THE DEVELOPMENTAL ROLE OF SEROTONIN:

A COMPARISON OF SHORT- AND LONG-TERM BLOCKADE OF THE SEROTONIN TRANSPORTER ON BEHAVIOURAL OUTCOMES IN ADULTHOOD

BY

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Abstract

Serotonin is an important neurotransmitter that regulates a range of processes within the brain and is implicated in several psychiatric disorders. In addition, serotonin acts as a developmental signal during critical periods of prenatal development, influencing processes such as neuronal proliferation, migration, and synaptogenesis (Gaspar et al., 2003). The serotonin transporter (5-HTT) plays a key role in regulating extracellular serotonin levels and is the main target of selective-serotonin reuptake inhibitors (SSRIs), a class of drugs that have anti-anxiety and anti-depressive activity. SSRIs cause an acute increase in extracellular serotonin and are commonly prescribed as a treatment for depression and anxiety during pregnancy (Tran & Robb, 2015). Given that these drugs alter serotonin transmission and can pass to the developing fetus via the placenta, it is vital that the outcomes of prenatal SSRI exposure are investigated. In humans, a genetic variant of the gene that codes for the 5-HTT (*SLC6A4)* has been linked to increased risk for developing depression and anxiety (Caspi et al., 2003). The functional consequences of this genetic polymorphism are life-long alterations in 5-HTT activity, resulting in increased extracellular levels of serotonin (Nakamura et al., 2000). Given prenatal SSRI exposure results in a time-locked blockade of 5-HTT during critical periods of development, it follows that alterations in serotonin during development might similarly result in enhanced risk for depression and anxiety later in life.

Outcomes in children prenatally exposed to SSRIs are difficult to study due to confounds of pre-existing maternal depression. Therefore, the current thesis presents two experiments that aimed to further investigate the role of altered extracellular serotonin levels during development in an animal model. Experiment one aimed to develop a method of voluntary oral administration of the SSRI fluoxetine to pregnant rat dams. This method was then applied in experiment two to create

a time-locked blockade of 5-HTT during critical periods of development in an animal model of life-long 5-HTT blockade. The aim of experiment two was to directly assess the contribution of short- and long-term 5-HTT blockade on anxiety and depression phenotypes in adult male offspring. In addition, maternal behaviour was assessed to determine whether fluoxetine treatment had an influence on mother-pup interactions that could confound results. To test for anxiety and depression phenotypes, the novel affective disorder test (ADT) was used to assess anxiety behaviour and the deficits in anticipatory pleasure indicative of anhedonia. In the current study, fluoxetine treatment did not have an effect on litter outcomes or mother-pup interactions. Crucially, no significant group differences were found indicating that neither short- nor long-term blockade of 5-HTT resulted in increased anxiety- or depressive-like behaviours in the current experiment. However, limitations with methodological design limit the translatability of these results to the broader literature, and validation of the ADT is required before these results can be generalised beyond this thesis.

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The Developmental Role of Serotonin

Serotonin (5-hydroxytryptamine) is a monoamine neurotransmitter that plays a central role in mood regulation and stress reactivity and has been implicated in several psychiatric disorders such as anxiety and depression. The serotonergic system is the main target for selective serotonin reuptake inhibitors (SSRI), a class of drugs that have anti-anxiety and anti-depressant activity. SSRIs are the main pharmacological option available for the treatment of depression and anxiety disorders, and are commonly prescribed during pregnancy (Tran & Robb, 2015). In addition to its role as a neurotransmitter, it has now been well established that serotonin acts as a signal during development, influencing a wide variety of processes within the developing brain (Gaspar et al., 2003). As SSRIs can pass from the mother through to the developing child via the placenta, it is vitally important that the effects to offspring are investigated.

Extracellular serotonin levels are tightly regulated through a variety of proteins, and the serotonin transporter (5-HTT) is central to this regulation in both the developing and mature brain. In adulthood, 5-HTT is primarily expressed on presynaptic terminals and under normal physiological conditions is the primary method of clearing serotonin from the synapse. Once released, the 5-HTT can bind this extracellular serotonin and pump it back into the presynaptic terminal, thereby limiting its effects on the postsynaptic cell. In humans, a polymorphism in the serotonin transporter gene (*SLC6A4*) has been linked to increased risk for developing depression and anxiety (Caspi et al., 2010; Lesch et al., 1996). This serotonin transporter gene-linked polymorphism (5-HTTLPR) results in approximately a 50% reduction in the amount of 5-HTT expressed, and therefore reduced ability to remove serotonin from the synapse (Nakamura et al., 2000). Without the timely removal of extracellular serotonin, serotonin can continue to bind to postsynaptic receptors, thereby prolonging its effects. This appears to be paradoxical, as this is also

the mechanism of action for SSRIs. SSRIs cause an acute increase in extracellular serotonin by blocking the transporters ability to remove extracellular serotonin from the synapse, similarly prolonging effects on postsynaptic cells. However, the key difference is that in carriers of this polymorphism, these alterations to serotonin signalling are present from early embryogenesis. Given the role of serotonin during development, it follows that these alterations in serotonin levels during critical periods of development may lead to long-term consequences which persist beyond childhood.

In this thesis, I present two experiments that aim to further investigate how alterations to the serotonergic system during development may contribute to risk for depression and anxiety in adulthood. In experiment one, I aim to validate a method for the administration of the SSRI fluoxetine in pregnant rat dams. In experiment two, this method of dosing is used in order to compare long- and short-term alterations in extracellular serotonin on behavioural indices of anxiety and depression in adult male rats.

Development of the serotonergic system

Serotonin influences a wide variety of processes during development. In order to understand what periods of development are most susceptible to alterations in serotonin signalling, it is important to understand the development of the serotonin system itself, along with the expression of 5-HTT in the developing brain.

The expression of 5-HTT is more widespread during development than in adulthood and occurs prior to the development of the serotonin neurons themselves. 5-HTT expression in fetal peripheral tissue begins from gestational day 10.5 in mice (Pavone et al., 2008) and may regulate a maternal source of serotonin (Kliman et al., 2018) which passes to the embryo via the placental barrier, as the fetus at this stage of development is not capable of producing serotonin itself.

The first serotonergic neurons appear in the raphe nuclei during gestational week 5 in humans/gestational day 12.5 in mice (Narboux-Nême et al., 2008; Pavone et al., 2008; Verney et al., 2002) and project 5-HTT-positive fibres towards the forebrain, setting up the major serotonergic pathways that mediate transmission of serotonin to the cerebral cortex, the limbic system and the basal ganglia. Migrating 5-HTT-positive fibres projecting from the raphe nuclei reach the diencephalon (the developing thalamus and hypothalamus) by gestational day 13.5 in mice, the telencephalon by gestational week 8 in humans/gestational day 16.5 in mice (Hansson et al., 1998; Pavone et al., 2008; Sodhi & Sanders-Bush, 2004; Witteveen et al., 2013), the subplate by gestational week 10 in humans, and the cortical plate by gestational week 13 in humans/gestational days 18-20 in mice (Hansson et al., 1998; Sodhi & Sanders-Bush, 2004; Verney et al., 2002; Zhou et al., 2000). During this period of migration and development, 5-HTT is highly expressed on both the soma and dendrites of serotonergic neurons in the raphe nuclei, as well as along the migrating axons (Zhou et al., 2000). In contrast, mature serotonergic neurons predominately restrict expression of 5-HTT to their presynaptic terminals instead. By gestational week 5 in humans the serotonin cell bodies have reached mature arrangement within the raphe nuclei (Takahashi et al., 1986). 5-HTT is also transiently expressed in non-serotonergic cells, including neural crest cell derivatives (Gaspar et al., 2003), and both the rostral and caudal limbs of the internal capsule (Verney et al., 2002). The consequence of such prolific expression of 5-HTT is highly regulated levels of extracellular serotonin and suggests the importance of maintaining specific levels during critical periods of development.

Serotonin has numerous roles during development. As a trophic factor, serotonin modulates developmental processes such as cell proliferation, neuronal division, differentiation, migration, and synaptogenesis (Gaspar et al., 2003). In addition, serotonin also plays an important role in the

morphogenesis of developing neurons and the development of other neurotransmitter systems. For example, in the development of the glutamatergic system, serotonin facilitates the proliferation and differentiation of early progenitor cells into glutamatergic neurons in the developing cortex (Lavdas et al., 1997) and regulates their migration (Edgar & Price, 2001; Vitalis & Parnavelas, 2003; Whitworth et al., 2002). It also influences the development of the GABAergic system, influencing the speed and trajectory of migrating GABAergic neurons (Murthy et al., 2014). In a morphogenetic role, serotonin enhances both primary neurite length, the amount of branching points, and the formation of dendritic spines in ventroposterior thalamic neurons (Persico et al., 2006; Sodhi & Sanders-Bush, 2004; Vitalis & Parnavelas, 2003).

Genetic modulation of the serotonergic system

The most common variant of the 5-HTTLPR polymorphism is a 44 base-pair insertion/deletion mutation in the promotor region of the serotonin transporter, resulting in a short (S) and long form (L) (Kraft et al., 2005; Nakamura et al., 2000). These variants differentially alter the transcription of 5-HTT, with the S variant resulting in reduced SERT expression and therefore function compared to carriers with two copies of the L variant (Lesch et al., 1996; Mortensen et al., 1999). In 2003, Caspi and colleagues published a study showing that when exposed to a stressful life event, carriers of the S variant were more susceptible to depression than those with an LL genotype. Since this publication, a range of personality traits related to anxiety and depression have been reported in carriers of the S variant, including higher levels of neuroticism (Greenberg et al., 2000; Lesch et al., 1996) and stress-reactivity (Mueller et al., 2011). Increased emotional reactivity has also been reported. In fMRI studies, S-carriers show greater amygdala activation in emotion-related tasks than subjects with LL-genotype

(Canli et al., 2005; Hariri et al., 2002), suggesting alterations in inhibitory processes involved in emotion regulation.

Several animal models have been developed that provide a way to understand how alterations in the serotonin transporter during development might predispose the brain to greater risk for developing mental illness. Rhesus monkeys have an orthologue of the 5-HTTLPR that similarly results in reduced transcription of 5-HTT (Barr, 2003) and slowed reuptake of serotonin from the synapse (Singh et al., 2012). In these monkeys, the S allele is associated with increased anxiety, agitation, and exaggerated HPA responses when combined with early life stress (Watson et al., 2009). Several morphological changes within the brain has also been found in these monkeys. These alterations include abnormal cortico-limbic structure and function and reduced grey matter volume in several key brain areas that mediate emotion regulation (Jedema et al., 2010).

Studies in rodents provide further insights. While no genetic orthologue of the 5-HTTLPR exists, a serotonin transporter gene-knockout model (SERT-KO) has been developed in both mice (Bengel et al., 1998) and rats (Smits et al., 2006). Such rodents lack either one (heterozygous) or both (homozygous) copies of the *Slc6a4* gene, resulting in either a 50% or 100% reduction in transporter expression. SERT-KO models have been studied extensively and are associated with a variety of structural and functional alterations within the brain. In line with the influence of serotonin on morphogenesis, SERT-KO animals show abnormal development of thalamocortical neurons (Gaspar et al., 2003; Persico et al., 2006), structural changes to the dendritic branches of pyramidal neurons within the limbic cortex, and density changes to dendritic spines in the basolateral amygdala (Wellman et al., 2007). Effects on cell migration can also be seen in these animals, with alterations in neuronal density as well as abnormal distribution of interneurons

within the cerebral cortex (Altamura et al., 2007; Riccio et al., 2009). Several alterations have also been found in the serotonergic system itself. A consistent finding in these animals is down-regulation of the 5-HT1A receptor (Kalueff et al., 2007; Riccio et al., 2009), a key receptor in serotonin transmission, as well as alterations in serotonergic innervations of the medial prefrontal cortex (Witteveen et al., 2013).

These morphological alterations are associated with several changes in behaviour as well. In mice, the homozgyous SERT-KO genotype is associated with delayed reflex development between PND 8 and 12, and delayed muscle development between PND 10 through 21 (Kroeze et al., 2016). In both mice and rats, homozygous SERT-KO animals show robust anxiety phenotypes when confronted with a variety of stress-inducing environments (Ansorge et al., 2004; Andrew Holmes et al., 2003; Olivier et al., 2008; Popa et al., 2008). In addition, several studies show increased depressive-like behaviours in SERT-KO animals (Olivier et al., 2008) although this is not always found (Altieri et al., 2014).

Pharmacological manipulations of serotonin

A limitation with genetic studies is that alterations in serotonin signalling are present from early embryogenesis through to adulthood and therefore the changes due specifically to alterations during development cannot be determined. One way to examine the influence on the critical periods of development in isolation is to manipulate serotonin levels using pharmacological means. SSRIs are a frontline treatment for depression and anxiety during pregnancy with around 8% of women prescribed an SSRI while pregnant (Huybrechts et al., 2014). SSRIs, along with serotonin produced by the mother, can pass to the fetus through the placenta and is detectable in amniotic fluid (Loughhead et al., 2006). This transmission results in substantial levels of the SSRI in infant serum (Brent & Wisner, 1998; Gentile et al., 2007; Gentile, 2005; Hendrick et al., 2003).

Population-based studies have shown no significant increase in birth defects following prenatal exposure to a range of SSRIs (Louik et al., 2007), however a postnatal adaptation syndrome is often reported in newborns (Levinson-Castiel et al., 2006), particularly from exposure during the third trimester (Costei et al., 2002; Hayes et al., 2012; Nordeng et al., 2007). This syndrome can last for up to one month (Nordeng et al., 2007), with symptoms including irritability, constant crying, disruptions in sleeping and eating, shivering, convulsions, increased muscle tone, and temperature instability (Haney & Spealman, 2008; Nordeng et al., 2007). The cause of this syndrome is often attributed to withdrawal from SSRI exposure following birth, and symptoms are similar to those reported by patients following cessation of SSRI treatment. However, SSRI exposure isn't necessarily finished at birth; SSRIs can pass to the newborn via breast milk, leading infant serum levels of fluoxetine and norfluoxetine to remain significant at 21 days old (Brent & Wisner, 1998). In addition, several of these symptoms can be detected prior to birth: altered motor activity in trimester 2 has been reported in fetuses with a high level of SSRI exposure, and non-REM sleep disruptions have been observed during trimester 3 (Mulder et al., 2011). An alternative explanation to a withdrawal hypothesis is that these symptoms may reflect an overstimulation to serotonin (Wisner et al., 2009). In support of this, Laine (2003) reported that postnatal adaptation symptoms decreased rapidly following cessation of SSRI exposure, which is inconsistent with a withdrawal hypothesis. Regardless of the direct cause, it is clear that SSRI exposure during development can lead to alterations in serotonin signalling that result in detectable changes in early behaviour.

Several longitudinal studies have now been conducted to examine more long-term behavioural effects of developmental SSRI exposure. In line with data from early observations, subtle alterations in motor development have been reported between 6 months and 3 years. These

differences include delayed motor skill development (Casper et al., 2003; Gentile & Galbally, 2011; Hanley et al., 2013) and lower scores on tests of psychomotor skills and motor control (Casper et al., 2003, 2011).

Importantly, several significant differences in socio-emotional behaviours have been found that may represent early indicators of later mental illness development. Internalising behaviours are the most consistently reported (Brandlistuen et al., 2015; Hanley et al., 2015; Hermansen et al., 2016; Klinger et al., 2011; Liu et al., 2017; Lupattelli et al., 2018; Malm et al., 2016). These behaviours can be described as emotional responses to stress that are directed internally and include anxiety and depressive behaviours, irritability, withdrawal, and somatic complaints. However, reports of this in the literature are conflicting, with multiple studies finding no differences on any neurobehavioural assessments (Misri et al., 2006; Pedersen et al., 2010, 2013). One explanation is that differences in socio-emotional behaviours may not be detectable until later in childhood. For example, of the four studies that failed to find any significant effect of prenatal SSRIs on neurobehavioural outcomes, three used samples under the age of two (Misri et al., 2006; Morrison et al., 2005; Pedersen et al., 2010). In contrast, of the remaining seven studies that found significant differences, six included children of five years or older (Hanley et al., 2015; Hermansen et al., 2016; Klinger et al., 2011; X. Liu et al., 2017; Lupattelli et al., 2018; Malm et al., 2016). This points to a delayed emergence in socio-emotional disruptions from SSRI exposure that might be missed at an early age. Internalising behaviours in childhood are associated with increased risk for later diagnosis of psychiatric disorders (Rutter et al., 2006). While only two studies published thus far have followed up with children in early adolescence, both reported increased risk of early diagnosis of depression and other psychiatric disorders (Liu et al., 2017; Malm et al., 2016). In spite of this evidence, it is difficult to separate the influence of SSRIs from confounding maternal

factors such as pre-existing psychiatric illness and lifestyle factors. Women prescribed with an SSRI during pregnancy are more likely to smoke (Colvin et al., 2011) and consume alcohol while pregnant (Nulman & Koren, 1996; Pedersen et al., 2010), and (untreated) maternal depression is similarly associated with an increase in internalising behaviour in children (Oberlander et al., 2010). While many of the population-based studies control for these issues by assessing the influence of maternal depression in the absence of pharmacological treatment, it is difficult to account for the effects of symptom severity. One option to overcome this limitation is by studying the effects of SSRIs during development in animal models.

The use of rodents in developmental studies circumvents many of the confounding factors inherently present in clinical studies. Animal models allow the researcher to target specific critical periods, assess dose-dependent relationships, and test a variety of SSRI drugs on behaviour and brain development. However, animal studies come with their own set of limitations. Rodents have a gestational period of 21-days and are born at a more premature point in development than humans, making it difficult to directly compare developmental stages in rodents and humans. The early postnatal period corresponds to trimester 3 development in humans.

Several studies have reported abnormal morphogenesis of neurons as a result of prenatal SSRI exposure. Mice exposed to the SSRI fluoxetine from gestational day (GD) 8 to 18 had significantly reduced dendritic complexity in layer 2/3 pyramidal neurons (Smit-Rigter et al., 2012), and treatment from GD 7 through postnatal day (PND) 7 delayed the maturation of interneurons (Umemori et al., 2015). Treatment with fluoxetine from birth (PND 0) through to weaning (PND 21) resulted in significantly reduced hippocampal cell proliferation (Rayen et al., 2011). Alterations in neuronal migration can also be seen; administration of paroxetine, another SSRI, from PND 0 to 8 resulted in altered organisation of thalamocortical fibres (Xu et al., 2004).

In addition, several studies report changes to the serotonergic system itself. Treatment with fluoxetine during PND 1-21 decreased both the number and cell body size of serotonergic neurons within the dorsal raphe nuclei, and reduced cell terminals in the hippocampal dentate gyrus (Mendes da Silva et al., 2010). Treatment with paroxetine from PND 8 through 21 reduced the rate-limiting enzyme tryptophan hydroxylase (an enzyme involved in the synthesis of serotonin) found in the dorsal raphe nuclei (Maciag et al., 2006). Lastly, prenatal fluoxetine treatment resulted in a down-regulation of 5-HTT in the cerebral cortex (Maciag et al., 2006) and raphe nuclei (Noorlander et al., 2008), and an up-regulation of 5-HTT expression in the hypothalamus (Pinheiro et al., 2017). Taken together, these studies demonstrate persisting changes to serotonergic circuitry as a result of SSRI exposure during development.

In addition to these structural alterations, several differences in behaviour have also been reported. In line with clinical studies, changes in motor activity have been reported in rodents exposed to SSRIs during the early postnatal period. Treatment from PND 5 through 21 resulted in reduced motor activity in adult offspring (Maciag et al., 2006; Popa et al., 2008), and increased the duration of REM sleep (Popa et al., 2008). Several studies have also reported increases in anxiety- and depressive-like behaviours (Maciag et al., 2006; Noorlander et al., 2008; Sprowles et al., 2016), although there have also been studies where no significant difference was found (Rayen et al., 2011; Salari et al., 2016; Toffoli et al., 2014). Surprisingly, some studies also show that developmental exposure to SSRIs decreases anxiety- and depressive-symptoms in adult offspring (Avitsur et al., 2016; Kiryanova et al., 2016; Kiryanova & Dyck, 2014; McAllister et al., 2012). These inconsistent findings may be attributed to variations in methodological design such as species used, period of development targeted, type and dose of SSRI, route of administration, as well as specific tests used in assessment of behaviour. Because of these issues, it remains unclear

as to whether alterations in serotonin levels during these specific periods of development lead to functional differences in behaviour that persist through to adulthood.

One possible contributing factor for these conflicting findings comes from the literature on maternal stress and neonatal outcomes. Maternal stress increases the risk of psychiatric illness in offspring (Kinney et al., 2008) and is associated with long-term changes within the brain (Kiryanova et al., 2013). Intriguingly, in preclinical studies of maternal stress, concurrent SSRI treatment has been shown to counteract some of these negative effects. Salari et al. (2016) investigated the combined effect of maternal stress and fluoxetine treatment on anxiety- and depressive-like behaviours in adult offspring. Pregnant mice were exposed to daily stress (restraint in bright light), and fluoxetine was administered orally to the treatment group from GD 10 through PND 20. While adult male offspring of maternally stressed dams showed increased anxiety- and depressive-like behaviours compared to offspring of non-stressed, non-treated dams, concurrent treatment with fluoxetine reversed these effects. Thus, while SSRI treatment on its own may have distinct effects, there also appears to be an interaction when treatment is combined with maternal stress. Many routes of drug administration (injection or oral gavage) involve handling and restraint of the animal, and show significantly increased markers of stress in the animal (Pryce & Fuchs, 2017). In studies that adopt these methods, it is possible that the control group is actually a model of maternal stress. From this perspective, it would be expected that treatment with an SSRI would reduce maternal stress, and that offspring in this group would show less anxiety- and depression-like behaviours relative to the group that did not receive treatment. This might explain some of the mixed findings in the literature, however it is clear that further work needs to be done.

Current Experiments

Experiment one aims to develop and validate a method of drug administration in pregnant rat dams. Due to the conflicting reports in the literature, and the potential interaction between maternal stress and concurrent SSRI treatment, a novel method of oral administration of fluoxetine through gelatine pellets was developed and tested for use in further studies. Oral administration methods are less direct and may result in varying serum levels compared to other methods that circumvent first-pass metabolism. To validate this method, levels of fluoxetine and norfluoxetine were analysed in pup tissue.

This dosing method was then employed in experiment two, which aimed to assess the effect of both genetic and pharmacological manipulations of extracellular serotonin during development on behavioural outcomes in adult offspring. Male and female heterozygous SERT-KO rats were bred in order to produce litters of all three genotypes for testing. Pregnant dams were randomly assigned to either the fluoxetine or control group and treated with gelatine pellets from GD 7 through to PND 7. Male offspring were tested in a novel behavioural assay called the affective disorder test (ADT) that enables the assessment of anxiety- and depressive-like behaviours concurrently.

In line with previous literature, we predict that heterozygous and homozygous SERT-KO animals will show an increase in anxiety- and depressive-like behaviours compared to wildtype controls. Crucially, if these changes are due to alterations to serotonin levels specifically during development, then wildtype and heterozygous SERT-KO animals exposed to fluoxetine during gestation and the early postnatal period will show an increase in these same behaviours compared to their untreated counterparts.

Experiment 1

The motivation for experiment one was to develop a method for administering fluoxetine to pregnant rat dams. Due to mixed findings reported in the literature, it was important first to establish that the pharmacological manipulation is effective, and that fluoxetine is indeed passing through to the developing offspring. In addition, it was important to establish a route of administration that limited handling and manipulation of the pregnant female to rule out any possible interaction between maternal stress and SSRI treatment. Oral administration of fluoxetine has been successfully transferred via drinking water (Bairy et al., 2007; McAllister et al., 2012; Salari et al., 2016), however this method can be difficult for controlling the dose each female gets. In addition, bottle leakages and issues with dissolving fluoxetine made this method of dosing inappropriate for the current experiment. Previously in our lab, a novel route of administration through gelatine pellets was successfully used for the administration of valproate in pregnant rat dams (Pettie, 2020). This administration method was based on a study by Zhang et al. (2011) that developed an alternative method of dosing in mice by creating drug-containing gelatine sweets that the animals would accept voluntarily. After a short training period, these mice would readily consume the treats, providing an effective method of dosing without the stress caused by other oral dosing methods such as gavage. This method has also been used in rhesus monkeys with success (Zhang et al., 2012). Thus far, this method has yet to be used for the administration of SSRIs in rats and is a novel approach that could minimise stress in the animal (compared to methods that require restraint) and allow more specific dosing than other voluntary methods (such as through the drinking water).

Fluoxetine was chosen as the SSRI in this experiment for several reasons. The primary metabolite of fluoxetine is norfluoxetine, which also acts as an SSRI. The half-life for fluoxetine and norfluoxetine in rats is approximately 5 and 15 hours respectively (Caccia et al., 1990). Both

fluoxetine and norfluoxetine and can pass through the placenta to the developing offspring at a transfer rate of 83% in rats (Olivier et al., 2011) and 69% in mice (Noorlander et al., 2008) leading to substantial levels of fluoxetine and norfluoxetine in pup serum (Ansorge et al., 2004; Olivier et al., 2011) and brain tissue (Kiryanova et al., 2016, 2017; Kiryanova & Dyck, 2014; Olivier et al., 2011). Because of fluoxetine's long half-life and rate of transfer, it is the most used drug in studies investigating the developmental effects of SSRIs (Altieri et al., 2014; Ansorge et al., 2004, 2008; Avitsur et al., 2016; Bairy et al., 2007; Boulle et al., 2016; Karpova et al., 2009; Ko et al., 2014; Lee, 2009; Lee & Lee, 2012; Lisboa et al., 2007; McAllister et al., 2012; Mendes-da-Silva et al., 2002; Noorlander et al., 2008; Popa et al., 2008; Rayen et al., 2011; Salari et al., 2016; Sarkar et al., 2014; Smit-Rigter et al., 2012; Sprowles et al., 2017), and was thus chosen for use in the current experiments.

To confirm successful passage of fluoxetine and norfluoxetine to the pups, we decided to test levels in the brain and the serum of pups at two time points: postnatal day 0, and postnatal day 7. This allowed us to get an estimate of placental passage of the SSRI during gestation (PND-0) as well as passage through the milk over the first postnatal week (PND-7).

Method

Animals

Animals were bred and housed in the Victoria University TTR Small Animal Facility and maintained on a 12-hour reverse light-dark cycle (lights on at 1900) in a temperature- and humidity-controlled environment (kept at 21°C ± 2, 55 - 60% humidity). Food and water were available *ad libitum*. Three female heterozygous SERT KO rats were used in the initial testing of the fluoxetine/gelatine pellets. In addition, seven female and three male heterozygous SERT KO rats were used for breeding. All animals were housed in same-sex groups of two or three. Of the

seven females that were mated, only four became pregnant and carried to term, resulting in a pilot experiment of four litters.

Gelatine Protocol

Development of the gelatine pellets occurred over three non-consecutive days. The initial drug delivery vehicle was based on a previous solution from this lab and contained a mixture of gelatine, table sugar, flavouring and water. Females were given a single exposure to the vehicle two days prior to testing.

Each day during testing, females were removed from their home cage and placed into a clean cage with standard wood chip bedding and given a three-gram weight of lime-flavoured fluoxetine gelatine. Following a two-hour period, females were returned to their home cages and the amount of gelatine remaining was weighed to determine the amount consumed.

On the first day, each female was randomly assigned to a different concentration of fluoxetine; 2.0mg per gram of vehicle, 1.5mg per gram of vehicle and 1.0mg per gram of vehicle.

The second day tested the addition of Equal artificial sweetener to the original mixture. The addition of sucrose alone to various bitter substances has been shown to suppress the perception of bitterness (Mennella & Bobowski, 2015). However, sweeteners differ in sweetness as well as in their intensity temporal profile (the onset of sweetness as well as how quickly the perception of sweetness decreases). This is important in bitterness-masking as bitterness has a longer temporal profile than sucrose. Equal contains the artificial sweeteners maltodextrin, aspartame and acesulfame potassium (Ace-K). Ace-K has a high-intensity, early-onset sweetness profile compared to sucrose, but does not linger as long (Walsh et al., 2014). In contrast, aspartame has a slower onset, but lingers longer than sucrose. By using a range of sweeteners with different

temporal profiles, the perception of sweetness is extended and may assist in bitterness-masking, helping with better consumption of fluoxetine in rats. Three grams of Equal was added to the original mixture; the concentrations of fluoxetine in the mixture and flavours given to each female were kept constant.

On the third day, all females were given the original vehicle at a concentration of 1.0 mg per gram of vehicle. This was to test a principle of positive behavioural contrast on consumption. Positive behavioural contrast is an increase in reward-response following exposure to a less-rewarding stimulus. As an example, compared to only giving less-concentrated pellets, the same less-concentrated pellet would appear to taste sweeter when the subject is initially exposed to a more-concentrated pellet, resulting in greater consumption overall.

Based on the outcomes of these tests, a final vehicle mixture solution and dosing protocol was determined.

Breeding and Administration

Breeding females were habituated for five days prior to mating. During this time, females received daily exposure to vehicle gelatine pellets and handled for 15 minutes daily to habituate them to being picked up and weighed. Pairs were bred according to a timed-mating protocol in order to determine gestational day 0. Breeding pairs were placed together in transparent polycarbonate cages containing metal subfloors and checked twice daily for the presence of sperm plugs. The vagina was also checked for the presence of sperm plugs, and cages were changed every day during this time. The day a sperm plug was found was labelled gestational day 0. Males were used to sire multiple litters, with at least five days between pairings.

Pairs were kept on the metal subfloors for a maximum of five days. If no plugs were observed during this time, pairs were separated and then reunited after a week if the female was determined not pregnant.

Pairs were separated following identification of a sperm plug. Females were moved to standard cages and kept in isolation. Each female was given at least one piece of enrichment (either a red polycarbonate tunnel, a wooden chew block, or both). Nesting material was added to the cage on gestational day 20, and day of delivery was designated as postnatal day 0.

Fluoxetine was administered orally via the sweetened and flavoured gelatine pellets at a dose of 12mg/kg. Out of the four females that became pregnant, one was randomly chosen as a control. Presentation of gelatine pellets occurred from gestational day 7 through until postnatal day 6. Females were weighed daily in order to determine the amount of fluoxetine (or control) to be administered, and gelatine was administered once-daily between 0900 and 1100 (during the dark phase of the light-dark cycle). The amount of gelatine administered was recorded, and cages were searched in the afternoon for any remaining gelatine in order to get an estimate of the day's dosage.

Based on the outcomes of the gelatine testing, a concentration of 1.5mg per gram of vehicle was chosen as the starting dose. The flavour of the vehicle was shifted if the previous dose consumed dropped below 7mg/kg. In addition, if a change in flavour did not lead to increased consumption the female would be shifted to a lower concentration of fluoxetine per gram of gelatine; first to 1mg/g and then to 0.5mg/g if consumption dropped again.

The control female received the same amount (in grams) of vehicle as the fluoxetine treated females, and the flavour was shifted periodically.

Tissue Extraction

Pregnant females (dams) were checked twice daily for pups from gestational day 20. Day of delivery was counted as postnatal day 0. To confirm successful passage of fluoxetine to the pups, half of each litter were sacrificed for tissue collection at postnatal day 0, and the remaining pups sacrificed for tissue collection at postnatal day 7. Tissue samples were then tested for levels of fluoxetine and norfluoxetine.

Extractions occurred during the dark phase of the light-dark cycle (0800 - 1100). Litters were kept in their home cages in the housing room, and pups were randomly selected and transported individually into an adjacent room for extractions. In order to keep maternal and pup stress low, pups were transported by a helper who did not come into contact with any tissue. Pups were weighed, and the sex of the pup was recorded.

Extraction of brain tissue was done by decapitation using surgical scissors. Brain tissue was then placed into 2mL Eppendorf tubes. At postnatal day 0, extraction was done by pressing down on the dorsal surface of the head. For postnatal day 7 pups, extraction was done by using surgical scissors to incise the posterior surface of the skull.

To measure the amount of fluoxetine and norfluoxetine in the dam's milk, the stomach contents were extracted from a single fluoxetine litter for analysis. For this extraction, an incision was made on the body's ventral surface and the stomach removed. A small incision was then made in the stomach and the contents were deposited into a 2mL Eppendorf tube. Collections of brain tissue and stomach contents were placed directly into an insulated container of dry ice and moved to -80º (Celsius) storage at the end of the session.

Additionally, blood serum samples were taken from each pup. Blood was placed in 2mL Eppendorf tubes and samples were left at room temperature for a minimum of 1 hour. These

samples were then centrifuged at 7800 RPM for 10 minutes, and the supernatant was extracted into a clean 2mL Eppendorf tube before being moved to -80º storage.

The posterior tip of the tail was severed using small surgical scissors and placed into a well-plate for genotyping the litters.

The processing and analysis of tissue samples was outsourced to the New Zealand Institute of Environmental Science and Research for detection of fluoxetine and norfluoxetine levels using High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS).

Results and Discussion

Fluoxetine Dose

The daily doses of fluoxetine can be seen for each dam in Figure 1. The combined average dose for dams during each week of development were as follows: gestational week 2 (M = 9.63, SEM = 0.59), gestational week 3 (M= 8.11, SEM = 0.87), and postnatal week 1 (M = 6.29, SE = 0.86). These doses were lower than the 12mg/kg/day we were trying to achieve. It is unclear whether aversion to the gelatine was due to failure to sufficiently mask the bitterness of the fluoxetine in the gelatine pellets, or whether a conditioned aversion based on physiological symptoms associated with treatment occurred.

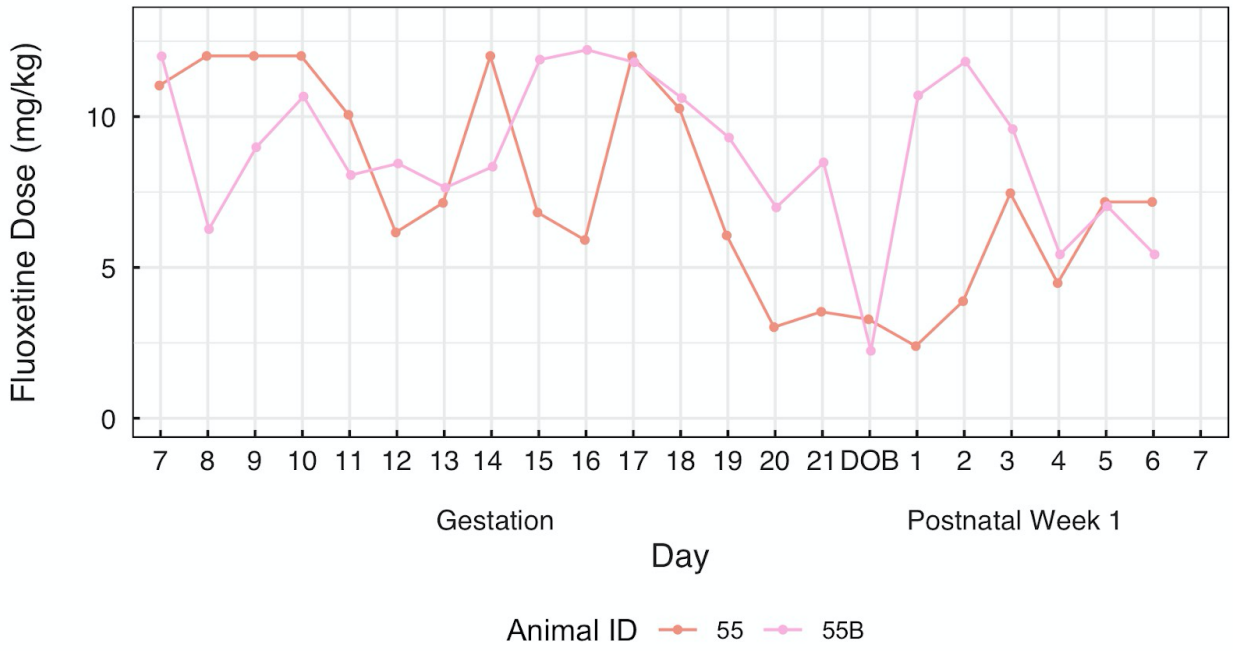

Figure 1: shows the daily dose in mg/kg for the two dams used in the tissue analysis study.

Tissue Analysis

To determine whether the doses of fluoxetine achieved through the gelatine protocol resulted in successful transmission of the drug to the developing pups, brain tissue and serum samples from the pups were sent for further analysis. In addition, the stomach contents from one of the litters were also analysed to get an estimate of drug levels in the milk. Mean ± SEM for each litter at each of the two time points are reported in Table 1. The detection limit for fluoxetine and norfluoxetine was 0.01 mg/L. Only values above this level are reported.

Table 1

Mean ± SEM levels of fluoxetine and norfluoxetine in pup brain tissue, pup serum, and milk for each litter at postnatal day 0 and postnatal day 7. All values are reported in mg/L.

	Brain		Serum		Milk	
	Fluoxetine	Norfluoxetine	Fluoxetine	Norfluoxetine	Fluoxetine	Norfluoxetine
M55						
PND 0	-	0.19 ± 0.0294	-	0.0683 ± 0.0075	*NA*	*NA*
PND 7	-	0.0533 ± 0.0067	-	0.02 ± 0.000	*NA*	*NA*
M55B						
PND 0	0.4825 ± 0.21	2.68 ± 1.12	0.062 ± 0.0022	0.240 ± 0.027	0.78 ± 0.24	3.68 ± 1.22
PND 7	-	0.44 ± 0.08	-	0.16 ± 0.025	0.128 ± 0.03	1.56 ± 0.312

The fluoxetine doses for litter M55 were below detection limits for both brain tissue and serum samples. Stomach contents were not extracted for this litter. The levels of norfluoxetine in this litter were above detection limits and indicate that successful passage of the drug was occurring. Fluoxetine has a much shorter half-life than norfluoxetine, and levels will have been substantially influenced by the time of extraction. For both litters, pups were born overnight, and the consumption of dams substantially dropped during this time. This could explain the low levels of fluoxetine at PND 0 in M55 offspring. Additionally, extractions on PND 7 were done in the morning, approximately 12 hours following the last presentation of gelatine to the dams. Dams would typically not consume the gelatine pellets in a single go. Because we did not observe them overnight, there is no way to know exactly how long it had been since dams last consumed the fluoxetine. Thus, it is likely that the levels that fell below detection at PND 7 are due to the drug being metabolised before samples were taken.

The levels of drug present in PND7 samples are substantially smaller than those found in PND 0 samples. These lower levels are likely due to the combination of lower doses during the postnatal week along with reduced transmission of fluoxetine and norfluoxetine through the milk. However, norfluoxetine levels at PND 7 do indicate that passage of fluoxetine was successful during this postnatal week.

A limitation of experiment one is that these levels are difficult to compare to the broader literature. Previous studies that have reported levels of fluoxetine and norfluoxetine in pups report levels as a function of sample weight, not volume like we have. Because we outsourced this analysis, I was unable to obtain these details in order to convert our results into comparable units. While our results demonstrate successful passage of fluoxetine and norfluoxetine to pups during the prenatal and early postnatal period, without comparing these to previous literature we cannot determine what the functional effect these levels may have had on extracellular serotonin levels. However, because of time constraints, and the promising results we obtained, this dosing method was employed for experiment two.

Experiment 2

The purpose of experiment two was to investigate the effects of developmental fluoxetine exposure on behavioural outcomes of male SERT-KO rats. In addition to assessing anxiety- and depression-like behaviours in adulthood, an assessment of the early rearing environment was included to assess the influence of fluoxetine on mother-pup interactions.

The rearing environment of rodents, and in particular mother-pup interactions, has been shown to influence a variety of behaviours in offspring (Weaver et al., 2004). The amount of time dams spend in arch-back nursing (ABN) posture, along with time spent licking and grooming (LG) pups, is a strong predictor of stress reactivity in adult offspring (Meaney, 2001). In ABN posture, the mother is positioned on all four limbs with an actively raised back while her pups suckle beneath her. This posture gives the pups full access to her nipples, along with the ability to switch between them. In addition to ABN, a variety of other nursing postures are observable in dams, including blanket nursing where the mother is on top of her pups without an arched-back, and passive nursing posture where the mother lays on her side. Individual

differences in dams can be seen in the preference for different nursing postures, and offspring of mothers with high ABN-LG behaviours show reduced anxiety behaviours compared to offspring of low ABN-LG mothers (Caldji et al., 1998).

Serotonin is a modulator of social behaviour, and there is some indication that administration of fluoxetine increases ABN-LG behaviours in dams (Johns et al., 2005; Pawluski et al., 2012). An increase in these behaviours could counteract the effects of the SERT-KO genotype and developmental SSRI treatment, rescuing any anxiety or depressive phenotype in these animals. Only a few studies that have investigated neurobehavioural effects of developmental SSRIs have included assessments of maternal behaviour. Of these studies, most assess an amount of time too brief to determine true differences. Undetected changes in maternal behaviour from SSRI treatment could explain the lack of significant findings in some studies. In the current experiment we include multiple assessments of maternal behaviour over the first postnatal week in order to assess for systematic differences in mother-pup interactions as a result of fluoxetine treatment.

Preclinical Tests of Depression

A variety of behavioural tests exist to assess depression-like behaviours in rodents. The forced swim test (FST) has been frequently used in the assessment of behavioural despair in SERT-KO mice (Altieri et al., 2014; Holmes et al., 2002; Lira et al., 2003) and rats (Olivier et al., 2008). In addition, the FST is the most cited test in the assessment of depressive-like behaviours in studies investigating the neurobehavioural effects of prenatal fluoxetine (Altieri et al., 2014; Avitsur et al., 2016; Boulle et al., 2016; Karpova et al., 2009; Lisboa et al., 2007; McAllister et al., 2012; Mendes-da-Silva et al., 2002; Rayen et al., 2011; Salari et al., 2016; Sarkar et al., 2014; Sprowles et al., 2017). In this test, animals are placed in a deep pool of water, and their

behaviour monitored for five minutes. Behavioural despair is characterised by an increase in time an animal floats immobile in the water and is thought to represent resignation to the situation.

Increased immobility in the FST has been demonstrated in SERT-KO models, consistent with a depression phenotype (Holmes et al., 2002; Lira et al., 2003; Olivier et al., 2008). However the SSRI literature is much more inconsistent, with studies sometimes finding increases in immobility (Boulle et al., 2016; Glover et al., 2015; Hansen et al., 1997; Hilakivi & Hilakivi, 1987; Lisboa et al., 2007; Popa et al., 2008; Sarkar et al., 2014; Sprowles et al., 2016; Zohar et al., 2016), decreases in immobility (Avitsur et al., 2016; Karpova et al., 2009; McAllister et al., 2012; Mendes-da-Silva et al., 2002), and no significant differences in adult animals exposed prenatally to SSRIs (Altieri et al., 2014; Boulle et al., 2016; Coleman et al., 1999; Lisboa et al., 2007; Rayen et al., 2011; Salari et al., 2016; Sprowles et al., 2017). Several criticisms of the FST have been made regarding its validity for testing depressive-like behaviours. For example, an animal expending less energy by spending more time immobile could have a survival advantage over those that continuously attempt to escape. In addition, the test is highly affected by manipulations that alter locomotor activity (Ko et al., 2014; Popa et al., 2008).

A more valid assessment of depression may be the assessment of anhedonia. Anhedonia is one of the major diagnostic criteria for major depressive disorder and is typified by an inability to find pleasure in things that were once enjoyed. Anhedonia can be further differentiated as a deficit in either anticipatory pleasure (pleasure elicited in anticipation of an upcoming reward) or consummatory pleasure (the rewarding stimulus itself). Clinical studies of patients with major depressive disorder show that the anhedonia seen in these patients is predominantly due to a deficit in anticipatory pleasure (Hallford & Sharma, 2019). Several paradigms have been developed in order to assess anticipatory pleasure in animal models. In these paradigms, time

spent in a novel arena is paired with a food-reward over multiple sessions. With consistent pairing, rodents will come to expect the food-reward, and both locomotor activity and rearing behaviour will increase in anticipation of the food (Barbano & Cador, 2006). Anticipatory locomotor activity is an evolutionary conserved behaviour (Storch & Weitz, 2009); reductions in these behaviours characterise a deficit in anticipatory pleasure and indicate anhedonia in these animals.

Anticipatory vocalisations can also be assessed using this same paradigm (Buck et al., 2014). Both mice and rats emit sounds in the ultrasonic range and use vocalisations as a means of communication under a variety of situations. Both the call rate and the frequency of the calls can be altered in response to environmental manipulations (Simola, 2015). In adult animals, two distinct call types have been described that differ in call frequency. Calls within the 50-kHz range are emitted under appetitive situations such as in response to food rewards, positive social interaction with a conspecific, and mating. Calls within the 22-kHz range are predominantly emitted under aversive situations such as in response to an aggressive conspecific. As with anticipatory locomotor activity, increases in the number of 50-kHz calls are seen in anticipation of a food-reward. A reduction in these anticipatory calls is interpreted as a deficit in anticipatory pleasure and indicates anhedonia. An advantage of using ultrasonic vocalisations in the assessment of anticipatory pleasure is that it does not rely on locomotor activity and is therefore resistant to manipulations that may alter this. A recently published study by Olivier et al. (2019) show alterations in 50-kHz calls in SERT-KO animals. While SERT-KO animals did not differ from controls in the number of 50-kHz calls emitted during social interaction, there were significant differences in other morphological parameters of the calls. Thus far neither

anticipatory locomotor activity or ultrasonic vocalisations have been assessed in adult offspring treated with an SSRI during the prenatal or early postnatal period.

Preclinical Assessments of Anxiety

Assessment of anxiety-phenotypes in rodents are predominantly based on concepts of conflict testing. In these paradigms, the animal is presented with an environment consisting of multiple areas that actively engage both the drive to explore a novel environment and the drive to avoid open areas that could expose them to predators. A measure of anxiety can be obtained by assessing behaviour in the open and exposed areas of the environment versus the more sheltered areas. The elevated plus maze (EPM) is a test of anxiety based on these principles. The arena is in the shape of a plus sign, with four perpendicular arms coming off a central area: two of these arms are protected by tall walls and are the sheltered 'safe' areas of the arena; the other two arms of the arena are open and exposed. In validation studies of the EPM using principal components analysis, two distinct measures of behaviour have been extracted: an anxiety measure that includes duration within the open arms and frequency of entering the open arms; and a measure of locomotor activity that includes frequency of entrance into the sheltered arms (Altieri et al., 2014; Fernandes & File, 1996). Anxiety phenotypes are characterised by reduced time spent in the open arms as well as reduced entries into these areas.

Similarly, the novelty-suppressed feeding paradigm assesses the conflict between avoiding open spaces against retrieving food. In these tests, animals are food-restricted prior to testing to increase food-related motivation. On the day of testing, the animal is faced with the conflict of leaving the sheltered peripheral areas in order to retrieve the food placed in the center of the arena. Animals that take longer to retrieve the food are said to show greater anxiety-type behaviours than animals that retrieve it more quickly. Olivier et al. (2008) conducted a

systematic assessment of anxiety phenotypes in SERT-KO rats, and found that, compared to controls, SERT-KO animals spent less time on the open arms of the EPM, and had a higher latency to eat in the novelty-suppressed feeding paradigm. In developmental SSRI studies, the EPM is the predominant test used in the assessment of anxiety. For the most part, these studies have found no significant differences between groups on measures of anxiety using the EPM (Altieri et al., 2014; Ansorge et al., 2004, 2008; Avitsur et al., 2016; Bairy et al., 2007; Coleman et al., 1999; Glover et al., 2015; Kiryanova & Dyck, 2014; Lee & Lee, 2012; Lisboa et al., 2007; Popa et al., 2008; Salari et al., 2016), with a few studies finding increased anxiety (Noorlander et al., 2008; Sarkar et al., 2014; Zohar et al., 2016), and some studies finding decreased anxiety compared to controls (Kiryanova et al., 2014; 2016; McAllister et al., 2012). More consistent findings have been reported with the novelty-suppressed feeding paradigm; there were significant increases in latency to eat in animals treated with an SSRI during development compared to controls in all studies that used this test (Ansorge et al., 2004, 2008; Noorlander et al., 2008; Smit-Rigter et al., 2012).

Because these conflict-based tests rely on changes in behaviour relative to a control group, the experimental conditions of the test are vitally important in order to protect against both floor and ceiling effects. For example, testing conditions that increase the anxiogenic properties of the arena (such as testing under bright light) can result in floor effects; under these conditions the control group may not explore the open areas, making increases in anxiety behaviours undetectable. The binary nature of the EPM means that it is even more susceptible to these effects compared to arenas that have more variability in spaces.

As a result of some of the critiques made of the EPM, Deacon (2013) created a modified version called the successive alleys test (SAT). This modified version eliminated the central

region that has caused some confusion in interpretability, and instead included four distinct regions joined end-to-end that increase in anxiogenic properties. In comparative tests between the EPM and the SAT, the SAT was able to detect similar changes in anxiety as a result of the anti-anxiety drug chlordiazepoxide (Deacon, 2013). In addition, because of the increased number of distinct regions, the test may better detect subtle changes in anxiety that might be missed using the EPM.

A major limitation of all preclinical tests of anxiety is that they are only sensitive to group differences on the first trial and can thus only measure state anxiety. Termed the one-trial tolerance, any differences between groups detected on day one of testing are eliminated by trial two. For example, day two control animals show decreased activity on the EPM's open arms; following an initial exposure to the arena, the test no longer stimulates an exploratory drive in the animals (Fernandes & File, 1996). The one-trial tolerance of preclinical anxiety tests limits their usefulness in the research of trait-anxiety and the translatability of results to anxiety disorders in humans. Recently, our lab created a modified version of the successive alleys test. This modified version combines principles from the original successive alleys test with those from the novelty-suppressed feeding test. Each area is baited with a food-reward in order to maintain the drive to enter anxiogenic areas of the arena over multiple trials. This also gives an additional measure of anxiety in latency to retrieve the food from each area. Preliminary results from our lab using this test suggest that stable genotype differences can be detected in SERT-KO animals for up to six days of testing (Kidwell, 2018). These preliminary results suggest that this could be the first test of anxiety that is robust to the one-trial tolerance and allows the assessment of trait-anxiety in preclinical models.

In this study, we combine the anticipatory pleasure paradigm with our modified version of the successive alleys test to create a novel test that can assess depression and anxiety concurrently. This affective disorder test (ADT) combines the anticipatory arena with food-rewards obtained in the successive alleys arena and has been used previously in our lab (Kidwell, 2018). We predict that heterozygous and homozygous SERT-KO rats will show increased anxiety-behaviours compared to control rats as indicated by decreased time spent in the more anxiogenic areas of the successive alleys arena, and increased latency to enter these areas, as well as anhedonia-like behaviours as indicated by reduced increases in locomotor activity and 50-kHz ultrasonic vocalisations in the anticipatory arena over time. Crucially, if these anxiety and depression phenotypes are due to alterations in serotonin levels during development, we predict that both SERT-WT and heterozygous SERT-KO animals treated with fluoxetine during development will show further alterations in these behaviours compared to their untreated counterparts.

In addition to 5-HTT, fluoxetine has varying affinity for other receptors within the brain (Cooper et al., 1996). If the predicted alterations in behaviour are due specifically to fluoxetine's action at the 5-HTT, we should see no effect of fluoxetine on homozygous SERT-KO animals that lack the serotonin transporter.

The validity of the SAT, and our own ADT have yet to be rigorously tested. Because of the multiple measures of anxiety that can be obtained from these tests, the possibility of type I errors is high. In order to reduce the dimensionality of measures obtained from the ADT, an exploratory principle components analysis has been included to assess behaviour on this arena over time.

Method

Animals

All procedures were approved by the VUW Animal Ethics Committee (AEC number 25840) in accordance with the Animal Welfare Act (1999). Animals were bred and housed in the Victoria University TTR Small Animal Facility and maintained on a 12-hour reverse light-dark cycle (lights on at 1900h) in a temperature (21°C ± 2) and humidity (55-60 %) controlled environment. Rat chow and water were available *ad libitum*. Animals used for breeding were female (n = 20) and male (n = 19) heterozygous SERT KO rats (PND 60 - 90), housed in same-sex groups of 2 to 3 prior to breeding and acclimated to the housing room for a minimum of 2 weeks. All animals were housed in Animal Care Systems Optirat® GenII caging systems, in polycarbonate cages measuring 356mm in length, 485mm wide, and 218mm high.

Of the 39 animals used in the study, 5 females and 5 males were excluded due to unsuccessful breeding attempts. In addition, one fluoxetine litter was excluded due to abnormally large number of pre-weaning deaths (greater than 50%). This resulted in 8 control litters and 8 fluoxetine litters for the study.

Pups were weaned on gestational day 21 and split into same-sex groups of littermates. Each pup was genotyped (Transnetyx, Cordova, USA) by taking a tissue sample from the ear, and RFID tagged.

Breeding

Habituation of the females occurred over the five days prior to mating. During this time, females received daily exposure to the control gelatine pellets and handled for 15 minutes each day to habituate them to being picked up and weighed. Pairs were bred according to a timed-mating protocol in order to determine gestational day 0. Breeding pairs were placed together in transparent polycarbonate cages containing metal subfloors and checked twice daily for the presence of sperm plugs. Cages were changed daily during this time. Gestational day 0 was

determined by the day that a sperm plug was observed (either beneath the subfloor or still within the vagina).

Pairs were kept on the metal subfloors for a maximum of five days. If no plugs were observed, pairs would be separated and then reunited after a week once it had been determined the female was not pregnant. In situations where the second round of mating was also unsuccessful, the female was paired with a different male.

Following the presence of a sperm plug, pairs were moved to standard cages and each male was kept with the paired female until gestational day 6 when the males were removed, and females kept in isolation. Each female was given at least one piece of enrichment (either a red polycarbonate tunnel, a wooden chew block, or both). Nesting material was added to the cage on gestational day 20, and day of birth was designated as postnatal day 0.

The habituation and breeding protocol was altered following the first 2 cohorts of breeding pairs. During the habituation period, the vagina was also visually inspected in order to track the estrus cycle, and pairs were placed together on the metal subfloors when it appeared the female was in estrus. Visual inspection of the vagina was done twice daily during dark phase (0700 to 1900), and the following attributes were noted; dilation of the vagina, moistness, colour and prominence of striations (Byers et al., 2012; Champlin et al., 1973). Ear-wiggling behaviours and responsiveness to rump thigmotaxis stimulation were used as additional behavioural indicators of estrus. This change to protocol was made to overcome issues with an initially low breeding success rate. Pairing when the female was in heat increased the likelihood of successful pairing and reduced the amount of time the pair needed to stay on the metal subfloors.

Administration

Females were randomly assigned to either fluoxetine or control conditions, and treatment occurred from gestational day 7 through to postnatal day 7. Fluoxetine was administered orally via sweetened and flavoured gelatine pellets given twice daily. This was chosen as the preferred method of administration as it is less stressful than methods that involve restraint (Stuart & Robinson, 2015) and results in a slower onset of action than parenteral administration methods (Turner et al., 2011). In theory this would lead to a more stable and less episodic blockade of the serotonin transporter (Kiryanova et al., 2014), which is more analogous to the constant reduced 5-HTT activity in the heterozygous and homozygous SERT-KO.

The amount of fluoxetine administered was calculated daily in order to maintain a dose of 8 mg/kg across the weight changes inherent in pregnancy. Due to the potential interaction between maternal stress and SSRI exposure during pregnancy, handling was limited to one weekly weighing during gestation. A baseline weight was recorded for each female before mating, and females were weighed on gestational day 6 to confirm pregnancy before treatment began. Additional weighing occurred on gestational days 14 and 20, and postnatal days 0 and 7.

The amount of gelatine administered each day was based on the weight of the animal plus an estimated percentage daily weight gain that was obtained from the pilot study. Following weighing, the previously estimated weights were adjusted in order to give a more accurate estimate of dose.

Gelatine pellets (fluoxetine or control) were administered twice daily during the dark phase of the light-dark cycle. The pellets were cut from a larger block immediately before administration. The cages were searched before each additional administration and the remaining pellet was gathered and weighed to obtain an estimate of the dose consumed. Dams assigned to

the control group consumed the sweetened gelatine pellets in their entirety, but cages were searched equally to maintain a similar level of disturbance across groups.

Both the flavour and the concentration of fluoxetine was shifted periodically and depended on the dams prior-consumed dose. The concentration of the fluoxetine pellets ranged between 1.5 and 0.5 mg of fluoxetine per gram of gelatine-mixture. Females in the treatment group were started on a concentration 1.5mg/g. This was reduced to 1.0mg/g on gestational day 15 and 0.5mg/g on postnatal day 0, when we saw a major drop off in consumption in the pilot study. For some animals this shift occurred earlier than gestational day 14 and postnatal day 0 if individual consumption dropped off. The flavour of the fluoxetine pellets was shifted for each individual when their estimated dose dropped below 7mg/kg per day. Females in the control condition received the same amount (in grams) of untreated gelatine pellets as the fluoxetine-treated dams, and the flavour was shifted periodically.

Dams were checked twice daily for pups and the day of delivery was designated as postnatal day 0.

Maternal Behaviour

Maternal observations were conducted on postnatal days 0, 4 and 7. Each day there were three observation periods conducted during the dark phase of the light-dark cycle; one during the morning (0830 to 1100), one during the middle of the day/early afternoon (1100 to 1500) and one during the later portion of the afternoon (1500 to 1800). Observations occurred with at least one hour between observation periods, and at least one hour following administration of gelatine pellets.

Each observation period was 44-minutes long, and observations were taken at 2-minute intervals, giving 23 observations per session. Behaviour was monitored for a five-second

snapshot every two minutes, and behaviours occurring during this time were recorded (Kiryanova et al., 2016). The following behaviours were scored:

1. Away from the nest (defined as the dam not being in contact with more than 50% of litter),
2. Pup retrieval (dam is moving pups from one location to another),
3. Nest building (dam is manipulating the nesting material or the bedding),
4. Maternal contact (dam is in physical contact with at least one pup but is not nursing, or licking/grooming the pup),
5. Licking/grooming pups,
6. Passive nursing (dam is laying on its side while pups nurse),
7. Low-ABN nursing/blanket nursing (dam is laying over its pups with a flat back, and either her front paws/back paws or all four are collapsed underneath her),
8. Mid-ABN (dam is laying over its pups nursing with all four limbs partially extended),
9. High-ABN (dam is laying over its pups nursing with all four limbs fully extended).

In situations where the dam was exploring the cage while pups held on and continued to nurse, this was counted as passive nursing. Maternal observations occurred in the home cages under red-light. Due to the difficulty of observing in this environment, mid- and high-arch back nursing were combined into a single mid/high-arch back nursing behaviour.

Behavioural Testing

Animals

Behavioural testing of the offspring occurred from early adulthood (postnatal day 60). Seventy male SERT-KO rats were used in the experiment; 38 from fluoxetine-treated litters (13 SERT-WT, 13 heterozygous SERT-KO, 12 homozygous SERT), and 32 from control litters (10 SER-WT, 12 heterozygous SERT-KO, 10 homozygous SERT).

Males were housed in pairs, with littermates where possible, otherwise with an age-matched male from a litter in the same treatment condition. Genotypes of each animal were randomised when pairing. Animals were maintained on 12-hour reverse light-dark cycle (lights on 1900).

Animals were placed on food-restriction from the onset of the habituation phase for the duration of behavioural testing (14-15 days). *Ad libitum* food access ceased, and animals were fed once daily (normal rat food at 15-20g per animal, between the times of 1630 and 1900). Animals were weighed every two days to monitor weight loss and ensure their weight was maintained above 95% of their free-feeding weight.

Apparatus

The ABT consisted of two arenas; the anticipatory arena and the successive alleys arena. The anticipatory arena was a square open-air chamber (40cm x 40cm x 40cm) made of black polycarbonate, placed on the floor of the experimental room. This arena was used for measuring anticipatory behaviour in the rats prior to being tested in the successive alleys arena and contained an ultrasonic microphone above the arena and pointed into the arena's centre to record ultrasonic vocalisations.

The successive alleys arena consisted of four open-air compartments, each 45cm in length and joined end-to-end (total length 180cm). Each compartment varied in width and height, so that from the first alley to the fourth they became narrower and lower; Alley 1 was 9 cm wide with 29cm high walls, Alley 2 was 8cm wide with 6cm high walls, Alley 3 was 6.5cm wide with

2.5cm high walls, and Alley 4 was 3.5cm wide with 1cm high walls. An example can be seen in Figure 2. The entire arena was elevated 88cm above the floor and firmly supported. For a food-reward during testing, half of a piece of sugary cereal (Kellogg's® Froot Loops®) was placed in the centre of each compartment. The lighting in the experimental room was set to dim so that each alley had the following lighting conditions; 1.9 ± 1 lux in alley 1, 10 ± 1 lux in alley 2, 18.6 ± 2 lux in alley 3 and 25.1 ± 2 lux in alley 4.

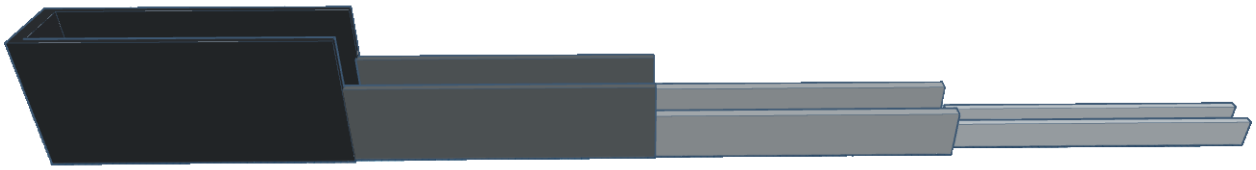

Figure 2: Shows an example image of the successive alleys arena as viewed from the side.

Procedure

Habituation. Habituation occurred one week prior to testing and included once-daily sessions over five-days, with a 2- to 3-day break between the habituation phase and the testing phase of the experiment. During habituation, animals were transported in their home cages into the experimental room and handled individually for two minutes in order to familiarise them to the experimental setting and to being handled. Habituation occurred during the dark phase of the light-dark cycle in dim lighting conditions (28.4 ± 5 lux). The order and time of habituation was randomised.

During this phase, animals were also exposed to the cereal used as food-rewards in the successive alleys arena. Exposure to these food-rewards occurred in the housing room at a random interval after habituation. This was done to ensure the animals made no premature

association between the experimental room and the food-rewards. Animals were given one piece of cereal each day, broken into halves, and observed to ensure all animals developed a preference. Habituation to the cereal stopped following the five days of habituation.

Testing. Testing occurred over seven days between 0830 and 1900 during the dark phase of the light-dark cycle. The order of testing each day was counterbalanced and pseudo-randomised using the following method: for each cohort, each cage was assigned to one of two testing groups. Cages from treatment and control litters were distributed evenly. The testing order within each group was pseudo-randomised prior to testing, and the testing order of the groups each day were counterbalanced in an 'ABAB' fashion. This led to a random interval of food-restriction with a period of 13.5 to 26 hours following feeding.

On the day of testing, animals were transported in their home cages to the experimental room. Testing of each cage occurred over a 30-minute period; this consisted of a 10-minute habituation period, a 10-minute testing session and a 10-minute wait-period while the cage mate was being tested. Testing occurred one cage at a time, to maintain at most one cage in the experimental room during testing.

During the habituation phase, pairs were left undisturbed in their home cages. This was to acclimate them to the experimental setting and to reduce the disruption transport might have on behaviour. Following this, an animal was selected at random for testing.

During the testing phase, animals were first placed in the anticipatory arena for five minutes. During this time, USVs were recorded to an audio file with Audacity® software, and rearing counts were scored manually. Rearing was defined as the animal raising both front paws off the ground. A single rearing behaviour ended when the animal next placed both front paws on the ground. Following anticipatory testing, the animal was placed into alley 1 of the successive

alleys arena facing the back wall. Behaviour was recorded for an additional five minutes. During this time, latency until eating and the alley each cereal piece was eaten in was recorded manually. The alley of consumption for each cereal piece was based on the location of the centre-point of the animal. In situations where the centre-point was on the boundary between two alleys, the alley they were facing was counted as alley of consumption.

Video was recorded for the entire 10-minute testing phase by a USB camera mounted above to give a birds-eye view of both arenas. Video was recorded using NHC Debut® Video Capture Home Edition software. Duration in each alley, the latency to reach alleys 2 through 4, and the number of entries into alleys 2 through 4 were extracted from the video recording using EthoVision XT9 software. These measures were all based on the centre-point of the animal.

Additionally, distance moved was assessed in the successive alleys arena to test for global reductions in locomotor activity.

Both arenas were cleaned with F10™ SC Veterinary Disinfectant between each trial and the arena baited with fresh cereal.

Results and Discussion

Dose and Breeding Outcomes

An average weekly dose was calculated for each female and can be seen in Table 2. Doses are displayed in mg/kg. Average doses achieved for each week were as follows: gestational week 2 (M = 8.41, SE = 0.39), gestational week 3 (M = 7.01, SE = 0.39), and postnatal week 1 (M = 6.05, SE = 0.40). Comparisons of doses between experiment one and experiment two are displayed in Figure 3. Due to the small sample size of experiment one, no statistical analysis could be run. However, based on the graph the doses across experiments appear comparable and

therefore experiment one fluoxetine and norfluoxetine levels reported in pups may be generalised to this sample.

Table 2

Mean ± SEM dose of fluoxetine (mg/kg) for each week for individual subjects.

Subject	GW 2 dose (mg/kg)	GW 3 dose (mg/kg)	PNW 1 dose (mg/kg)
M39	6.59 ± 1.21	6.07 ± 0.68	5.62 ± 0.85
M72	7.28 ± 1.36	7.12 ± 1.16	6.54 ± 0.47
M73	7.91 ± 0.51	4.61 ± 0.78	4.83 ± 1.06
M84	8.19 ± 0.93	8.88 ± 1.00	6.43 ± 1.33
M86	12.03 ± 0.33	10.48 ± 0.75	9.37 ± 1.06
M87	7.99 ± 1.2	8.34 ± 1.04	6.02 ± 0.87
M88	9.28 ± 1.09	7.55 ± 1.18	5.58 ± 1.31
M89	7.29 ± 1.22	5.7 ± 0.99	6.72 ± 1.16

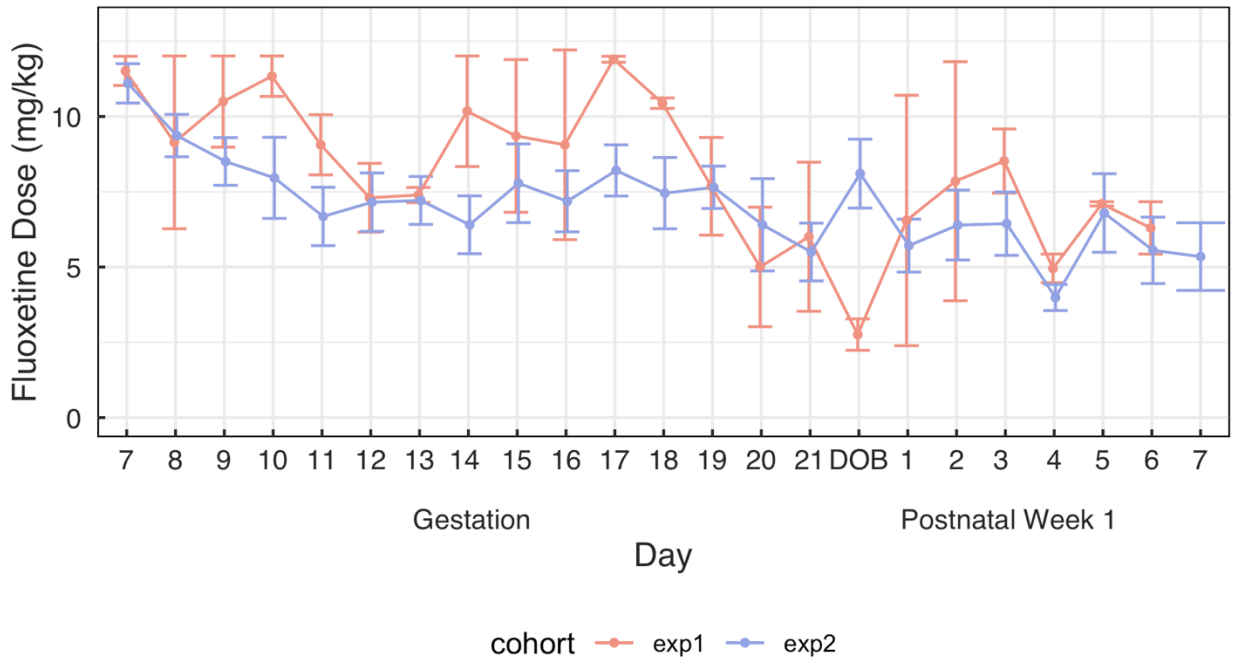

Figure 3: shows the average daily dose (mg/kg) of fluoxetine for dams in experiment one versus dams in experiment two. Error bars are standard error of the mean.

To assess whether fluoxetine-treatment had any effect on litter size or pre-weaning deaths, data was analysed using Welch's (or unequal variances) *t*-test. This approach was chosen due to violations of the assumption of homogeneity of variance making independent-samples t-testing inappropriate for this data.

Fluoxetine-treatment had no significant effect on litter size ($M = 12.67$, $SE = 0.53$), compared to untreated litters ($M = 12.38$, $SE = 1.39$), $t(9.00) = -0.19$, $p = 0.85$. Fluoxetine-treatment also had no significant effect on number of pre-weaning deaths ($M = 1.11$, $SE = 0.87$), compared to untreated litters ($M = 0.25$, $SE = 0.25$), $t(9.29) = -0.95$, $p = 0.37$. One litter from the fluoxetine group showed an abnormal number of pre-weaning deaths. However, as this was the exception, and doses for this dam were less than doses for other dams in this study, the attrition in this litter was unlikely due to fluoxetine treatment. This is in line with several other studies that have

reported no significant differences in litter size (McAllister et al., 2012), and indicate that at these levels fluoxetine does not have any toxicity effects in rats.

Maternal Behaviour

To assess whether fluoxetine-treatment altered maternal behaviour, the three observation sessions for each dam were combined for PD1, PD4 and PD7. To get an overview of mother-pup interactions, a combined score for pup-oriented behaviours was calculated for analysis. Many of the individual behaviours were so infrequent that we could not run statistics on them individually. Pup-oriented behaviours were any observations where the dam was directing her behaviour towards her pups or nest and was the sum of each of the following: pup retrieval, nest building, maternal contact, licking-grooming, passive nursing, blanket nursing, active low arch, and mid/high arch. These include instances where the dam may be engaged in more than one behaviour at a time (eg. LG and ABN, or where there was a switch in behaviours within the 5-second window of observation). Because of their specific associations with anxiety outcomes in offspring, LG and active nursing behaviour (the sum of active-low arch and mid/high arch) were also analysed separately. Active-ABN was analysed as proportion of total nursing observations. The number of observations for each behaviour are shown in Table 3, in addition to the summed behaviours used in analyses. Data in tables and figures are reported as mean ± standard error of the mean.

Table 3

Mean ± SEM observation count for each behaviour assessed, along with summed behaviours used in analysis.

	Postnatal day 1		Postnatal day 4		Postnatal day 7	
Behaviour	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
Away from nest	27.83 ± 6.06	25.57 ± 5.36	38.50 ± 3.55	36.86 ± 7.60	36.17 ± 5.02	41.86 ± 2.77
Pup retrieval	0.00 ± 0.00	1.71 ± 0.64	1.33 ± 0.76	0.86 ± 0.55	0.33 ± 0.33	0.71 ± 0.29
Nest building	4.33 ± 0.95	3.86 ± 0.96	4.83 ± 1.90	4.29 ± 1.69	5.67 ± 1.58	2.57 ± 0.97
Maternal contact	3.17 ± 0.60	4.29 ± 1.13	2.17 ± 0.60	2.00 ± 0.49	2.5 ± 0.56	1.57 ± 0.61
LG	6.67 ± 1.33	7.29 ± 1.04	7.33 ± 1.12	6.14 ± 0.99	8.67 ± 1.02	8.43 ± 1.00
Passive nursing	7.50 ± 4.46	4.00 ± 1.50	3.33 ± 1.82	1.71 ± 1.08	8.00 ± 3.19	2.14 ± 1.03
Low ABN	14.67 ± 3.20	16.71 ± 3.78	10.83 ± 1.47	12.71 ± 3.64	10.33 ± 2.92	9.43 ± 1.65
Active low ABN	8.17 ± 0.79	13.29 ± 2.72	7.50 ± 2.77	6.86 ± 2.44	11.00 ± 1.29	8.57 ± 1.77
Mid/high ABN	7.17 ± 1.78	5.86 ± 1.99	9.17 ± 2.68	9.14 ± 3.19	6.33 ± 1.80	4.43 ± 1.49
Pup-oriented behaviours	51.67 ± 6.44	57.00 ± 5.72	46.50 ± 5.80	43.71 ± 9.51	52.83 ± 6.68	37.86 ± 3.88
Total nursing	37.50 ± 5.35	39.86 ± 6.17	30.83 ± 3.68	30.43 ± 7.44	35.67 ± 6.18	24.57 ± 2.85
Active nursing	15.33 ± 2.39	19.14 ± 3.08	16.67 ± 2.60	16.00 ± 4.19	17.33 ± 2.12	13.00 ± 1.27

Pup-oriented behaviours, LG, total nursing, and active-ABN behaviours were each analysed using a mixed-effects model to overcome violations of sphericity in the data. All analyses met assumptions of normality as assessed by Shapiro-Wilk's test and assumptions of variance as assessed by Levene's test.

There was no significant main effect of treatment group, $\chi2(7) = 0.639$, $p = .424$ or postnatal day, $\chi2(6) = 2.96$, $p = .227$ on number of pup-oriented behaviours. In addition, there was no significant interaction, $\chi2(9) = 2.71$, $p = .259$. The number of licking-grooming behaviours for each day can be seen in Figure 4. For number of licking-grooming behaviours, there was no significant main effect of treatment group, $\chi2(7) = 0.108$, $p = .743$ or postnatal day, $\chi2(6) = 3.69$, $p = .1587$, and there was no significant interaction, $\chi2(9) = 0.817$, $p = .665$. For total nursing observations, there was no significant main effect of postnatal day $\chi2(6) = 3.41$, $p = .18$, or treatment group $\chi2(7) = 0.50$, $p = .48$, and no treatment-day interaction $\chi2(9) = 1.86$, $p = 0.39$.

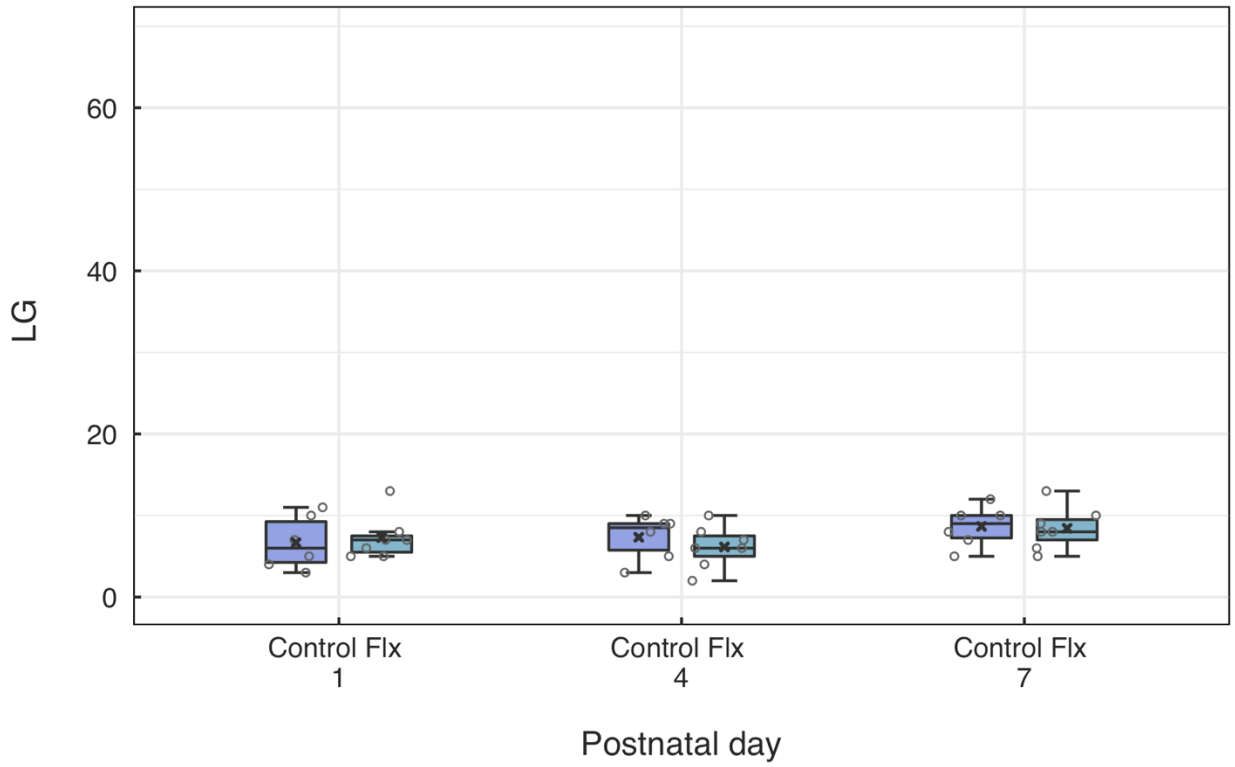

Figure 4: shows the number of licking-grooming observations in fluoxetine treated (Flx) and untreated (control) dams for each day. Observation periods for each day were summed, giving a total of 69 possible observations.

To test whether the preference for active versus passive nursing postures were significantly different across treatment groups, the proportion of nursing behaviours that were active (the sum of active low ABN and mid/high ABN observations) were analysed using a mixed effects model. Proportion of nursing behaviours that were active are displayed in Figure 5. There was no significant main effect of postnatal day $\chi2(6) = 1.90$, $p = 0.39$ or treatment group $\chi2(7) = 0.09$, $p = .77$ on proportion of nursing behaviours that were active. In addition, there was no treatment-day interaction $\chi2(9) = 0.63$, $p = 0.44$.

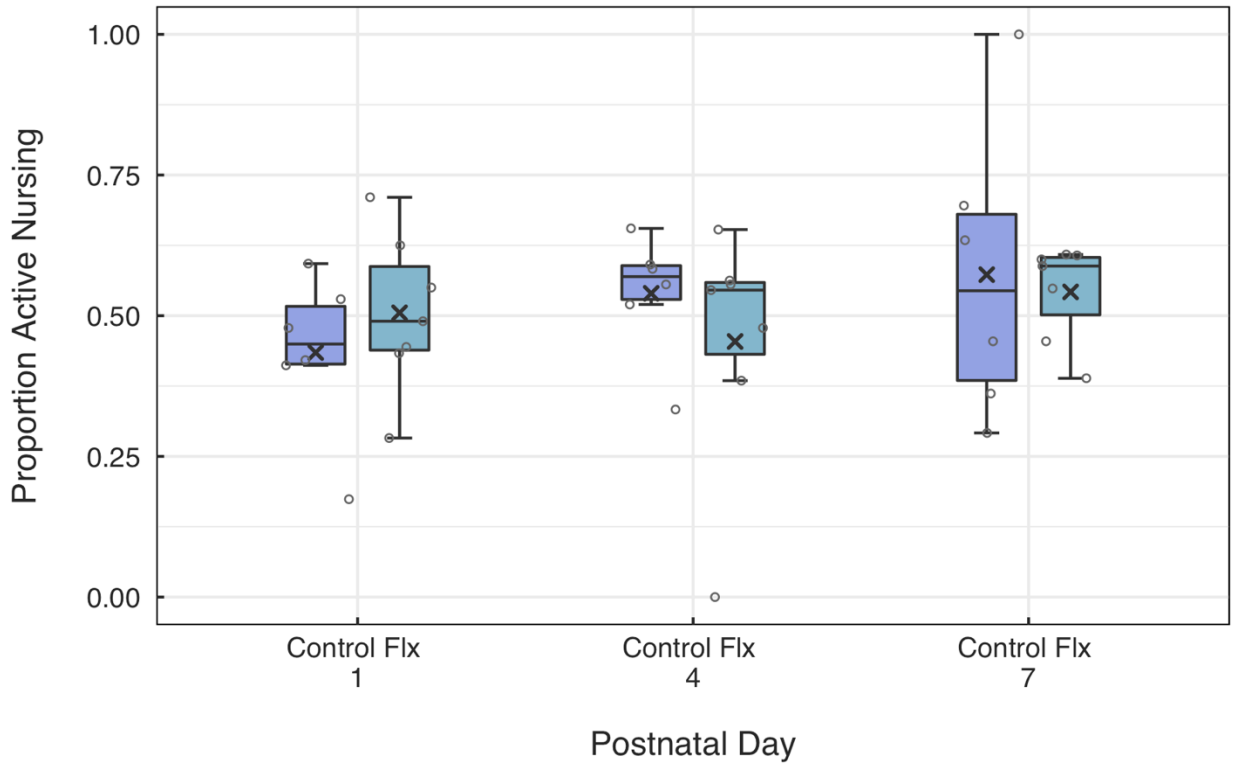

Figure 5: shows the proportion of total nursing behaviours that were spent in active nursing postures for fluoxetine treated (Flx) and untreated dams for each day of testing. The average for each group is indicated by the x.

Together these findings indicate that the doses of fluoxetine achieved in the current study did not significantly influence mother-pup interactions. Dams did not differ in the amount of contact they had with pups as measured by pup-directed behaviours and spent similar amounts of time nursing. In addition, dams did not differ in LG behaviours or the proportion of nursing time spent in active nursing postures. Because of these findings we can be confident that any systematic treatment differences in subsequent analyses are not as an artefact of altered early-life rearing environment.

Anticipatory Pleasure

The number of 50-kHz USVs emitted on each day are displayed in Figure 6. Anticipatory ultrasonic vocalisation data were analysed using a generalised linear mixed effects regression

using Poisson distribution. Our data had two major issues when it came to analysis: the first was missing values due to technical issues with recording software on one of the days of testing for one of the cohorts, and the second was overdispersion in the data (the variability was greater than the mean). The data failed to fit a negative binomial regression which would typically be used in this situation, so instead a random effect of observation was entered to correct for the overdispersion.

The fixed effects entered into the model were genotype, treatment condition, and day, in addition to their interaction terms. A random intercept of animal ID was entered into the model to account for the large variability in the baseline call rate of the animals in this experiment. P-values were obtained from likelihood ratio tests of the full model compared to the model minus the effect in question.

There was no main effect of genotype, $\chi2(5,2) = 1.46$, $p = 0.48$, or treatment group, $\chi2(6,1) = 0.31$, $p = 0.58$ on number of ultrasonic vocalisations over time. There was a significant main effect of day, $\chi2(12,6) = 56.46$, $p < .001$. There was no significant genotype-condition, $\chi2(14,2) = 0.69$, $p = 0.71$, genotype-day, $\chi2(26,12) = 17.45$, $p = 0.13$, or genotype-condition-day, $\chi2(44,12) = 8.58$, $p = 0.74$, interactions.

Contrasts were used to investigate the main effect of day, comparing each day to the first day call count. Contrasts revealed a significant difference between the number of calls emitted on day six compared to the number of calls on day one, ($t(388) = 2.60$, $p = 0.009$). All other contrasts were non-significant.

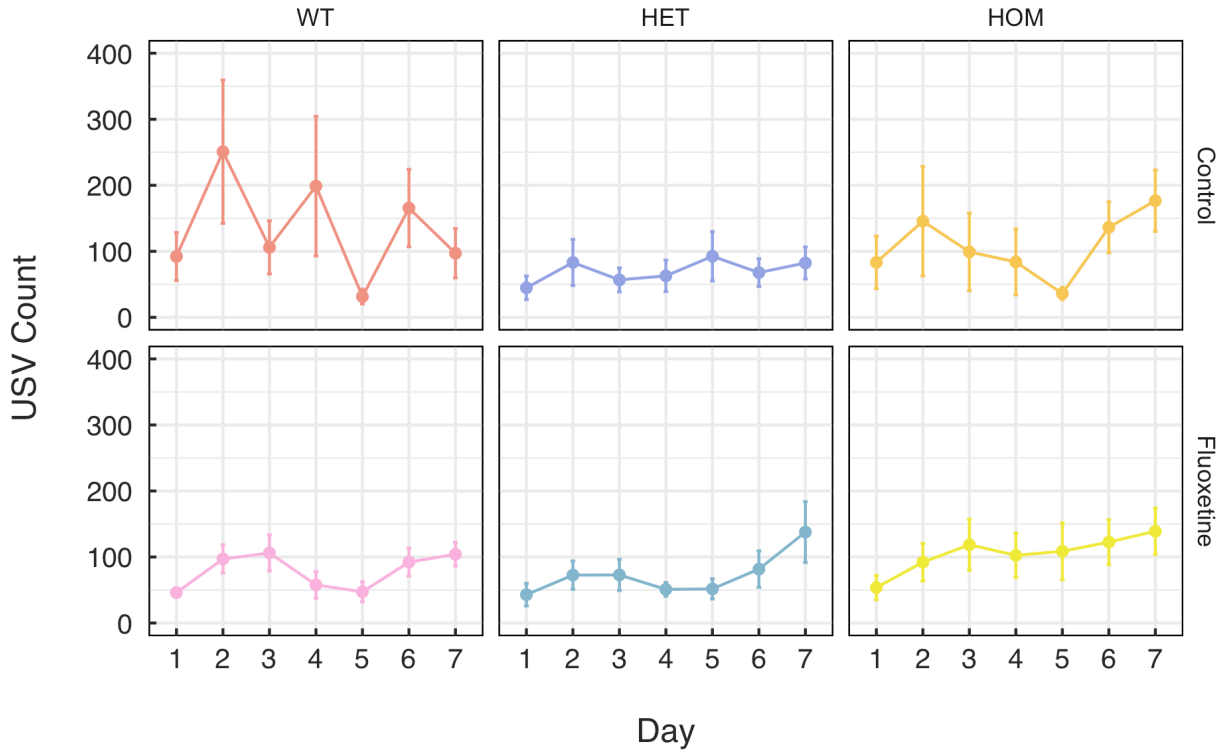

Figure 6: shows the average number of 50-kHz calls emitted on each day of testing for each experimental group. Error bars are standard error of the mean.

These findings indicate that animals did not systematically differ in call rate as a function of genotype or treatment condition. Specifically, the failure to find a significant interaction between genotype and day is inconsistent with our hypothesis that homozygous and heterozygous SERT-KO animals would show reduced anticipatory USVs compared to controls. The significant increase in call rate between day one and day six might suggest the emergence of anticipatory USV responses, however as this was not found on day seven it is unclear whether the conditioning processes required for detecting anticipatory USVs had occurred.

For the second measure of anticipatory pleasure, rearing data was analysed using a generalised linear mixed effects model using Poisson distribution. The number of rearing behaviours scored for each experimental group are displayed in Figure 7. The fixed effects

entered into the model were genotype, treatment condition, and day, along with their interaction terms. A random effect of animal ID was added to account for differing baseline rates of locomotor activity that may vary at the level of the individual.

P-values were obtained from likelihood ratio tests of the full model compared to the model minus the effect in question. There was a significant main effect of day ($\chi2(11,6) = 322.22$, $p < .0001$), however genotype ($\chi2(11,2) = 1.19$, $p = 0.55$) and treatment condition ($\chi2(11,1) = 1.09$, $p = 0.30$) did not have a significant overall effect on rearing behaviour. In addition, there were no significant interactions. Contrasts were used to investigate the main effect of day. These contrasts compared rearing scores each day to the rearing scores on day three. Day three was chosen for comparison as it was the day mean rearing reached its turning point (see Figure 7), indicating a reduction in novelty-induced locomotor activity following habituation to the arena.

The first contrast revealed a significant difference between day one and day three rearing scores ($t(383) = 2.25$, $p = .03$). The second contrast revealed a non-significant difference between day two and three rearing scores ($t(383) = 0.53$, $p = 0.60$), indicating there was no difference in locomotor activity between days two and three. The third contrast on days three and four was also nonsignificant ($t(383) = 0.99$, $p = 0.32$), as was the fourth contrast between days three and five ($t(383) = 1.47$, $p = 0.14$). The fifth contrast comparing days three and six rearing scores revealed a significant difference ($t(383) = 4.19$, $p < .001$). The final contrast also revealed a significant difference in rearing between days three and seven ($t(383) = 2.94$, $p = 0.003$).

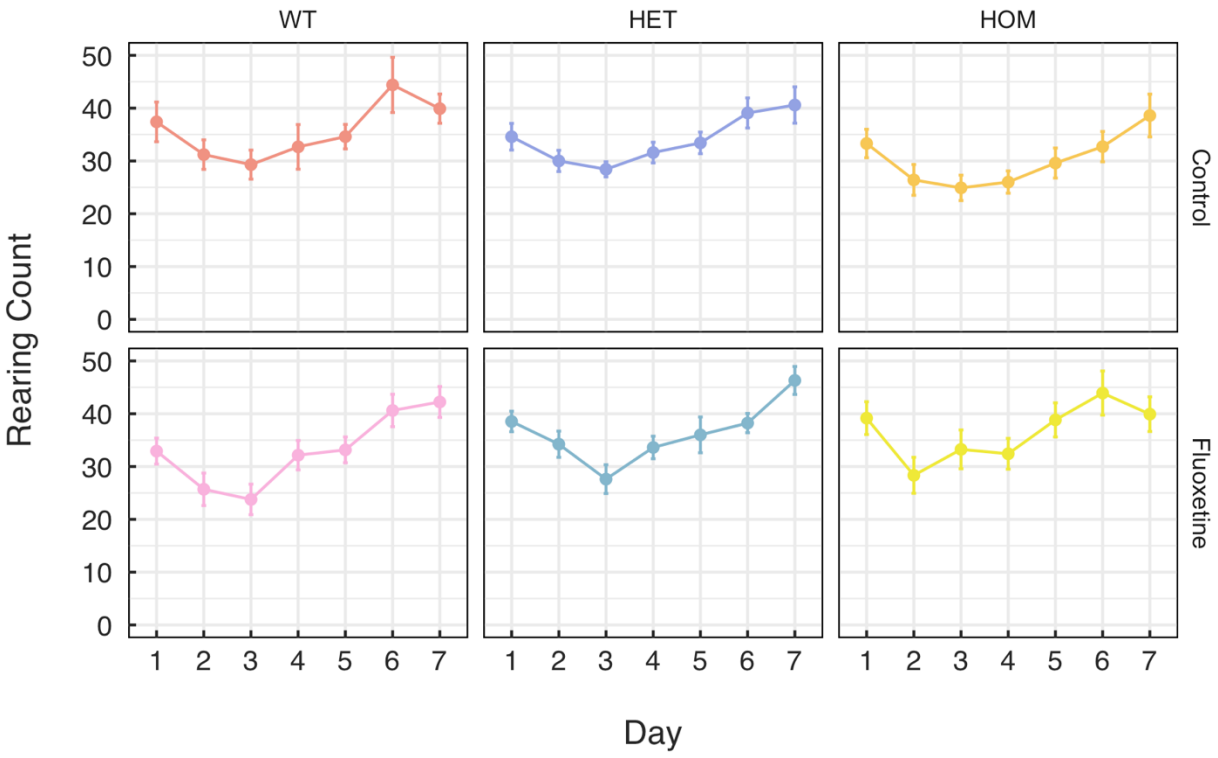

Figure 7: shows the average rearing behaviours scored each day for each of the experimental groups. Error bars are standard error of the mean.

Rearing significantly increased over time compared to baseline scores at day three, and suggests that irrespective of genotype or treatment, animals showed normal anticipatory locomotor activity. This is inconsistent with our hypothesis that heterozygous and homozygous SERT-KO animals would show reduced anticipatory locomotor activity compared to controls. Both the USV and rearing data suggest that there were no differences in depressive phenotypes as measured by deficits in anticipatory pleasure. However, it is difficult to make interpretations due to lack of robust day effects, particularly for USV data, and indicate the lack of findings may be due to methodological issues.

Successive Alleys Test

Only the first four minutes (240 seconds) of video data in successive alleys were analysed due to technological issues for one of the cohorts, resulting in data corruption over the fifth minute of every video file. For latency to enter an alley, animals that did not reach that alley were assigned a maximum value of 240 seconds. Latency to eat was recorded manually, so animals that did not consume any cereal were assigned a maximum value of 300 seconds. For a table outlining the mean ± SEM scores for each of the individual variables measured on the SAT see Appendix A.

The data collected in the successive alleys arena were first analysed using a principle component analysis (PCA) followed by orthogonal varimax rotation. Each day was analysed separately, and the variables included in the PCA were determined by Kaiser-Meyer-Olkin (KMO) analysis (KMO > 0.5 overall and for each variable). In addition, Bartlett's test of sphericity was used to determine sufficiently large correlations between variables for PCA ($p >$ 0.5).

The number of factors extracted for each day was determined by the Scree test (the point of inflection on a scree plot with eigenvalues ranked from largest to smallest), and factors with eigenvalues greater than 0.75 were considered. Only factor loadings above 0.5 were kept and are presented in Table 4.

Table 4

Orthogonal factor loadings for behaviour in successive alleys arena for each day of testing.

	Day 1			Day 2				Day 3	
	factor 1	factor 2	factor 3	factor 1	factor 2	factor 3	factor 4	factor 1	factor 2
Distance moved	0.82			0.78				0.73	
Alley 1 duration		-0.84			-0.71				-0.78
Alley 2 entries	0.84			0.87				0.87	
Alley 3 duration		0.79			0.68				0.79
Alley 3 latency		0.82			-0.62				-0.58
Alley 3 entries		0.75			0.78			0.51	0.61
Alley 4 duration			0.87			0.85			0.85
›Alley 4 latency			0.84			0.82			-0.77
Alley 4 entries			-0.77			-0.77			0.77
Consumption score									0.56
Consumption latency							0.93		

	Day 4			Day 5			Day 6		
	factor 1	factor 2	factor 3	factor 1	factor 2	factor 3	factor 1	factor 2	factor 3
Distance moved	0.82			0.84			0.9		
Alley 1 duration		-0.58	-0.56		-0.63	-0.55		-0.84	
Alley 2 entries	0.78			0.87			0.8		
Alley 3 duration		0.54			0.84			0.66	
Alley 3 latency		-0.68			-0.64			-0.54	
Alley 3 entries			0.74	0.58			0.64		
Alley 4 duration		0.79				0.72		0.82	
Alley 4 latency		-0.86			-0.55			-0.6	
Alley 4 entries		0.73				0.65		0.53	
Consumption score			0.6			0.77		0.81	
Consumption latency									0.72

	Day 7		
	factor 1	factor 2	factor 3
Distance moved	0.73		
Alley 1 duration		-0.73	
Alley 2 entries			
Alley 3 duration		0.72	
Alley 3 latency	-0.69		
Alley 3 entries	0.75		
Alley 4 duration			0.74
Alley 4 latency	-0.59		
Alley 4 entries			0.86
Consumption score		0.83	
Consumption latency			

Day 1: Duration in alley two, consumption score, and consumption latency were excluded from the analysis due to KMO values below criterion. The overall KMO score following the removal of these variables was KMO = 0.75, indicating adequate sampling. Bartlett's test of sphericity was non-significant, $\chi 2(66) = 591.05$, $p < .001$, indicating correlations between the remaining variables were sufficiently large for PCA. An initial analysis was run to determine the number of factors. Three factors had eigenvalues above 1, and together explained 77% of the variance. In addition, the scree plot showed inflections at both two and three factors. Based on these results, three factors were retained for the final analysis. Factor one includes variables reflecting behaviour in alley three (alley one duration also loaded negatively on this factor) and factor two includes variables reflecting behaviour in alley four. The third factor is made up of the number of entries into alley two, as well as the total distance moved, and could indicate a measure of locomotor or exploratory activity in the arena.

Day 2: Alley two duration and consumption scores were excluded from analysis due to unacceptable KMO scores. The overall KMO score following removal of these variables was KMO = 0.81, indicating acceptable sampling adequacy. Bartlett's test was non-significant, $\chi 2(45) = 279.08$, $p < .001$, indicating sufficiently large correlations between variables for PCA. An initial analysis run to determine the number of factors revealed four factors with eigenvalues greater than 1 that explained 76% of the cumulative variance. The scree plot showed inflection points at both two and four factors; thus, four factors were retained in the final analysis. The factors extracted mirror what was found with day one data: factor one included variables related to behaviour in alley four; factor two included variables related to behaviour in alley three, along with duration in alley one loading negatively on this factor; factor three included number of

entries into alley two as well as distance moved. A final factor contained only consumption latency, which was excluded from day one analysis.

Day 3: Alley two duration and consumption latency were excluded from analysis due to unacceptable KMO scores. The overall KMO score following removal of these variables was acceptable, KMO = 0.83. Bartlett's test was non-significant, $\chi2(45) = 395.12$, $p < .001$. Initial analyses revealed three factors with eigenvalues greater than 0.75 that explained 74% of the variance. The scree plot showed a single point of inflection at two factors, so two factors were retained in the final analysis. Variables relating to behaviours in alleys three and four loaded onto a single factor, along with consumption score. This merging of previously independent factors could be due to habituation of the animals to the successive alleys arena, resulting in less distinct regions between alleys three and four. The second factor included variables that may be related to exploratory or locomotor activity, similar to factors extracted for previous days. In addition, entries into alley three loaded heavily on both factors.

Day 4: Alley two duration and consumption latency were excluded from analysis due to unacceptable KMO scores. The overall KMO score was acceptable following removal of these variables, KMO = 0.77. Bartlett's test was non-significant, $\chi2(45) = 270$, $p < .001$. An initial analysis revealed four factors with eigenvalues greater than 1, explaining 75% of the cumulative variance. The scree plot showed a single point of inflection at three factors (explaining 66% of the cumulative variance), so three factors were retained in the final analysis. Similar to day three results, a single factor was extracted for most variables measuring behaviour in alleys three and four. As in previous analyses, a second factor included variables that could be related to locomotor activity or exploratory behaviour in the arena. The third factor included consumption

scores and number of entries into alley three. Duration in alley one loaded heavily on factors one and three.

Day 5: Alley two duration and consumption latency were excluded from analysis due to unacceptable KMO scores. The overall KMO score was acceptable following removal of these variables, KMO = 0.77. Bartlett's test was non-significant, $\chi2(120) = 782.38$, $p < .001$. Initial analyses revealed four factors with eigenvalues greater than 1, together explaining 78% of the variance. The scree plot showed a single point of inflection at three factors, so three factors were retained in the final analysis (explaining 70% of the cumulative variance). Along with previous days, a factor containing variables that could be related to exploratory behaviour was extracted. In addition to distance moved and number of entries into alley two, entries into alley three also loaded on this factor. This could be a result of further habituation to the arena. The second factor is made up of duration and latency to enter alley three, as well as latency to enter alley four. Factor three includes duration in alley four, entries into alley four, and consumption score. Alley one duration loaded negatively on both factors two and three.

Day 6: Alley two duration was excluded from analysis due to a KMO score less than 0.5. The overall KMO was 0.83, and Bartlett's test was non-significant, $\chi2(55) = 312$, $p < .001$. Initial analyses revealed four factors with eigenvalues above 1 that together explained 75% of the variance. The scree plot showed a single inflection point at three factors, so three factors were retained in the final analysis (explaining 66% of the cumulative variance). Factor one contained variables related to behaviour in alleys three and four and consumption score. Alley one also loaded negatively on this factor. The second factor was the same as extracted in day five and included entries into alleys two and three, along with distance travelled, reflecting exploratory behaviour. Factor three contained only latency to begin eating.

Day 7: Alley two duration, number of entries into alley two, and consumption latency were excluded from analysis due to unacceptable KMO values. The overall KMO value was acceptable following removal of these variables, KMO = 0.78. Bartlett's test was non-significant, $\chi 2(36) = 264.28$, $p < .001$. Initial analyses revealed three factors with eigenvalues above 1 that explained 72% of the cumulative variance. The scree plot showed a point of inflection at three factors, so three factors were retained for the final analysis. Factor one included distance travelled, entries into alley three and latency to enter alleys three and four. Together, this could reflect increased exploratory behaviour following habituation to the arena. Factor two included consumption score and duration in alleys three and four. The final factor included entries and duration in alley four.

Overall, the most consistent factor extracted was general locomotor activity or exploration of the arena and included number of entries into alley two along with total distance travelled. This appears to be analogous with the measure of motor activity in the EPM, which includes number of entries into the closed arms and sometimes time spent in the central region (Fernandes & File, 1998). In the current study several other measures began to load onto this factor, which may reflect habituation to the arena. Distance moved and number of entries into alley two load consistently onto a single factor on day one. Over time, entries into more anxiogenic alleys begin to load on this factor, along with latency to enter alley four by the final day of testing. Habituation to the arena is also seen with behaviour in alleys three and four merging onto a single factor over time. Over the first two days, behaviours in alleys three and four remain on separate factors, providing evidence that animals are distinguishing between these two distinct areas. By day three, these variables are loading onto a single factor. This could be interpreted as habituation to the arena and the loss of distinct regions for at least some of the animals.

An advantage of PCA is that factors can be used to produce a composite score for each subject. Composite or factor scores are calculated by multiplying the standardised score for each variable by the factor loading and summing the products. These scores can then be used as outcome variables in subsequent analyses. This approach is suited for tests such as ADT where a number of correlated variables are measured. Differences may be missed when running individual analyses on each measure separately, in addition to increasing the Type I error rate.

Scores for each group were analysed by MANOVA using Wilks' lamda statistic and Type III sum of squares (Carola et al., 2002). Factor scores were only analysed for days one and two, as habituation to the arena is evident from day three, making factors more difficult to interpret. Each day was analysed separately.

There were no significant main effects of genotype or treatment group on any of the three factors extracted from day one data ($F(2,61) = 0.47$, $p = 0.82$; $F(1,61) = 0.94$, $p = 0.43$). In addition, there was no significant genotype-treatment interaction, $F(2,61) = 0.30$, $p = 0.94$.

For factor scores extracted from day two data there was a significant main effect of treatment, $F(1,64) = 2.58$, $p = 0.046$, but not of genotype, $F(2,64) = 0.30$, $p = 0.25$, and no significant genotype-treatment interaction, $F(2,64) = 0.79$, $p = 0.61$. Separate univariate ANOVAs on each factor revealed a significant main effect of treatment on the factor containing only consumption latency, $F(1,64) = 7.01$, $p = 0.01$. Irrespective of genotype, offspring of fluoxetine-treated dams showed slower latency to eat ($M = 106.42$, $SE = 16.41$) than non-treated offspring ($M = 56.03$, $SE = 11.15$). There were no other significant main effects or interactions.

These findings partially support our hypothesis; however, the lack of significant genotype-findings make the significant main effect of treatment difficult to interpret, and it is unclear why

fluoxetine treatment would influence latency to eat in isolation of a significant genotype difference.

Across all PCA analyses, duration in alleys three and four remained independent from factors relating to exploratory or locomotor activity and did not load on more than a single factor over each day. These variables were chosen for subsequent analyses. As these measures sometimes loaded together onto a single factor, durations in alleys three and four were combined for each day and analysed using a multilevel model.

There was no significant main effect of genotype, $\chi2(2) = 0.22$, $p = 0.90$, or of condition, $\chi2(3) = 0.86$, $p = 0.35$ on duration in alleys three and four. Analyses revealed a significant main effect of day, $\chi2(12) = 240.71$, $p < .0001$, and a significant genotype-day interaction, $\chi2(26) = 24.89$, $p = 0.015$.

Contrasts were used to investigate the significant genotype-day interaction. These contrasts compared duration in alleys three and four for each day to duration on day one, and this was done for the heterozygous and homozygous SERT-KO groups compared to the wild type group. Contrasts revealed a significant difference between day one and day five duration when comparing wild type animals to homozygous animals ($b = -25.89$, $t(393) = -2.58$, $p = 0.01$); while all animals increased the amount of time they spent in alleys three and four over time, this increase was significantly larger for homozygous animals compared to wildtype animals,

irrespective of treatment condition. All other contrasts were non-significant.

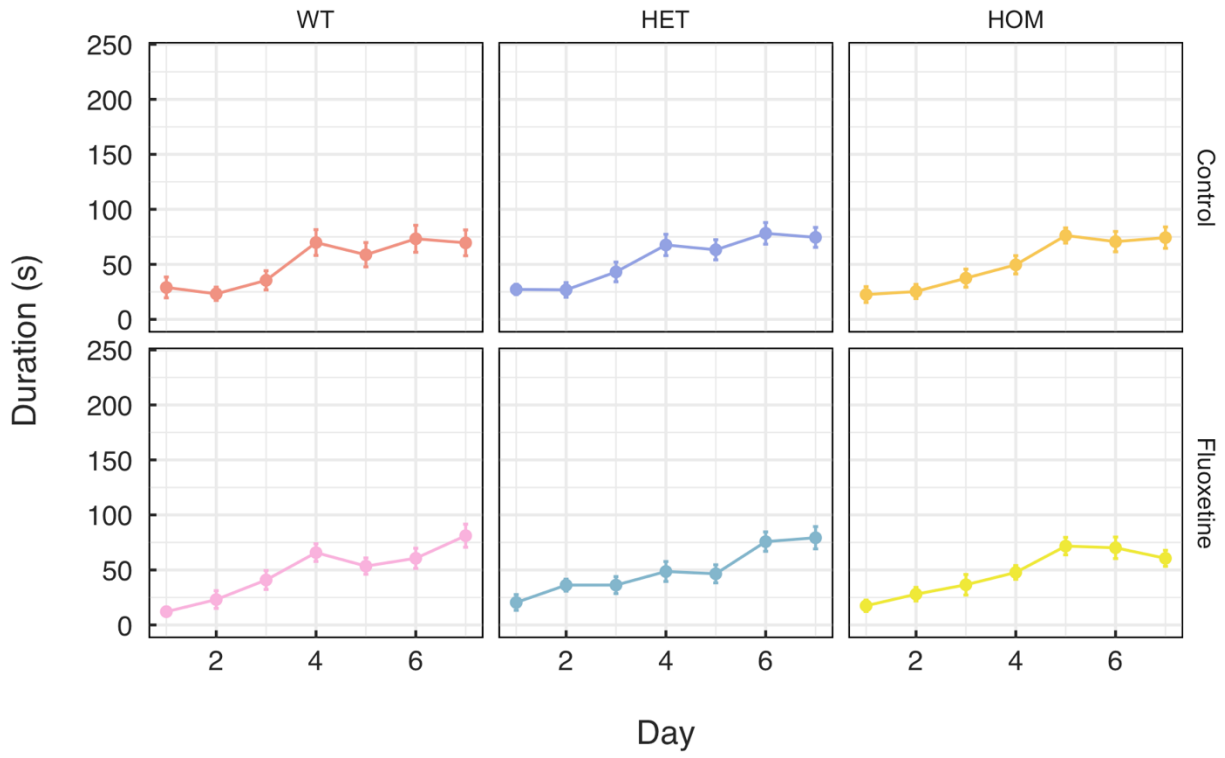

Figure 8: shows the average duration (s) spent in alleys 3 and 4 combined for each experimental group. Error bars are standard error of the mean.

Overall, data from the SAT do not support our hypothesis that heterozygous and homozygous SERT-KO animals showed increased anxiety behaviours.

General Discussion

The current thesis aimed to assess the contribution of short-term developmental blockade of 5-HTT to the depression and anxiety phenotypes seen in animals with life-long deficits in 5-HTT activity. It has been suggested that the anxiety and depression phenotypes reported in these SERT-KO animals are due to changes occurring specifically during development as a result of altered extracellular levels of serotonin. While a multitude of studies have been published investigating the contribution of 5-HTT blockade during development to anxiety- and

depressive-like behaviours, methodological differences between studies and inconsistent findings means a clear relationship has yet to be established. In addition, a potential interaction between maternal stress and SSRI treatment and changes in early rearing environment could be contributing to these mixed findings.

In this thesis I aimed to directly assess effects of developmental fluoxetine exposure in the SERT-KO strain. Experiment one aimed to establish a method of voluntary oral dosing of fluoxetine in pregnant rat dams. In experiment two, this dosing method was used to create a time-specific blockade of 5-HTT during the prenatal and early postnatal periods. As most behavioural tests of anxiety are only effective at testing for state anxiety, we used the novel affective disorder test (ADT) developed to assess trait-anxiety and anhedonia concurrently. Thus, the first step was to assess genotype differences in SERT-KO animals using the ADT. We predicted that heterozygous and homozygous SERT-KO animals would show increased anxiety and deficits in anticipatory pleasure indicative of anhedonia. More importantly, if these differences in genotype were specifically due to blockade of the 5-HTT during development, SERT-WT and heterozygous animals should show increased anxiety behaviours and greater deficits in anticipatory pleasure compared to untreated control and heterozygous groups. However, we failed to find significant genotype differences in behaviours assessed by the ADT, making further modulation of behaviour through SSRI treatment unable to be assessed.

Inconsistent with predictions, rearing showed a significant increase over time irrespective of genotype or treatment, indicating that there were no deficits in anticipatory pleasure in any of the groups. Due to the ADT being a novel test, it is difficult to interpret whether the failure to find genotype differences is due to methodological issues, or whether there are truly no genotype differences in measures of anticipatory pleasure.

In the ADT, anticipatory processes are directly linked to reward motivation: in order to gain food-rewards, the animal must first overcome a fear response. The addition of reward motivation to this paradigm could have impacted our anticipatory results. The anticipatory paradigm relies on respondent conditioning processes through contingent and contiguous pairing of the food-reward with the arena. The introduction of motivational factors could reduce the saliency of this pairing and disrupt the learning process. While the increase in rearing seen from day six indicates that associations between the arena and rewards occurred, it is possible that genotype differences may have emerged with extended testing and stronger conditioning. In support of this argument, anticipatory locomotor paradigms are usually conducted over two weeks. For example, in the protocol outlined in Barbano and Cador (2006), male Wistar rats were first habituated to the arena for several days to reduce novelty-induced locomotor activity and acquire a stable baseline. Animals were then trained, with their daily food being presented following a 30-minute session in the arena. With this protocol it took 10-days of training before anticipatory activity became stable. In the protocol used in experiment two, food rewards were smaller in comparison and required work to obtain, and the number of pairings were less. Thus, it is unlikely that anticipatory locomotor activity would have stabilised in our animals by the final day of testing. The USV data did not show a systematic increase in call rate over time, further suggesting that the strength of the association was not robust enough to see these changes over 7 days. Because of these limitations in methodology, firm conclusions about our lack of findings cannot be made.

The combination of the anticipatory paradigm with the modified successive alleys test in the ADT is both a strength and a limitation when it comes to validity of the constructs they are designed to measure. In spite of the issues addressed above, introducing reward motivation into the anticipatory paradigm increases the face validity of this preclinical test of depression. The

link between anticipatory pleasure and motivation has been well established in human studies. In studies that include distinct measures for consummatory and anticipatory pleasure, anticipatory pleasure is found to be linked to behavioural activation (a measure of motivation; Carver & White, 1994; Gard et al., 2007). Day-to-day functioning becoming significantly impaired is a major criterion for diagnosis of most mental disorders. In classic tests of anticipatory pleasure, it is difficult to interpret what the functional consequences of reduced anticipatory pleasure might be. In contrast, the introduction of motivational factors in the ADT in which an animal has to work to obtain the food-rewards allows us to directly assess these consequences; if an animal shows reduced anticipatory behaviour and food-retrieval is subsequently impaired in spite of the food restriction, this may better reflect a meaningful deficit that is significantly impairing day-to-day life or processes. However, this still requires a strong conditioned response to be developed first, and further work is needed before the ADT can be established as a valid model.

Inconsistent with our predictions on anxiety phenotypes, we also failed to find any genotype or treatment differences in anxiety behaviours as measured by the ADT. The novel ADT test for detecting trait differences in anxiety behaviours has yet to be validated and has only been used once previously with SERT-KO animals. In addition, the indices of anxiety-like behaviours that can be obtained from the test are either novel (such as consumption score) or have not been validated as a stable measure over time. Because of these limitations, we chose to run an exploratory PCA to get an idea of the specific constructs being measured and examine how these behaviours related to each other over time. The most notable limitation with our PCA was that all groups were combined. This was done to give the PCA enough statistical power, as our control group alone did not contain enough subjects for this test. Due to this limitation our results cannot be generalised to other samples.

Due to potential habituation seen with measures such as number of entries and latency to enter alleys three and four, duration in alleys three and four appears to be the most stable behaviour obtained in this test. Because of this consistency in the PCA, duration in alleys three and four were analysed to test for genotype and treatment differences on this potential measure of trait anxiety. We did not find any significant group or treatment differences in the amount of time spent in alleys three and four, indicating no differences in anxiety phenotypes in this sample. The SAT has been used previously with SERT-KO animals in our lab (Kidwell, 2018). Consistent with our findings, there were no significant genotype differences on duration in alley 4. However, in the 2018 study, significant differences between homozygous and wildtype SERT-KO animals on latency to enter alley four was found, with homozygous SERT-KO animals consistently slower to enter alley four compared to controls. In addition, a significant dose-dependent reduction in consumption scores was found, with SERT-WT animals consistently scoring highest and homozygous SERT-KO animals consistently scoring lowest. These differences in findings could be due to alterations in protocol between the previous study and the current experiment. In the version of SAT run by Kidwell (2018), reward-baiting of alleys was dependent on the animal's prior behaviour. Alleys were not equally baited with cereal, but instead were based on the animal's individual consumption score on previous trials. Thus, once a food-reward was consumed in the alley it was presented in, this reward would be absent on subsequent trials. Latency measures in subjects that have alleys baited based on previous trial consumption will differ based on the amount of treats present: an animal with less food-rewards will be faster to reach alley 4 due to less time spent consuming treats in prior alleys compared to animals that encounter treats in every alley. Because of the group-level differences in consumption scores present from trial one, this confound could be driving the stable genotype

differences seen over time. In the current experiment each alley was baited fully on each trial, which was important for establishing associations between the anticipatory arena and these food-rewards. Latency to enter alley four was not explicitly analysed, however analysis of factor scores for day one and two data support results from duration data that animals in the current experiment did not differ in anxiety phenotypes as a function of genotype or fluoxetine treatment.

This lack of anxiety behaviours in the SERT-KO animals is inconsistent with many previous studies. Anxiety phenotypes in SERT-KO animals have been studied in single trial tests such as the EPM, the OFT, and the novelty-suppressed feeding test, which can be compared to our trial one results. In both rats and mice, the homozygous SERT-KO genotype in males is associated with significantly longer latency to eat in the novelty-suppressed feeding test, significantly reduced time in the open arms of the EPM, and significantly reduced time in the central region in the OFT (Holmes et al., 2003; Olivier et al., 2008). In these tests, heterozygous SERT-KO groups did not significantly differ from control animals. Altieri et al. (2014) used factor analysis to reduce dimensionality of the EPM and compared SERT-WT mice anxiety factor scores to factor scores for heterozygous and homozygous SERT-KO animals. In the EPM, both heterozygous and homozygous SERT-KO animals differed in anxiety factor scores compared to controls, indicating this global approach to behaviour analysis may be more sensitive in detecting differences compared with a more isolated approach to assessing anxiety behaviours. In the current experiment, factor scores obtained for day one data did not differ across any of the genotypes. Similarly, duration in alleys three and four were similar across the genotype groups as well. As with the anticipatory pleasure findings, the use of a novel test makes it difficult to

interpret whether the failure to find genotype differences is due to methodological issues or a true null finding.

Assessment of maternal care in experiment two revealed that fluoxetine treatment did not alter maternal behaviour. Dams did not differ in the amount of contact they had with pups as measured by pup-directed behaviours and spent similar amounts of time nursing. In addition, dams did not differ in LG behaviours or the proportion of nursing time spent in active nursing postures. This is in contrast to studies that have found that fluoxetine treatment increases the amount of ABN-LG behaviours in dams. Pawluski et al. (2012) assessed nursing behaviours twice daily for continuous 5-minute observation periods between PND 2 and PND 7. A dose of 5mg/kg/day of fluoxetine administered from P1 via osmotic minipump resulted in an increase in ABN behaviours compared to controls. Johns et al. (2005) similarly found an increase in the frequency of LG behaviours during a single 30-minute observation period on PND 0 in dams exposed to a high dose of fluoxetine (8mg/kg/day) when compared to the low dose (4mg/kg/day) and control groups. Observation periods in the current study were conducted for longer periods of time, and captured a larger proportion of the early postnatal period compared to the protocols employed by Johns et al. (2005) and Pawluski et al. (2012), thus Type I errors are less likely in the current study and we can be more confident in our results. The current study used a protocol similar to Kiryanova et al. (2016), who similarly found no systematic differences in maternal care as a result of fluoxetine treatment, further strengthening the conclusion that fluoxetine treatment during gestation and the early postnatal period does not alter dam-pup interactions.

While dams in the current study did not differ from each other on measures of maternal care, as a group they showed high levels of ABN-LG behaviours relative to observation percentages reported in the literature. In one study, high and low ABN-LG dams spent 26% and 20% of

observations in active nursing posture respectively; additionally, high ABN-LG dams spent 11% of observations licking and grooming pups, whereas low ABN-LG dams spent 6% of observations licking and grooming pups (Caldji et al., 1998; Liu et al., 1997). In the Caldji et al. (1998) study, offspring of high ABN-LG dams were reported to have significantly reduced levels of anxiety compared to low ABN-LG offspring. In the current study, group means for percentage of active nursing observations ranged from 19-27% and were 9-12% for LG observations. While differences in methodology limit any conclusions that can be made, these data suggest that dams in the current study trended towards high ABN-LG. This could partially explain the failure to find genotype differences on measures of anxiety; high ABN-LG behaviours could have resulted in a rescuing of anxiety phenotypes in SERT-KO offspring. Issues with statistical power prevented us from systematically examining whether genotype differences could be detected in dams that scored the lowest on these behaviours.

The use of the novel ADT in the current study makes it difficult to compare our results to the broader literature. It also makes it difficult to speculate about what our null findings mean for understanding how developmental changes in serotonin contribute risk for mental illness in later life. Validation studies directly comparing the modified successive alleys test to classic tests of anxiety such as the EPM need to be conducted before the current findings can be generalised to the broader literature. The EPM has been well established as a measure of anxiety in rodents. Exposure to the open arms is associated with elevated plasma corticosterone levels, increased freezing, and increases in fecal boli. In addition, pharmacological studies further validate the EPM, with anxiolytic drugs such as benzodiazepines decreasing aversion to the open arms. The assessment of these measures in the successive alleys arena, along with a direct comparison of behaviour in the EPM and SAT, is required before the successive alleys test of trait anxiety is

validated for use in future research. Principle component analyses may further determine which of the measures on the ADT are most robust for detecting differences in trait-anxiety.

Additionally, further work needs to be done to refine the anticipatory paradigm in the combined ADT. An improvement on the protocol used in the current experiment would be to conduct habituation and training sessions prior to testing in the ADT. These sessions would be similar to those conducted by Barbano and Cador (2006); animals would be given a food-reward following time spent in the anticipatory arena. These sessions would occur in the absence of reward motivation, and animals would not be exposed to the successive alleys arena. Once a stable baseline of anticipatory activity had been established, testing in the combined ADT would begin; these testing sessions would occur as outlined in experiment two. By establishing differences in anticipatory pleasure prior to testing, we can then directly examine how differences in anticipatory pleasure influence behaviour once factors of reward motivation are introduced.

Summary and Conclusions

The aim of the current thesis was to investigate the role of increased extracellular serotonin levels during development on anxiety and depression phenotypes in adult rats. Short-term blockade of 5-HTT during development was achieved by administering the SSRI fluoxetine to heterozygous SERT-KO dams during the prenatal and early postnatal period. In experiment one, we developed a method of voluntary oral dosing in pregnant rat dams. In experiment two, heterozygous and homozygous SERT-KO and SERT-WT offspring from treated and untreated litters were tested in adulthood for anxiety behaviours and for deficits in anticipatory pleasure, as measured by the novel ADT. Fluoxetine treatment did not show any toxicity effects as measured by litter size and pre-weaning deaths. Fluoxetine also showed no influence on maternal-pup

interactions. Behaviour analysis of offspring revealed no significant genotype differences on any of the key measures, indicating that anxiety and depression phenotypes were not present in any of the groups. The lack of genotype-differences is at odds with many previous studies and meant that in the current study further modulation of behaviour as a result of fluoxetine treatment could not be detected.

The use of the novel ADT in the current thesis limits the translatability of results to the broader literature. The failure to find significance could be due to fundamental methodological flaws in the design of the experiment. It may be that with refinement of the ADT and validation of these tests, the novel ADT could be an important contribution to preclinical research methods. However, due to these limitations, the contribution of altered extracellular serotonin levels to the development of anxiety and depression remains to be established.

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Appendix A

The means ± standard error of the means of each individual variable obtained in the successive alleys test. Results are displayed for each experimental group, for each day of testing.

			Day 1			
	Wildtype		Heterozygous		Homozygous	
Behaviour	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
A1 duration (s)	113.92 ± 11.13	127.93 ± 7.59	110.63 ± 8.58	123.29 ± 8.47	131.34 ± 13.14	140.91 ± 9.61
A2 duration (s)	97.09 ± 7.37	100.02 ± 6.04	102.19 ± 8.37	96.27 ± 5.83	86.11 ± 11.78	75.02 ± 6.16
A2 entries (n)	8.7 ± 1.37	6.25 ± 0.48	6.42 ± 0.48	7.75 ± 1.09	7.44 ± 1.48	6.75 ± 0.84
A2 latency (s)	10.35 ± 2.34	14.54 ± 3.33	12.76 ± 2.68	11.57 ± 3.08	13.97 ± 4.53	18.55 ± 4.92
A3 duration (s)	23.67 ± 8.16	11.19 ± 3.59	21.88 ± 3.78	17.1 ± 5.08	19.86 ± 5.99	16.72 ± 4.88
A3 entries (n)	2.3 ± 0.75	2.33 ± 0.96	2.92 ± 0.62	3.17 ± 0.98	1.67 ± 0.5	1.92 ± 0.6
A3 latency (s)	132.74 ± 32.95	168.51 ± 23.47	102.13 ± 19.75	117.54 ± 24.94	141.07 ± 27.94	161.02 ± 22.81
A4 duration (s)	5.34 ± 2.84	0.86 ± 0.54	5.32 ± 2.12	3.34 ± 2.4	2.69 ± 1.34	0.73 ± 0.58
A4 entries (n)	0.8 ± 0.39	0.42 ± 0.26	0.83 ± 0.3	0.33 ± 0.26	1.22 ± 0.6	0.17 ± 0.11
A4 latency (s)	185.78 ± 24.94	229.68 ± 7.43	192.64 ± 19.9	225.47 ± 11.87	191.37 ± 26.1	234.62 ± 4.22
Consumption latency (s)	166.4 ± 20.45	165.62 ± 22.6	185 ± 31	223.62 ± 24.24	193.4 ± 26.54	172.83 ± 24.62
Consumption score	2.7 ± 0.47	2.15 ± 0.36	2.17 ± 0.65	1.23 ± 0.47	3 ± 0.58	2.83 ± 0.75
			Day 2			
	Wildtype		Heterozygous		Homozygous	
	Control	Fluoxetine	Control		Control	Fluoxetine
A1 duration (s)	114.9 ± 6.88	114.45 ± 11.79	109.28 ± 9.38	97.71 ± 9.54	137.56 ± 13.45	127.89 ± 12.41
A2 duration (s)	101.89 ± 7.08	101.96 ± 8.74	103.95 ± 7.67	102.7 ± 7.14	76.95 ± 8.56	84.12 ± 8.84
A2 entries (n)	7.1 ± 1.3	6.92 ± 0.58	6.75 ± 0.71	6.85 ± 0.64	5.6 ± 0.73	8.33 ± 1.29
A2 latency (s)	17.12 ± 11.39	8.21 ± 2.17	10.68 ± 3.19	8.07 ± 2.61	19.74 ± 11.27	18.1 ± 6.91
A3 duration (s)	21.25 ± 5.58	16.74 ± 5.03	19.83 ± 3.84	25.53 ± 3.91	21.33 ± 4.81	20.1 ± 4.18
A3 entries (n)	3.9 ± 1.12	2.46 ± 0.73	3.08 ± 0.51	4.85 ± 1.1	3.9 ± 1.1	3.25 ± 1.05
A3 latency (s)	113.55 ± 23.49	152.43 ± 22.08	71.68 ± 10.77	96.44 ± 14.44	141.62 ± 21.69	127.15 ± 15.54
A4 duration (s)	1.95 ± 1.29	6.33 ± 3.63	6.94 ± 3.26	10.67 ± 3.07	3.97 ± 2.15	7.86 ± 3.63
A4 entries (n)	0.6 ± 0.22	0.54 ± 0.24	1.08 ± 0.31	2.23 ± 0.54	1.4 ± 0.78	1 ± 0.33
A4 latency (s)	192.81 ± 20.52	216.84 ± 10.65	185.31 ± 19.53	165.04 ± 19.55	205.29 ± 13.53	205.28 ± 14.19
Consumption latency (s)	55.7 ± 14.93	102.62 ± 28.95	64.5 ± 24.46	135.54 ± 30.57	46.2 ± 15.77	79 ± 24.93
Consumption score	3.7 ± 0.45	2.85 ± 0.58	3.83 ± 0.61	2.46 ± 0.49	5.3 ± 0.5	3 ± 0.44
			Day 3			
	Wildtype		Heterozygous		Homozygous	
	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
A1 duration (s)	108.04 ± 9.37	105.27 ± 7.27	104.2 ± 12.72	97.14 ± 12.12	115.52 ± 13.7	110.83 ± 9.71
A2 duration (s)	96.53 ± 6.37	93.85 ± 3.89	92.13 ± 6.93	106.06 ± 7.78	86.96 ± 9.21	92.56 ± 5.38
A2 entries (n)	7.3 ± 0.65	8 ± 0.55	7.5 ± 0.9	9.46 ± 0.88	8.8 ± 1.06	9.58 ± 1.22
A2 latency (s)	4.86 ± 1.05	8.76 ± 3.59	8.29 ± 3.95	9.51 ± 3.04	5.49 ± 2.01	6.14 ± 2.48
A3 duration (s)	29.63 ± 5.38	27.64 ± 4.47	30.47 ± 5.54	24.52 ± 5.02	24.14 ± 4.16	26.96 ± 6.77
A3 entries (n)	3.6 ± 0.56	3.46 ± 0.74	3.58 ± 0.47	5.54 ± 1.14	3.3 ± 0.62	5.5 ± 1.32
A3 latency (s)	68.74 ± 22.23	99 ± 16.6	94.76 ± 11.48	91.45 ± 19.66	51.34 ± 11.7	83.03 ± 22.08
A4 duration (s)	5.82 ± 4.81	13.25 ± 5.12	12.63 ± 4.62	11.71 ± 3.58	13.29 ± 4.98	9.57 ± 2.9
A4 entries (n)	0.7 ± 0.26	1.54 ± 0.43	1.25 ± 0.35	1.46 ± 0.43	2 ± 0.56	1.83 ± 0.59
A4 latency (s)	203.42 ± 18.64	169.49 ± 21.18	162.35 ± 20.42	149.71 ± 21.33	162.52 ± 20.44	157.17 ± 23.4
Consumption latency (s)	27.2 ± 4.33	88.92 ± 26.96	45.17 ± 10.13	95.15 ± 26.82	28.7 ± 6.17	38.67 ± 8.14
Consumption score	5.1 ± 0.72	4 ± 0.74	4.83 ± 0.52	3.92 ± 0.75	5.7 ± 0.73	5.08 ± 0.71

	Day 4					
	Wildtype		Heterozygous		Homozygous	
	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
A1 duration (s)	81.99 ± 9.75	90.69 ± 9.07	86.59 ± 10.12	86.75 ± 9.65	102.3 ± 8.29	105.59 ± 8.17
A2 duration (s)	88.18 ± 8.35	79.1 ± 4.13	85.8 ± 6.14	104.37 ± 10.1	88.1 ± 4.59	81.91 ± 7.4
A2 entries (n)	8.9 ± 1.19	8.92 ± 0.75	8.92 ± 0.9	9.08 ± 1.31	8.4 ± 0.54	10.2 ± 0.71
A2 latency (s)	8.05 ± 2.01	9.08 ± 2	7.16 ± 2.29	5.1 ± 1.3	12.53 ± 4.66	9.08 ± 2.42
A3 duration (s)	34.6 ± 5.34	34.59 ± 2.92	33.58 ± 3.55	31.28 ± 5.14	30.48 ± 3.91	28.14 ± 3.01
A3 entries (n)	5.5 ± 1.12	6.31 ± 1.36	5.33 ± 0.57	5 ± 0.87	4.2 ± 0.57	6.6 ± 1.18
A3 latency (s)	53.33 ± 10.36	55.64 ± 7.69	42.21 ± 10.89	51.38 ± 7.28	56.51 ± 10.25	44.29 ± 8.16
A4 duration (s)	35.24 ± 8.82	31.18 ± 5.84	34.04 ± 8.27	17.34 ± 5.16	19.12 ± 6.27	19.61 ± 4.67
A4 entries (n)	2.9 ± 0.64	3.38 ± 0.75	2.75 ± 0.62	2.23 ± 0.47	1.8 ± 0.36	3.8 ± 0.89
A4 latency (s)	143.24 ± 21.17	113.73 ± 16.19	122 ± 19.72	152.07 ± 20.04	138.99 ± 21.99	127.3 ± 20.94
Consumption latency (s)	15.9 ± 3.1	47.08 ± 21.56	23.33 ± 5.87	73.23 ± 25.17	18.3 ± 4.64	18.25 ± 4.81
Consumption score	6.3 ± 0.75	5 ± 0.79	4.92 ± 0.48	4.69 ± 0.58	5.6 ± 0.56	5.58 ± 0.7
	Day 5					
	Wildtype		Heterozygous		Homozygous	
	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
A1 duration (s)	105.01 ± 14.56	81.6 ± 9.82	98.62 ± 11.07	89.86 ± 11.15	82.21 ± 8.78	81.1 ± 7.84
A2 duration (s)	75.61 ± 7.12	102 ± 6.74	78.18 ± 6.67	100.36 ± 6.29	81.58 ± 10.04	85.21 ± 5.66
A2 entries (n)	7.1 ± 0.69	8.15 ± 0.58	7.73 ± 1.1	9.83 ± 0.75	9.7 ± 1.34	11.42 ± 0.97
A2 latency (s)	16.62 ± 3.33	9.72 ± 2.73	8.21 ± 2.56	6.64 ± 2.39	11.98 ± 4.33	5.31 ± 1.31
A3 duration (s)	30.18 ± 4.09	32.68 ± 4.66	37.69 ± 5.73	25.61 ± 3.71	37.5 ± 3.9	35.71 ± 5.22
A3 entries (n)	4.9 ± 1.19	5.69 ± 1.02	5.27 ± 0.79	5.42 ± 1.06	6.7 ± 1.04	8.92 ± 1.35
A3 latency (s)	56.64 ± 3.53	45.99 ± 8.75	59.42 ± 11.26	47.49 ± 8.03	47.06 ± 5.71	31.17 ± 6.15
A4 duration (s)	28.55 ± 9.06	20.81 ± 5.46	25.52 ± 6.42	20.89 ± 4.85	38.71 ± 5.88	35.98 ± 5.26
A4 entries (n)	2.7 ± 0.58	2.85 ± 0.64	2.27 ± 0.51	2.83 ± 0.71	4.2 ± 0.57	3.42 ± 0.26
A4 latency (s)	103.98 ± 16.68	107.57 ± 22.5	123.4 ± 17.81	113.14 ± 20.22	99.92 ± 14.49	77.17 ± 11.18
Consumption latency (s)	10 ± 1.92	67 ± 27.99	17.92 ± 3.94	29 ± 8.66	11 ± 1.98	16.33 ± 3.41
Consumption score	6.4 ± 0.87	4.77 ± 0.61	5.5 ± 0.54	4.85 ± 0.85	5.7 ± 0.68	5.25 ± 0.33
	Day 6					
	Wildtype		Heterozygous		Homozygous	
	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
A1 duration (s)	90.5 ± 13.9	79.38 ± 12.22	84.61 ± 9.99	78.16 ± 12.57	88.92 ± 10.71	92.74 ± 11.53
A2 duration (s)	76.28 ± 5.97	98.49 ± 10.38	75.67 ± 5.34	85.55 ± 7.62	80.43 ± 8.42	76.22 ± 5.07
A2 entries (n)	7.3 ± 0.8	9.62 ± 1.03	6.83 ± 0.3	8.23 ± 0.94	10 ± 1.61	12.25 ± 1.29
A2 latency (s)	7.29 ± 2.83	7.97 ± 2.7	12.09 ± 2.37	6.87 ± 2.82	11.62 ± 3.79	7.84 ± 1.7
A3 duration (s)	36.09 ± 4.76	33.37 ± 5.4	34.22 ± 4.3	35.62 ± 4.75	36.58 ± 6	42.31 ± 5.71
A3 entries (n)	5.1 ± 0.77	5.77 ± 0.74	7.5 ± 1.42	6.85 ± 1.41	7.4 ± 1.03	10.25 ± 1.55
A3 latency (s)	37.62 ± 4.97	45.51 ± 7.54	43.58 ± 6.29	40.56 ± 5.9	36.46 ± 6.78	37.05 ± 4.97
A4 duration (s)	37.15 ± 9.78	27.21 ± 5.5	43.95 ± 6.82	40.11 ± 5.19	34.07 ± 5.2	27.86 ± 4.94
A4 entries (n)	2.5 ± 0.4	3 ± 0.73	3.75 ± 0.33	3.85 ± 0.77	4.9 ± 1.12	4 ± 0.56
A4 latency (s)	88.65 ± 14.65	133.08 ± 21.02	89.46 ± 14.16	98.12 ± 15.3	93.51 ± 11.32	82.81 ± 15.82
Consumption latency (s)	14.1 ± 3.81	18.77 ± 5.14	11.17 ± 3.36	26.92 ± 13.26	10 ± 2.16	10.08 ± 2.1
Consumption score	6.2 ± 0.73	6.08 ± 0.6	5.75 ± 0.54	5.31 ± 0.33	5.5 ± 0.48	5.5 ± 0.42
	Day 7					
	Wildtype		Heterozygous		Homozygous	
	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
A1 duration (s)	91.87 ± 15.9	73.41 ± 9.8	82.17 ± 9.13	73.67 ± 10.31	80.19 ± 9.91	86.47 ± 9.5
A2 duration (s)	78.59 ± 7.55	84.9 ± 11.31	83.29 ± 5.93	86.97 ± 5.44	82.22 ± 7.48	91.06 ± 5.13
A2 entries (n)	9 ± 1.07	9.08 ± 0.71	8.83 ± 0.82	9.08 ± 1.26	8.6 ± 0.83	13.17 ± 1.17
A2 latency (s)	12.76 ± 4.38	4.88 ± 1.45	7.82 ± 1.89	5.35 ± 2.79	3.79 ± 1.59	13.97 ± 2.6
A3 duration (s)	27.24 ± 5.65	34.12 ± 5.37	35.58 ± 5.44	41.5 ± 5.83	34.29 ± 6.93	34.39 ± 4.78
A3 entries (n)	5.2 ± 0.68	6.92 ± 1.49	5.67 ± 0.91	7.62 ± 1.12	6.8 ± 0.8	8.67 ± 1
A3 latency (s)	37.65 ± 2.64	38.93 ± 4.33	34.89 ± 6.38	30.28 ± 4.33	24 ± 4.35	32.87 ± 5.38
A4 duration (s)	42.32 ± 7.24	47.01 ± 7.63	38.96 ± 5.48	37.76 ± 5.19	39.98 ± 5.32	26.18 ± 4.13
A4 entries (n)	3.7 ± 0.78	3.62 ± 0.5	3.08 ± 0.29	3.62 ± 0.5	3.8 ± 0.42	4.75 ± 0.92
A4 latency (s)	77.57 ± 18.33	90.12 ± 17.18	74.4 ± 8.86	79.02 ± 14.89	62.3 ± 8.32	60.41 ± 9.15
Consumption latency (s)	8.2 ± 2.15	18.85 ± 6.05	8.75 ± 1.99	31.85 ± 17.74	9.3 ± 2.36	6.25 ± 1.19
Consumption score	5.8 ± 0.68	5.23 ± 0.32	5.25 ± 0.48	4.85 ± 0.48	5.5 ± 0.45	5.5 ± 0.51