^{-/-}$ rats. In line with our hypothesis, neonatal TRP depletion led to an increase in time spent in the centre of the open field in male SERT$^{-/-}$ rats. Intriguingly however, while there was no effect of neonatal TRP depletion, female SERT$^{-/-}$ rats spent more time in the centre of the open field, implying the female SERT$^{-/-}$ rats show less anxiety-like behaviour compared to their SERT$^{+/+}$ counterparts. These alterations in time spent in the centre of the open field were not mediated by increased exploratory behaviour in the open field, however females did display an increase in distance travelled compared with male rats. These findings contrast with those observed in the NSF task, in which both male and female SERT$^{-/-}$ rats exhibited a longer latency to feed. However, while there was no effect of neonatal TRP depletion in male rats, it did lead to increased latency to feed in females. Finally, neither genotype, nor neonatal TRP depletion, affected the mRNA levels of either 3'UTR BDNF or 5-HT1a, in either sex.

In contrast to a previous study assessing the anxiety-like behaviours of SERT$^{-/-}$ rats (Olivier et al., 2008), the current data do not reveal the anticipated genotype effect on time spent in the centre of the open field. Olivier et al. (2008) found that SERT$^{-/-}$ rats spent *less* time in the centre of the open field, and there was no difference between sexes. In contrast, while there was no genotype effect observed for male rats in the current data, female rats paradoxically displayed *increased* time spent in the centre of the open field, implying *decreased* anxiety-like behaviour compared with their SERT$^{+/+}$ counterparts. Methodological differences likely underlie these incongruent findings. For instance, the open field used in the present study was much smaller than that used in Olivier et al. (2008), which measured 100 cm x 100 cm. Moreover, the open field in the current study was placed in a sound-attenuated chamber with a relatively low ceiling (approximately 15 cm above the walls), while Olivier et al. (2008) placed the open field in a normal room (i.e. the ceiling was more than a meter above the open field walls). It is therefore quite possible that the centre of the particular open field task used in the current study may not have produced such anxiety provoking conditions compared with that used by Olivier

et al. (2008). Nonetheless, the results show that mild anxiety was reversed by neonatal TRP depletion in SERT$^{-/-}$ males.

Moreover, in contrast to the decreased anxiety-like behaviour displayed by females in the open field, both male and female SERT$^{-/-}$ showed increased anxiety-like behaviour in the NSF task. Any conclusions that female SERT$^{-/-}$ rats display less anxiety-like behaviour in the open field than their SERT$^{+/+}$ counterparts are therefore limited. These findings are partially consistent with those previously described by Olivier et al. (2008) who found a genotype effect in male but not female SERT$^{-/-}$ rats in this task. Compared to their male counterparts, Olivier et al. (2008) found that female SERT$^{+/+}$ rats exhibited an increased latency to feed (i.e. were more "anxious"), while female SERT$^{-/-}$ rats showed a decreased latency to feed (were less "anxious"). Consequently, the difference between genotypes apparent in males was absent in females. In the current study however, considering control TRP diet rats only, while female SERT$^{-/-}$ rats were also less "anxious" than their male counterparts, the latency to feed in SERT$^{+/+}$ rats was consistent between sexes. Overall then, the differences between the two studies may be mediated by the performance of SERT$^{+/+}$ females. This is possibly due to methodological differences. For instance, while Olivier et al. (2008) isolated animals for 24 hours prior to the NSF task, this was not possible in the current study given space constraints. Consequently, it may be that this isolation increased the sensitivity of SERT$^{+/+}$ females to the anxiogenic conditions of the NSF task in Olivier et al. (2008) compared to the current study.

Given that the open field task used in the current experiment was not as anxiety inducing as previous iterations, it is likely that the NSF was much more anxiogenic. It appears therefore, in male SERT$^{-/-}$ rats at least, neonatal TRP depletion was able to rescue mild anxiety, but not able to rescue more severe anxiety. This is most likely due to the TRP treatment itself, which was only able to produce a mild reduction in 5-HT functioning, as indicated by the HPLC findings. Indeed, previous 'rescuing' of the altered phenotype in SERT$^{-/-}$ mice by decreasing 5-HT during neonatal development used the 5-HT neurotoxin pCPA (Alexandre et al., 2006), a much stronger 5-HT reducing manipulation. However, in addition to the confound evident in administering distressing

injections during early life (Girardi et al., 2014), in pilot testing prior to the current study pCPA led to blindness across all genotypes, and its use was consequently terminated for ethical reasons. TRP depletion was consequently performed as a less severe alternative. Unfortunately, this manipulation may not have been strong enough to rescue anxiety in the more anxiogenic NSF task. Indeed, while TRP depletion did lead to a decrease in 5-HIAA, there was no significant decrease in 5-HT, although there was a trend. Moreover, instead of direct administration to pups, as occurs with pCPA, TRP depletion is transmitted to the pups indirectly via lactation. In line with previous studies, the depletion protocol was only enacted from P0 to P20, a protocol that has been successful in previous studies by González et al. (2008). However, many other studies have introduced TRP depletion from much earlier in development, some from prior to conception (Del Angel-Meza, Ramírez-Cortés, Olvera-Cortés, Pérez-Vega, & González-Burgos, 2001). However, given that previous studies have successfully induced mood disorder-like behaviour in mice following a reduced exposure to TRP depletion (P0 to P8)(Zoratto et al., 2011; Zoratto et al., 2013) it is unclear whether a longer exposure would have had a greater effect. An alternative could be the 5-HT1a receptor antagonist WAY100635, which rescued depression-like behaviour in SERT$^{-/-}$ mice (Alexandre et al., 2006). While its administration also comprises injection-stress, it is likely to be less harsh on the pups than pCPA, given it is not a neurotoxin, and therefore may indeed be a viable alternative to rescue severe anxiety-like behaviour in the SERT$^{-/-}$ rat.

Further research is required to determine whether the increased sensitivity to psychostimulants displayed by SERT$^{-/-}$ rats can also be rescued through decreasing excessive extracellular 5-HT during early development. However, given that neonatal TRP depletion could only rescue mild anxiety in male SERT$^{-/-}$ rats, and that sensitivity to psychostimulants is a much more robust behaviour than anxiety, it is likely a stronger manipulation, possibly a neonatal 5-HT1a receptor antagonist treatment, will be required.

Unlike in the open field task, neonatal TRP depletion failed to rescue the anxiety-like phenotype displayed by male SERT$^{-/-}$ rats in the NSF task. However, there was an effect in females, with TRP depletion leading to an increased

latency to feed, regardless of genotype. These findings are in line with those observed in Sprague-Dawley rats in the pilot experiment (Experiment 1), which indicated that neonatal TRP depletion led to an increase in anxiety-like behaviour in the NSF task (but not in the open field task). These finding indicate that both increased as well as decreased neonatal 5-HT can lead to similar pathological phenotypes, and are in line with previous studies on both neonatal TRP depletion (Zhang et al., 2006; Zoratto et al., 2013) and SSRI exposure (Ansorge et al., 2008; Ansorge et al., 2004; Olivier et al., 2011). Taken together, these data demonstrate that both increasing and decreasing 5-HT during early development can lead to an increase in anxiety-like behaviours. Moreover, in addition to disruptions in emotion related behaviours, results from the pilot study (Experiment 1) indicate neonatal TRP depletion may lead to increased sensitivity to the psychomotor effects of amphetamine, which is in line with previous indications that neonatal SSRIs lead to increased sensitivity to the reinforcing effects of cocaine (Forcelli & Heinrichs, 2008). Thus, it is tempting speculate that an optimal level of 5-HT during development may be necessary for normal neuronal development.

Unexpectedly, there were no differences in mRNA expression for long 3'UTR BDNF or 5-HT1a, in either sex, between genotypes or following neonatal TRP depletion. This is in contrast with our hypothesis that the mRNA levels of both these genes would be down-regulated in SERT$^{-/-}$ rats, and unaffected in SERT$^{-/-}$ rats that had been exposed to neonatal TRP depletion. Previous studies in SERT$^{-/-}$ rats have indicated that long 3'UTR BDNF mRNA is decreased in male SERT$^{-/-}$ rats in the hippocampus from P0 and the PFC from P7 (Calabrese et al., 2013), and in the ventromedial, but not the dorsomedial, PFC in adults (Calabrese et al., 2015). Given that the current study measured long 3'UTR BDNF mRNA in the whole PFC, not differentiating between these two regions, it is possible any differences in the present study were averaged out. Future studies ought to examine different regions of the PFC, as well as the mRNA levels of different BDNF transcripts, for which differences between genotypes have also been observed (Calabrese et al., 2015; Calabrese et al., 2013; Molteni et al., 2010). Furthermore, although the total number of 5-HT1a receptors is down-regulated in SERT$^{-/-}$ rats (Homberg et al., 2008), given the results of the

current study, it is possible the transcription of 5-HT1a mRNA is unaffected. This suggests a regulatory mechanism for this down-regulation, such as receptor internalisation, increased degradation, or a failure to translate the receptor. Further research is required to determine the exact mechanism of this down-regulation.

Due to animal number constraints, monoamine levels following neonatal TRP depletion in $SERT^{-/-}$ rats were not assessed. Consequently, it is unknown precisely how neonatal TRP depletion affected the levels of monoamines in $SERT^{-/-}$ rats. However, given that $SERT^{-/-}$ rats have increased extracellular 5-HT, it's likely the effect of TRP depletion would have been much greater. In addition, as significant differences in anxiety-like behaviour were found between TRP-deprived and control $SERT^{-/-}$ rats, this manipulation appears to have had a successful behavioural effect. However, it is unknown precisely the extent to which 5-HT levels were decreased in $SERT^{-/-}$ rats. Further studies are required to determine whether their levels were normalised to the levels usually found in $SERT^{+/+}$ rats or just decreased.

Overall, the results from the current experiment indicate a mild, but not severe, anxiety-like phenotype in male $SERT^{-/-}$ rats can be rescued through decreasing 5-HT during neonatal development. While the mechanism for this alteration is as yet unknown, it is possible that an optimal level of 5-HT is necessary for normal brain development.

Chapter 5: General Discussion

1 in 4 people, like me, have a mental health problem.
Many more people have a problem with that.

— Stephen Fry

Although the lifetime rates for mental illness are staggering, as high as 1 in 3 in New Zealand (Oakley Browne et al., 2006), substantial stigma exists toward individuals suffering from mental illness. Indeed, drug addiction and alcoholism are commonly perceived as self-inflicted (Crisp, Gelder, Rix, Meltzer, & Rowlands, 2000), and a common mantra in New Zealand is that individuals with depression just need to 'harden up'. Moreover, individuals suffering from some forms of mental illness, such as schizophrenia and drug addiction, are thought to be dangerous and unpredictable (Crisp et al., 2000). This pervasive stigma provides significant barriers to recovery. For instance, it leads to lowered self-esteem, and makes it more difficult to obtain work and find stable housing (Corrigan, 2004; Link, Struening, Neese-Todd, Asmussen, & Phelan, 2001), in a population that already suffers from these difficulties due to their illness.

However, there is no scientific evidence to back up the notion that mental illness is a choice. Instead, complex interactions between an individual's genetic and environmental backgrounds lead to an increased predisposition for mental illness in most individuals. For instance, as opposed to being self-inflicted, addiction is better described as a complex brain disorder, characterised by a loss of control, leading to the compulsive use of psychoactive substances despite negative consequences. As outlined in Chapter 1, the neurotransmitter serotonin (5-HT) is thought to be implicated in many psychiatric disorders. For instance, antidepressants that inhibit the 5-HT reuptake transporter (SERT) are commonly prescribed to treat a multitude of psychiatric disorders, including depression, anxiety, and anorexia nervosa. Moreover, the loss of function allele of the 5-HTTLPR (the short (s-) allele) has been associated with narcissism, anxiety, bipolar disorder, major depression, and increased drug dependence

(Cao et al., 2013; Caspi et al., 2003; Enoch et al., 2011; Gerra et al., 2007; Heils et al., 1996; Lesch et al., 1996; Sen et al., 2004).

Consequently, the overall aim of the current thesis was to determine whether dysregulation of 5-HT is directly linked to the occurrence of psychiatric disorders, particularly drug dependence and anxiety. Studies using humans can only elucidate a correlation between variables, not causal relationships. Indeed, it is entirely possible that an independent variable leads to both 5-HT dysregulation and psychiatric disorders. However, rodent models, in which variables can be more tightly controlled and directly manipulated, can be used to establish causation. While low SERT expressing genotypes found in humans, such as the s-allele, do not naturally occur in rodents, genetic models have been created in which the SERT allele is knocked out ($SERT^{-/-}$) (Bengel et al., 1998; Smits et al., 2006). Unlike s-allele carrying humans, who only show a 50% reduction in SERT activity (Lesch et al., 1996), $SERT^{-/-}$ rats have no SERT protein whatsoever. However, there are similarities in their phenotypes: As with human that have low SERT expressing genotypes, $SERT^{-/-}$ rats demonstrate anxiety- and depression-related behaviours (Olivier et al., 2008), although the presentation of these behaviours is strain dependent in $SERT^{-/-}$ mice. For instance, while $SERT^{-/-}$ mice on a C57BL/6J background show no apparent depression-like behaviour (Holmes et al., 2002; Kalueff et al., 2006), on a 129S6 background, $SERT^{-/-}$ mice display a depression-like phenotype in the forced swim test, but an anti-depressant response in the tail suspension test (Holmes et al., 2002; Lira et al., 2003). Conversely, $SERT^{-/-}$ mice on a C57BL/6J background exhibit anxiety-like behaviour (Holmes et al., 2003), while on a 129S6 background, $SERT^{-/-}$ mice display no anxiety-like behaviour in the open field or elevated plus maze tasks, although they do in the novelty supressed feeding (NSF) task (Lira et al., 2003), indicating that both species and genetic background can influence phenotype.

Additionally, as with s-allele carriers, who show increased abuse of cocaine (Cao et al., 2013; Enoch et al., 2011), $SERT^{-/-}$ rodents show increased sensitivity to the reinforcing properties of cocaine (Homberg et al., 2008; Sora et al., 1998). Although, unlike $SERT^{-/-}$ rats, $SERT^{-/-}$ mice do not display increased cocaine-induced psychomotor activity (Homberg et al., 2008; Sora et al., 2001).

However, these increased effects of cocaine in SERT$^{-/-}$ rodents are not particularly dramatic, and are indeed only seen at low but not high doses (Homberg et al., 2008). This is likely due to the relatively strong reinforcing effects of cocaine. Consequently, determining genotype responses to a less reinforcing drug of abuse, such as (±) 3, 4-Methylenedioxymethamphetamine (MDMA), was proposed in the current thesis.

MDMA acts upon the reuptake transporters of 5-HT, dopamine (DA), and norepinephrine (NE) by both blocking the transporters and inducing reverse transport, to increase extracellular neurotransmitter levels (Crespi et al., 1997). However, unlike traditional psychostimulants such as cocaine and methamphetamine, which exert most of their effects on DAT, MDMA has a much higher affinity for SERT (Han & Gu, 2006). This increased propensity for the SERT compared with traditional psychostimulants is thought to underlie its reduced abuse potential (Callaway, Wing, & Geyer, 1990; Fantegrossi et al., 2002; Schenk et al., 2007). Although no studies have previously looked at the effects of MDMA in SERT$^{-/-}$ rats, there have been a limited number of studies using the mouse model. These studies show that both the self-administration and the psychomotor effects of MDMA are abolished in SERT$^{-/-}$ mice (Bengel et al., 1998; Trigo et al., 2007). However, there are a number of differences between rats and mice which may mean that these findings do not translate to the rat model or humans. For instance, MDMA acts as a selective DA neurotoxin in mice but a 5-HT neurotoxin in rats, which is more similar to humans, as the SERT is down-regulated in current MDMA users (Buchert et al., 2003; Buchert et al., 2004; Colado et al., 2004; Easton & Marsden, 2006; Kindlundh-Högberg et al., 2007; O'Callaghan & Miller, 1994; O'Shea et al., 2001; O'Shea et al., 1998). Consequently, it is important to assess the effects of MDMA in the rat model also. Given the complex behavioural profile of MDMA in rats compared to traditional psychostimulants, in addition to its influence on 5-HT, DA, and NE, the current thesis aimed to use SERT$^{-/-}$ rats to investigate which of the behaviours were mediated by SERT and which are not.

Consequently, Chapter 2 investigated the response of SERT$^{-/-}$ to several behavioural tasks that MDMA has previously been shown to disrupt. The results indicated that MDMA-induced disruption of startle habituation was abolished in

SERT$^{-/-}$ rats for 5 mg / kg, but not 10 mg / kg MDMA. The lack of effect for 10 mg / kg MDMA is likely to do with learning (see Chapter 2 for more detail). Indeed, subsequent data collected in our laboratory group has shown the same effect of MDMA-induced disruption of startle habituation in experimentally naïve SERT$^{+/+}$ rats with 10 mg / kg MDMA. These findings indicate that the SERT is necessary for MDMA-induced disruption of startle habituation, and are in line with previous pharmacological studies that showed these tasks are mediated by 5-HT (Kehne et al., 1992). Moreover, the increase in ambulation and stereotypy following MDMA was unaltered in SERT$^{-/-}$ rats, although there was a trend for a diminished stereotypic response following MDMA compared to SERT$^{+/+}$ rats. These findings indicate that the SERT is not required for MDMA-induced locomotor activity, in spite of previous pharmacological studies indicating that MDMA-induced locomotor activity is mediated by 5-HT as well as DA (Daniela et al., 2004; Kehne et al., 1996). However, these differences are likely mediated by the lack of target specificity in the pharmacological antagonistists used in these studies (see page 25 for further detail). Finally, in the drug discrimination paradigm, those SERT$^{-/-}$ rats that could discriminate between saline and low dose MDMA in the training phase fully generalised low dose MDMA to amphetamine, while only partial generalisation was observed in control rats. These findings indicate that 5-HT is essential for discriminating low dose MDMA, and also suggest that in SERT$^{-/-}$ rats the discriminative stimulus properties of MDMA are mediated via DA. However, only a small number of SERT$^{-/-}$ rats could learn to discriminate low dose MDMA from saline. This is likely due to the very small dopaminergic increase achieved following a low dose of MDMA, which did not enable most of the SERT$^{-/-}$ rats to reliably differentiate MDMA from saline. Overall, the findings from Chapter 2 indicate that the response to MDMA-induced disruption of tasks that pharmacological studies had previously indicated were predominately mediated by 5-HT are abolished in SERT$^{-/-}$ rats.

Given that those SERT$^{-/-}$ rats that could discriminate between saline and low dose MDMA could not discriminate between MDMA and amphetamine, it was postulated that SERT$^{-/-}$ rats might show an increased propensity to self-administer standard doses of MDMA. This was the focus for Chapter 3.

Strikingly, while only around half of SERT$^{+/+}$ rats learned to self-administer MDMA, a finding in keeping with previous studies (Schenk et al., 2003), all of the SERT$^{-/-}$ rats acquired MDMA self-administration. Moreover, upon acquisition, SERT$^{-/-}$ rats obtained higher amounts of MDMA, and expended increased efforts to obtain the drug. These results indicate that SERT$^{-/-}$ rats are indeed more sensitive to the reinforcing effects of MDMA. These findings are consistent with the theory proposed by Bradbury et al. (2013) that a reduction in normal MDMA produced increases in 5-HT neurotransmission is required for the acquisition of MDMA self-administration.

Overall, both humans and rodents with a down-regulated SERT function display pathological phenotypes in adulthood. However, the mechanism underlying this change is unclear. Consequently, Chapter 4 investigated the role of increased 5-HT during early brain development in the SERT$^{-/-}$ rats. It is paradoxical that humans and rodents with low functioning SERT genotypes would display increased risk of anxiety, depression, and drug abuse given that selective SERT blocking drugs (SSRIs) result in a decreases in these behaviours, e.g. (Carroll et al., 1990a). However, while individuals taking SSRIs do so largely in adulthood, individuals with low SERT expressing genotypes display increased extracellular 5-HT from very early on in development. Indeed, when extracellular 5-HT is normalised in SERT$^{-/-}$ mice in the two weeks following birth using the 5-HT neurotoxin para-chlorophenylalanine (pCPA), their disrupted rapid eye movement (REM) sleep is rescued (Alexandre et al., 2006). While the effect of normalising 5-HT has not yet been performed with emotion related behaviours, and never with SERT$^{-/-}$ rats, there are indications that preventing the activation of the 5-HT1a receptor by excessive 5-HT in SERT$^{-/-}$ mice rescues their depression-like behaviour (Alexandre et al., 2006). Moreover, the anxiety-like behaviour of 5-HT1a$^{-/-}$ mice is reversed when the 5-HT1a receptor is restored during early development, but not adulthood (Gross et al., 2002), supporting a significant role of 5-HT neurotransmission during development in mediating normal anxiety-like behaviour. While being a relatively new rodent model of low functioning SERT allele, the SERT$^{-/-}$ rat is distinct from the mouse model in a number of ways. In addition to the aforementioned species difference in the effects of MDMA, 5-HT1a receptor

function differs between mice and rats, with 8-OH-DPAT induced hypothermia mediated presynaptically in mice, and postsynaptically in rats (Bill et al., 1991). Consequently, it is imperative to investigate whether the pathological behaviours displayed by SERT$^{-/-}$ rats are due to increased neonatal 5-HT during development.

Accordingly, the aim of Chapter 4 was to determine whether neonatally lowering the excess extracellular 5-HT in SERT$^{-/-}$ rats would rescue their anxiety-like behaviour in adulthood as well as mRNA levels of both long 3'UTR Brain-derived neurotrophic factor (BDNF) and 5-HT1a in the prefrontal cortex, genes predicted to be down-regulated in SERT$^{-/-}$ rats. In order to avoid both injection stress in young pups, as well as the adverse effects of pCPA observed in a pilot study, neonatal TRP depletion, a more mild reduction of 5-HT, was selected. Following neonatal TRP depletion, male, but not female, SERT$^{-/-}$ rats showed increased time spent in the centre of the open field compared to their SERT$^{+/+}$ counterparts that was not mediated by an increase in exploration. However, while SERT$^{-/-}$ rats displayed an increased anxiety-like phenotype in the more anxiogenic novelty supressed feeding paradigm, this was unaltered by neonatal TRP depletion. These results indicate that decreasing neonatal 5-HT in male SERT$^{-/-}$ rats normalises their anxiety-like behaviour in low anxiety, but not high anxiety, situations. Future studies are required to determine whether normalising neonatal 5-HT will rescue addiction-like behaviour in these animals. However given that neonatal TRP depletion was unable to rescue high anxiety-like behaviour in these animals, it is likely a stronger manipulation will be required, such as neonatal 5-HT1a receptor blockade.

Unexpectedly, there were no differences in mRNA expression for long 3'UTR BDNF or 5-HT1a, in either sex, between genotypes, or following neonatal TRP depletion. This is in stark contrast to previous studies indicating that long 3'UTR BDNF mRNA is decreased in the ventromedial, but not dorsomedial PCF in adult male SERT$^{-/-}$ rats (Calabrese et al., 2015). Given that the current study measured long 3'UTR BDNF mRNA in the whole PFC, not differentiating between these two sections, it is possible any present differences were averaged out. Furthermore, these findings are also opposed to those indicating that binding of the 5-HT1a receptor is down-regulated in SERT$^{-/-}$ rats (Homberg

et al., 2008). However, given the results of the current study, it is possible the transcription of 5-HT1a mRNA is unaffected, suggesting a regulatory mechanism such as receptor internalisation, increased degradation, or a failure to translate the receptor. See table 5.1. for a summary of the findings of the current thesis.

Table 5.1. Overall findings for SERT$^{-/-}$ rats

Effect	Outcome
MDMA induced disruption of PPI	–
MDMA induced disruption of startle habituation	↓
MDMA induced locomotor hyperactivity	–
Discrimination of MDMA and saline	↓
Discrimination of MDMA and amphetamine	↓
MDMA self-administration	↑
Neonatal TRP depletion rescue of mild anxiety	↓
Neonatal TRP depletion rescue of severe anxiety	–
5-HT1a mRNA	–
long 3'UTR BDNF mRNA	–

↑ Increase in SERT$^{-/-}$ rats, ↓ Decrease in SERT$^{-/-}$ rats, – No change in SERT$^{-/-}$ rats. Note: due to the decreased discrimination of MDMA and saline, fewer SERT$^{-/-}$ rats met the discrimination criterion required to move on to the amphetamine probe trial.

Overall, the results of the current study suggest that at least some of the behaviours observed in SERT$^{-/-}$ rats are likely due to an increase in perinatal extracellular 5-HT levels, rather than to high levels of 5-HT during actual testing. This is consistent with other studies which have demonstrated that postnatal SSRI exposure in rodents leads to increased anxiety- and depression-like behaviours, as well as increased sensitivity to the reinforcing properties of cocaine (Ansorge et al., 2008; Ansorge et al., 2004; Forcelli & Heinrichs, 2008; Olivier et al., 2011), while SSRI use in adulthood is commonly used to decrease anxiety and depression. The ensuing question then is how excessive neonatal 5-HT leads to emotion- and drug-dependant related behaviours in adulthood. Although this was not specifically investigated in the current thesis, it may be

due to the neurotrophic role of 5-HT. Given the wide role 5-HT plays in neural development, particularly in neuronal migration, division, and differentiation (For review see (Gaspar et al., 2003)). For instance, postnatal SERT blockade leads to altered dendrite morphology, as well as altered excitability of pyramidal neurons associated with fear extinction, in the medial PFC (Rebello et al., 2014). Moreover, there is significant interaction between 5-HT and the neurotrophic growth factor BDNF, which also plays a significant role in neural development. For instance, SSRIs increase BDNF in both rats and humans (Chen, Dowlatshahi, MacQueen, Wang, & Young, 2001; Nibuya, Morinobu, & Duman, 1995). Moreover, BDNF is decreased in the PFC and ventral hippocampus of $SERT^{-/-}$ rats from very early on in development (P7-14) (Calabrese et al., 2013), while the addition of maternal separation (an early life stressor) reduces BDNF levels in the ventral hippocampus of mice heterozygous for the SERT gene ($SERT^{+/-}$) (Calabrese et al., 2015). Consequently, it is possible that alteration of BDNF by the genetic reduction of SERT in these rats plays a role in the development of pathological phenotypes.

More generally, the results of the current thesis suggest that neonatal imbalances of 5-HT lead to an increased predisposition to develop psychiatric disorders in humans. Given the wide role 5-HT plays in neuronal development (Gaspar et al., 2003) it is possible that increased 5-HT during development does not lead to gross brain abnormalities, but to structural and therefore functional disconnectivity between brain regions. Such functional disconnectivity is now widely proposed to underlie many psychiatric disorders such as schizophrenia and autism spectrum disorder (Geschwind & Levitt, 2007; Stephan, Friston, & Frith, 2009). Indeed, s-allele carriers show reduced functional connectivity between the perigenual anterior cingulate cortex and the amygdala, a circuit involved in emotional regulation (Pezawas et al., 2005). Moreover, the magnitude of disconnectivity was found to predict variation in harm avoidance, a trait associated with both anxiety and depression.

Additionally, given the results of Chapters 2 and 3, it is possible that individuals with low SERT expressing genotypes are predisposed to finding psychostimulants more reinforcing, which may explain why they are more likely to abuse cocaine and methamphetamine (Cao et al., 2013; Enoch et al., 2011;

Gerra et al., 2007). If this is indeed the case it would likely be recommended these individuals avoid consuming drugs in the first place. Of course, the flip side is that individuals who do not have low SERT expressing genotypes may interpret such advice as indicating they are immune from the risks of drug dependence. This would be a precarious conclusion however, given that there are indications the l-allele is sometimes the risk allele depending on ethnicity (Cao et al., 2013), as well as patently disregarding the probable multitude of other genetic and environmental causes of drug dependence. However, that is not to ignore the logical conclusion that if susceptibility to drug addiction could be accurately predicted, one would know precisely whether one would become drug dependent or not. It is possible this information would lead to increased drug taking by unaffected individuals. However, it is important to note addiction is not the only harm associated with drug taking, and the medical risks of ingesting illicit drugs should also be considered. This issue is particularly salient given the increasing popularity of direct-to-consumer genomic testing (e.g. 23 and me [www.23andme.com]). These test kits allow individuals to have their genome screened for known polymorphisms. Consequently, consumers are able to have access to more genetic information than ever before, and can proactively change their behaviour accordingly. However, these kits have come under considerable controversy, predominantly criticised for supplying access to medical information without accompanying guidance from a medical professional, potentially leading to gross misinterpretations. While the FDA no longer allows medical information to be provided in the US, raw data can be easily uploaded to an Internet database to obtain information on known medical associations. Consequently, information regarding polymorphisms will be more and more sought after.

Furthermore, the results imply that individuals should be aware of the potential risks of SSRI use during pregnancy. There have been suggestions from animal studies that neonatal SSRI use leads to increases in pathological behaviour, such as anxiety, depression, and sensitivity to cocaine, in adulthood (Ansorge et al., 2008; Ansorge et al., 2004; Forcelli & Heinrichs, 2008; Olivier et al., 2011). However, human studies have been unable to answer similar questions due to short follow up periods, although gestational SSRI has been

associated with increases in autism and behavioural problems in children (Harrington et al., 2014; Oberlander et al., 2010). Given that the results of the current thesis strengthen assertions from animal literature that neonatal SSRI exposure leads to adult pathology, there is a need for longitudinal studies to determine whether the same occurs for humans.

Given that the s-allele of the 5-HTTLPR has been linked to an increase in risk of psychopathology, its continued existence in the gene pool is puzzling. Indeed, given that the variation is particularly ancient, approximately 40 million years old (Lesch et al., 1997), it is unlikely that the variation evolved too recently to have been selected out. Consequently, it is likely that the s-allele is associated with positive outcomes in some circumstances. For instance, it has been proposed that, as opposed to displaying an overall increased risk for pathological behaviours, s-allele carriers display increased reactivity to the environment. For instance, an increase in the number of stressful life events has been positively correlated with depression in s-allele, but not l-allele, carriers (Caspi et al., 2003). Significantly however, follow up studies have demonstrated that this reactivity to the environment occurs with positive environments as well. For instance, supportive early life environments in s-allele carriers have been correlated with positive affect in children, and a decrease in depressive symptomology in young adults, compared with l-carriers (Hankin et al., 2011; Taylor et al., 2006). These findings indicate that, as opposed to being a 'risk allele', the s-allele is more likely a 'plasticity allele' given its responsiveness to changing environments, for better or for worse. The l–allele on the other hand is much more robust in the face of environmental changes.

It has been speculated that the mechanism underlying these changes is altered activity in corticolimbic structures in s-allele carriers (Homberg & Lesch, 2011). For instance, s-allele carriers display increased baseline amygdala reactivity that correlates positively with life stress (Canli et al., 2006). This is speculated to result in hyper vigilance to the environment (Homberg & Lesch, 2011). Indeed, individuals with the s-allele show bias toward both positive and negative stimuli (Beevers, Ellis, Wells, & McGeary, 2010; Beevers, Gibb, McGeary, & Miller, 2007), although when a triallelic distinction is used, individuals with the low functioning SERT genotype show a bias toward angry

faces, while high functioning SERT genotypes show a bias toward happy faces (Pérez-Edgar et al., 2010). This hyper vigilance is likely adaptive given a positive environment but maladaptive given a negative one. Interestingly, individuals with mood disorders show similar neuronal dysregulation in corticolimbic areas. For instance, depressed patients also show increased amygdala activity (Drevets et al., 1992; Sheline et al., 2001), giving strength to the argument that these neuronal dysregulations are causative. While it is currently unclear why some individuals with a low functioning SERT genotype become anxious, while others become depressed or addicted to drugs, it is possible this hyper vigilance to the environment plays a role. For instance s-variant individuals may demonstrate increased attention toward stressful life events, leading to an increase in rumination, followed by an increase in depression (Canli et al., 2006; Caspi et al., 2003). Additionally, following exposure to drugs of abuse, s-variant individuals may be biased toward the positive effects of these substances, thereby finding them more reinforcing and being biased toward taking more of them (Homberg & Lesch, 2011).

Finally, while the current thesis used a rat model of the 5-HTTLPR to model low functioning SERT in humans, this is not perfectly analogous to the human genotype. As the 5-HTTLPR does not normally occur in rodents, the SERT was genetically knocked-out to create a model of the polymorphism. While the heterozygous version of the model, like humans with the s/s genotype, have 50% lowered transcriptional efficiency of the SERT gene (Homberg et al., 2007; Lesch et al., 1996), the full knock-out, containing no SERT protein whatsoever, was used in the current thesis in order to ensure proof of concept. Given the increasing support for the idea that the environment is imperative to these pathological phenotypes, it is probable that with the full knock-out the genetic influence is so extreme that this is not required. Indeed, this would explain why the $SERT^{-/-}$ animals display increases in all measured pathological behaviours while humans with the low SERT expressing genotype do not. Consequently, the experiments ought to be repeated in the heterozygous model. Moreover, the addition of either negative or positive life events would allow it to be elucidated whether the s-allele is indeed a plasticity gene. While it is possible that the presence of one SERT allele is enough to compensate for decreased SERT gene,

and indeed, this seems to have mostly occurred with cocaine (Homberg et al., 2008), preliminary data from our lab indicated a gene dose effect occurs in the self-administration of MDMA in these rats (Brox & Ellenbroek, unpublished data).

Conclusions

The current thesis found that genetic removal of the SERT resulted in an alteration in the behavioural effects of MDMA. This genetic removal resulted in an abolishment of MDMA-induced disruption to startle habituation, but failed to affect MDMA-induced increases in locomotor activity, implying that the SERT is necessary for MDMA's disruption of startle habituation but not its psychomotor effects. Moreover, for those rats that could discriminate low dose MDMA from saline, genetic removal of the SERT resulted in the inability to discriminate MDMA from amphetamine, implying that, in these rats, MDMA was now subjectively indistinguishable from amphetamine. Indeed, this alteration also resulted in enhanced sensitivity to the reinforcing properties of MDMA, giving MDMA the qualities of traditional psychostimulants in $SERT^{-/-}$ rats. These findings support studies in humans with genetically reduced SERT that indicate these individuals demonstrate an altered response to MDMA, and imply they may be more sensitive to its reinforcing effects. Although the genetic removal of the SERT results in enhanced extracellular 5-HT, the pathological phenotypes present in this rat are likely due to this increase occurring in early development, not its continued presence in adulthood. Lowering the excessive 5-HT during neonatal development in $SERT^{-/-}$ rats led to a rescue of mild, but not high, anxiety-like behaviour in males. However, mRNA levels of long 3'UTR BDNF and 5-HT1a, genes associated with neurodevelopment, remained unchanged across genotypes and treatment groups. Overall, these findings contribute to the growing body of literature indicating that enhanced brain 5-HT during early development can lead to pathological behaviour in adulthood.

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