NEUROGENIC PLASTICITY IN A SEROTONIN TRANSPORTER KNOCKOUT MODEL: EFFECTS OF MATERNAL AND OFFSPRING GENOTYPE

BY

SARAH TAPNIKAR

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Table of Contents

Abstract

Major depressive disorder (MDD) is debilitating mental disorder that is increasing in prevalence. Many theories have tried to explain the aetiology of depression including the classic monoamine deficiency hypothesis and the newer neurogenic hypothesis. The finding that selective serotonin transporter inhibitors (SSRIs) work by increasing extracellular serotonin levels in the brain and have antidepressant effects has formed the basis of the most widely accepted theory of depression, the monoamine hypothesis. However, a genetic reduction in human and animal serotonin reuptake transporters, which also increases extracellular serotonin, is associated with depressive symptomology. This paradox is not explained by the monoamine hypothesis. The key difference between these two scenarios is that genetically induced increases in serotonin occur from development onward, while SSRIs increase serotonin only in adulthood. Furthermore, SSRIs typically take several weeks to confer a therapeutic effect. This finding has led to the hypothesis that, rather than acute monoamine-increasing effects, it is the downstream effects of such increases on neurogenesis and neural plasticity which confer antidepressant effects. To further elucidate the neurobiology of depression, this study sought to examine the effects of genetically increasing serotonin on early postnatal neurogenesis in a serotonin knockout rat model using BrdU immunohistochemistry. We examined both the offspring and maternal genotype effects. We found that SERT-/- offspring had the highest levels of neurogenesis compared with SERT+/- and SERT+/+ at postnatal day 7. In addition we found a maternal genotype effect with SERT+/+ offspring born and reared by SERT+/- mothers having lower neurogenesis compared to SERT+/+ offspring from SERT+/+ mothers. The potential effects of maternal caregiving, neuroplasticity in altered mood and stress responses and the role of 5-HT receptors are discussed.

CHAPTER ONE

SEROTONIN TRANSPORTER GENOTYPES' EFFECTS ON NEUROGENESIS

Introduction

Depression has become a forerunner in the ailments impacting large amounts of people today. It is a debilitating mental disorder characterised predominantly by a heterogenous spectrum of symptoms including pathologically depressed mood and loss of pleasure, as well as combinations of decreased energy levels, changes in appetite or weight, poor concentration, feelings of guilt or low self-worth and sleep disturbances (American Psychiatric Association [APA], 2013). It is highly prevalent, with the World Health Organization estimating that over 300 million people suffer from depression and that this rate is increasing globally (World Health Organization [WHO], 2017).

The lifetime prevalence of depression varies across countries with the rate in New Zealand at 1.5 cases per 100 adults and a mean age of onset of 18.2 years (Simon, Goldberg, Kroff, Üstün, 2002; Weissman et al., 1996). The diagnostic and statistical manual of mental disorders (DSM) puts the lifetime risk of major depressive disorder at 10-25% for women and 5-12% for men while the point prevalence is between 5-9% in women and 2-3% in men (APA, 2013). The lifetime prevalence rate appears to be on the rise and occurring earlier in life (Joyce, Oakley-Browne, Wells, Bushnell & Hornblow, 1990). In many countries, mood disorders are the second most common type of mental illness after anxiety disorders (Demyttenaere et al., 2004). The prevalence of depression in adolescents and young adults has increased recently (Mojtabai, Olfson & Han, 2016) and the total estimated number of people living with depression increased by 18.4% from 2005 to 2015 (Vos et al., 2016).

The gravest consequence of depression is unquestionably suicide. Overall, depression increases the risk of suicide ideation and attempt, as well as death by suicide (Ribeiro, Huang, Fox & Franklin, 2018). Studies find mortality rates of suicide to be up to 27-fold greater for those with affective disorders more generally, as well as depression specifically, when compared with the general population (Bostwick & Pankratz, 2000; Melchior et al., 2010; Ösby, Brandt, Correia, Ekbom & Sparén, 2001). The mortality risk associated with depression also increases with the severity of symptoms (Almeida, Alfonso, Hankey & Flicker, 2010). Antidepressant use is also associated with a 30% higher risk of mortality and this association is independent of the presence of clinical depression (Almeida et al., 2010). Suicide accounted for 1.5% of all deaths in 2015 bringing suicide into the top 20 leading causes of death (although many more attempts are made; WHO, 2017).

Higher levels of impairment are reported for mental disorders (particularly anxiety, mood and impulse control disorders) than other chronic medical conditions, with severe functional impairment reported by 42% of those with mental disorders and 24% with chronic medical disorders (Druss et al., 2009). While prevalence rates differ drastically across countries, depression is universally associated with disability (Simon et al., 2002). However, treatment is provided for a significantly lower proportion of mental disorders than medical conditions (Druss et al., 2009). Psychosocial and relationship impairment, in particular, is most commonly associated with mood disorders (compared with chronic medical conditions that are associated more with work and home functioning; Druss et al., 2009; Leader & Klein, 1996; Lee & Murray, 1988) and is associated with depressive symptom severity (Judd et al., 2000). While treatment improves functional impairment, some level of disability appears to persist even after remission (Kennedy, Foy, Sherazi, McDonough & McKeon, 2007; Mintz, Mintz, Arruda & Hwang., 1992). Longitudinal studies have also found that up to one-third of depressed patients die or suffer chronic incapacitation (Brodaty, Luscombe, Peisah, Astney & Andrews, 2001; Kiloh, Andrews & Neilson, 1988; Lee & Murray, 1988; Surtees & Barkley, 1994). Chronic depression is more common than initially thought with up to 21% of depressed patients suffering chronically (Gilmer et al., 2005), and Kocsis et al. (2008) found that the mean duration of chronic depression was 20 years. Chronic depressive episodes were also associated with greater illness burden, comorbidity and severity (Gilmer et al., 2005; Kessler, Zhao, Blazer & Swartz, 1997). Depression is a recurrent illness as longitudinal studies reveal that up to 86% of patients in remission undergo relapse (Brodaty et al., 2001). Older studies found even higher relapse rates of up to 95% (Kiloh et al., 1988; Lee & Murray, 1988; Mueller et al., 1999).

In terms of work-related disability, Broadhead, Blazer, George and Tse (1990) report that depressed individuals have up to 4.78 times higher risk of absenteeism and daily task impairment than asymptomatic individuals. Depressive symptomology is also associated with increased unemployment and decreased annual salary with studies finding annual income losses due to absenteeism and presenteeism of $4400 and $9600 USD for depressed and bipolar individuals respectively (Kessler et al., 2006; Whooley et al., 2002). Loss projections in the US estimate a $36.6 billion salary-equivalent lost productivity per year due to major depressive disorder (Kessler et al., 2006). Clearly depression is a disabling condition causing significant impairments in an individual's social functioning, work and for the wider society (Briley & Moret, 2010).

The Monoamine Hypothesis

Understandably, the neurobiological aetiology of depression is of great interest. The monoamine hypothesis is one of the most widely accepted theories of depression and has informed the use of most currently-used antidepressant medications (Boku, Nakagawa, Toda & Hishimoto, 2018).

The monoamine deficiency hypothesis of depression (aka the biogenic amine hypothesis) proposes that depressive and anxious symptoms arise from insufficient levels of the monoamine neurotransmitters serotonin (5-HT), norepinephrine (NE) and dopamine (DA; Massart, Mongeau & Lanfumey, 2012; Tran, Bymaster, McNamara & Potter, 2003). It arose from studies showing that the depletion of monoamines results in an induction of depressive symptomology while antidepressant drugs, such as selective serotonin reuptake inhibitors (SSRIs) and monoamine oxidase inhibitors (MAOIs), increase monoamine levels and alleviate depression (Belmaker & Agam, 2008; Coppen, 1967; Gingrich & Hen, 2001; Hamon & Blier, 2013; Holschneider et al., 2001; Salomon et al., 2003; Ruhé et al., 2000; Wong & Licinio, 2001). The SSRIs mode of action is a rapid and immediate increase in synaptic 5-HT availability while chronic treatment downregulates the serotonin transporter (SERT, aka 5-HTT) and the autoreceptor 5-HT1A and heteroreceptor 5-HT1B, although other 5-HT receptors are also involved in the antidepressant effects of SSRIs (Blier & Ward, 2003; Cryan, Valentino & Lucki, 2005; Descarries & Riad, 2012; Fabre et al., 2000; Gray et al., 2013; Hervás et al., 2001; Le Poul et al., 1995, 2000; Lesch et al., 1993; Moret & Briley, 2000; Nishi & Azmitia, 1999). There is also an association between reduced (or a complete lack of) 5-HT1A and 5-HT1B's inhibitory regulation of serotonergic signalling with depressive outcomes which lends further support for the notion of a group which is genetically vulnerable to depression due to a genetic dysregulation of their serotonergic system (Albert & Lemonde, 2004; Boldrini, Underwood, Mann & Arrango, 2008; Garcia-Garcia et al., 2014; Lemonde et al., 2003; López-Figueroa et al., 2004; Richardson-Jones et al., 2011; Xia et al., 2012; Zhuang et al., 1999).

Overall, these studies suggest an abnormality of serotonergic function in depression where a deficit leads to depression for which antidepressants can help in compensating (Neumeister, 2003).

One aspect of the monoamine hypothesis that remains unexplained is that, while antidepressants have an acute biochemical action in increasing monoamine levels, there is a delay between this and their clinical/ therapeutic effect of approximately four weeks or more (Hindmarch, 2001; Sapolsky, 2004; Wong & Licinio, 2001). Moreover, many depressed individuals are not responsive to antidepressants (Serretti et al., 2004). Thus, it has been argued that secondary, adaptive changes in the brain are underlying the clinical improvements rather than the primary/acute serotonin enhancing effect of the drug (Hindmarch, 2001). The focus of current research is investigating the underlying molecular and cellular mechanisms that occur due to downstream changes in the brain after chronic antidepressant treatment.

As 5-HT has also been shown to be a potent regulatory growth signal during neurodevelopment and facilitates neurogenesis in adulthood (Vitalis & Panavelas, 2003), one proposed downstream antidepressant mechanism is neurogenesis and changes in neuroplasticity. A multitude of studies show that SSRIs facilitate neurogenesis, increase neurotrophic factors' expression, and alter neural circuitry, mainly via an increase in extracellular 5-HT levels (Cai et al., 2013; Surget et al., 2008).

Another potential criticism of the monoamine hypothesis which is in sharp contrast to the beneficial effects of SSRIs in adulthood is that animal and human research also indicates that early developmental increases in 5-HT (either by pharmacological or genetic means) enhances anxiety, depression and emotional problems in later life (Ansorge, Morelli & Gingrich, 2008; Lira et al., 2003; Oberlander, Gingrich & Ansorge, 2009, Oberlander et al., 2010). These tenets of the monoamine hypothesis illustrate that the effects of 5-HT are multifaceted and need further study.

The SLC6A4 serotonin transporter gene

Early heritability studies have confirmed a genetic component of depression (Hamet & Tremblay, 2005; Shadrina et al., 2018). Monoaminergic transporters have been of particular interest because they are sites of action for most antidepressants as well as for certain psychostimulants (Borowsky & Hoffman, 1995). The serotonin transporter has a critical role in serotonergic neurotransmission and homeostasis as it is responsible for the active transport (sodium-dependent reuptake) of 5-HT back into the presynaptic neuron to reduce synaptic concentrations of 5-HT and terminate post-synaptic transmission (Murphy et al., 2004; Rudnick, 1977; Rudnick & Clark, 1993). Research into the genetic determinants of

depression has since focused heavily on a serotonin transporter polymorphism's (5-HTTLPR) contribution to the aetiology for depression (Cervilla et al., 2006; Furlong et al., 1998; Gutiérrez et al., 1998).

Within the human SERT gene's (termed solute carrier 6a4, *SLC6A4*) promoter region is a 5-HTT-linked polymorphic region (5-HTTLPR) which has an insertion/deletion variable nucleotide repeat (VNTR) polymorphism with a short (*s*) allele consisting of a ~44 base pair (bp) deletion of repeat elements 6-8 from bp 1,212 to bp 1,255 (Heils et al., 1996; Kraft, Slager, McGrath & Hamilton, 2005; Lesch et al., 1996; Wendland, Martin, Kruse, Lesch & Murphy, 2006). Thus, the 5-HTTLPR consists of a length variation of a repetitive sequence containing 20—23-bp-long GC-rich segments with 14 repeats in the short allele and 16 in the long allele (Heils et al., 1996).

This is a functionally significant polymorphism as these two common alleles of the SERT gene promoter region polymorphism impact its transcriptional activity (Heils et al., 1996; Lesch et al., 1996). The long (*l*) allele confers threefold greater transcriptional activity than the short allele which has reduced *SLC6A4* mRNA production and SERT binding (Bradley, Dodelzon, Sandhu & Philibert, 2005; Heils et al., 1996; Lesch et al., 1996; Stoltenberg et al., 2002). In humans, lymphoblast cells homozygous for the *l* 5-HTTLPR variant produced 1.4 to 1.7 times more *SLC6A4* mRNA, had 30-40% more SERT proteins and had 1.9-2.2 times more 5-HT uptake than cells containing one or two copies of the *s* variant (Lesch et al., 1996). The *s* allele variant also has reduced enhancer/promoter repression compared to the *l* variant (Heils et al., 1996).

Importantly, this polymorphism has been found to be associated with neuropsychiatric disorders, in particular the *s* allele is associated with a greater risk of developing affective and mood disorders (Furlong et al., 1998; Gutiérrez et al., 1998) and psychological traits such as neuroticism (Lesch et al., 1996). Naturally, because of its crucial role in serotonergic homeostasis as well as being the site of action of many antidepressants, the *SLC6A4* gene is a candidate for mood disorder aetiology (Kelsoe et al., 1996). It is important to note, however, that in addition to the *l* and *s* 5-HTTLPR variants, there are many more *SLC6A4* polymorphisms (both in the 5-HTTLPR itself, as well as in other regions of the gene). Several of these, which affect SERT levels, are also associated with affective disorders (Hu et al., 2005; Jarrett et al., 2007; Kraft et al., 2005; Ogilvie et al., 1996; Wendland et al., 2006).

Mounting evidence from studies of depressed individuals reveal a multitude of serotonergic system changes that occur in comparison to healthy controls (D'haenen, 2001). For example, studies have demonstrated decreased SERT binding sites in the brain of depressed individuals (Ellis & Salmond, 1994; Malison et al., 1998; Mann et al., 2000; Owens & Nemroff, 1994), reduced concentrations of the major 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) in depressed individuals and suicidal patients (Gibbons & Davis, 1986; Roy, Jong & Linnoila, 1989), decreased tryptophan concentrations in depressed patients (Quintana, 1992) which also led to rapid relapses/ return of symptoms in depressed patients (Morris et al., 1999; Neumeister et al., 2004).

Research into the 5-HTTLPR polymorphism's significance for depression, however, has been less consistent. Initially several studies found that the *s* allele is associated with a higher risk for developing affective disorders, particularly depression (Cervilla et al., 2006; Furlong et al., 1998; Gutiérrez et al., 1998). Cervilla et al. (2006) found that the association between the *s/s* genotype and depressive outcomes was stronger for more severe depression. The 5-HTTLPR *s* allele was also found to occur more in patients with Seasonal Affective Disorder (SAD) than controls (Rosenthal et al., 1998). SAD patients were less likely to have the *l/l* genotype but those that did had lower seasonality scores than patients with *s/l* or *s/s* genotypes (Rosenthal et al., 1998). The 5-HTTLPR *s/s* genotype is also associated with a poorer response to SSRI treatment than *s/l* and *l/l* genotypes, with the *s/s* genotype being associated with greater difficulties reaching remission and a lower response rate (Serretti et al., 2004, Serretti, Kato, De Ronchi & Kinoshita, 2007). On the other hand, there have been recent studies and meta-analyses that have been unable to confirm the association of the *s* allele with depression. Most recently a large population-based study involving over 100,000 participants found no evidence of a linkage between the *s* allele of the 5-HTTLPR and depression (Border et al., 2019). While all these data strongly implicate the serotonergic system in the pathophysiology of depression, its exact role and the genetic contribution of *SLC6A4* in particular remain to be fully elucidated.

The serotonin transporter knockout rat model of depression

Investigating the role of specific genes, such as the *SLC6A4* in humans has proven incredibly difficult, partly because of the strong genetic heterogeneity between individuals, even within the *SLC6A4* gene itself, as mentioned above and partly because of the

heterogeneity of depression itself. As a result, a direct link between a genetic variation in the *SLC6A4* gene (or any other gene for that matter) and depression has so far not been established in studies in patients. Animal research, on the other hand, allows us to selectively manipulate one gene in a predictable manner, to investigate the causal relationship between that gene and behavioural changes.

A genetic alteration in the rat *Slc6a4* gene was achieved by ENU-mutagenesis which induced a C to A transversion at position 3924 in the *Slc6a4* gene resulting in a premature stop codon in the third exon (Homberg et al., 2007; Smits et al., 2006). This produced viable animals in which the *Slc6a4* mRNA and functional protein are completely absent (referred to as SERT-/- rats) or reduced by about 50% (SERT+/- rats; Homberg et al., 2007). The heterozygous (SERT+/-) animals demonstrate a similar loss-of-function in SERT activity as the human 5-HTTLPR *s* allele and are therefore used as models for this condition (Kim et al., 2005; Lesch et al., 1996; Montañez, Owens, Gould, Murphy & Daws et al., 2003).

In the homozygous (SERT-/-) rats the maximum rate of 5-HT reuptake is reduced by approximately 72.2% while for SERT +/- rats this rate is reduced by 13.4%. The less than 100% reduction in SERT-/- rats is thought to be due to residual uptake by the dopamine and/or noradrenaline transporter (Homberg et al., 2007). Reducing or eliminating SERT activity results in an increase in extracellular levels of 5-HT and basal levels of extracellular 5-HT increase nine-fold in the hippocampus of SERT -/- rats (Homberg et al., 2007).

The SERT KO rat is therefore suitable as a model for investigating the role of the *Slc6a4* in depression and anxiety. SERT knockout mice also show similar depressive and anxious behaviours as humans (Holmes, Murphy & Crawley, 2003). Similarly, behavioural testing revealed that SERT-/- rats spent less time in the centre of an open field and on the open arm of the elevated plus maze, had higher latencies before starting to feed during novelty suppressed feeding, had higher latencies to feed in a bright novel arena and had higher latency in emerging from their home cages, were less mobile in the forced swim test and consumed less sucrose compared to SERT+/+ rats (Kalueff, Olivier, Nonkes & Homberg, 2010; Olivier et al., 2008). These behaviours are all indicative of greater anxiety and depression-like symptoms in the SERT-/- rats (Kalueff et al., 2010; Olivier et al., 2008). SERT -/- and SERT+/+ rats did not, however, differ in the number of immune-positive 5-HT neurons in the dorsal raphe nuclei (DRN), similarly, post-mortem studies of humans suffering

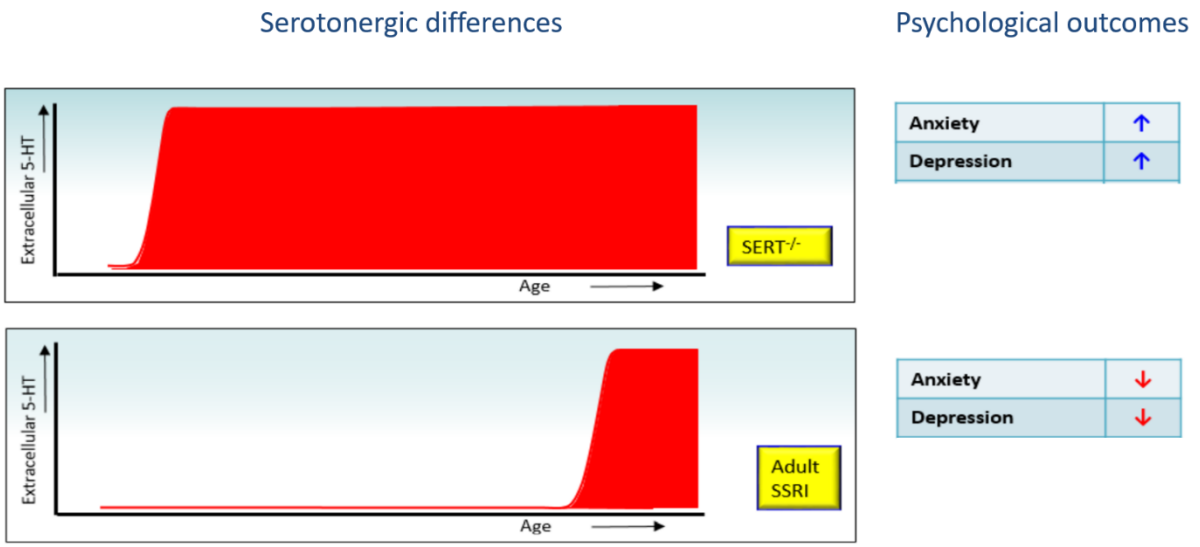
from depression did not show reduced serotonergic neurons in the DRN (Olivier et al., 2008). Unfortunately, very little research has been done on SERT+/- rats so far. It has been questioned whether an absence of SERT leads to neurodevelopmental abnormalities and cortical disorganisation and whether this is an explanation for why genetic inactivation of the SERT induces anxiety and depression-like symptoms (Olivier et al., 2008).

The Serotonin Paradox

Putting all this research together, the role of serotonin presents us with a paradox. It has been shown that the most common medications given to treat depression and anxiety are selective serotonin reuptake inhibitors (SSRIs) that work by selectively inhibiting the serotonin transporter function thereby increasing extracellular levels of serotonin and alleviating the depressive or anxiolytic symptoms (Homberg et al., 2010). However, a genetic mutation of the *SLC6A4* gene promoter region (5-HTTLPR) results in an allele (*s* allele) which similarly produces increased extracellular 5-HT levels through reduced SERT functioning (Heils et al., 1996) and is also linked to depressive and anxiolytic symptomology (Lesch et al., 1996; Furlong et al., 1998). Moreover, an increase in depression- and anxiety-like behaviours and emotional problems in later life has been reported in SERT knockout rodent models and after early exposure to SSRIs (Kalueff et al., 2010; Olivier et al., 2008). Thus, a major shortcoming of the widely accepted monoamine hypothesis of depression is that it does not account for this serotonin paradox. See figure 1.

Figure 1

The serotonin paradox



Note. A visualisation showing the opposing serotonergic differences across lifetime and the corresponding anxiolytic and depressive risk outcomes between individuals lacking the serotonin trasnporter and adult SSRI use.

One apparent difference between these two scenarios is that extracellular 5-HT is chronically increased from early development onwards in *s* allele carriers and SERT knockout models, while SSRIs only increase 5-HT in adulthood. This leads us to speculate that early exposure to increased 5-HT may affect brain development and result in depressive symptomology through altered neurocircuitry while individuals who develop depression in later life without this genetic background may have a reduction in their monoamines via mechanisms involving experience-induced changes to circuitry such as those associated with stress (Czéh et al., 2001; Djordjevic et al., 2012; Flugge, 1995; Manji, Drevets & Charney, 2001; McKittrick, Blanchard, Blanchard, McEwen & Sakai, 1995; McKittrick et al., 2000; Savitz, Lucki & Drevets, 2009; Watanabe, Sakai, McEwen & Mendelson, 1993). Indeed, it has been shown that depressed individuals do display altered neural connectivity (Greicius et al., 2007; Kaiser, Andrews-Hanna, Wager & Pizzagalli, 2015; Perlman et al., 2012; Zeng et al., 2012).

While 5-HT is known to be a mood-regulating neurotransmitter in the adult brain, it initially has a crucial role in neurodevelopment (Azmitia, 2001; Gaspar, Cases & Maroteaux, 2003). Ample evidence has shown that genetic and pharmacological disturbances in 5-HT signalling leads to the disruption of circuit formation, connectivity and altered cell morphology (Daubert & Caudron, 2010; Gaspar et al., 2003; Homberg et al., 2010; Vitalis et al., 2007). In their review of animal and human studies of developmental exposure to increased 5-HT via SSRIs, Homberg et al. (2010) report long term effects on neurodevelopment which can be different to SSRI effects in adulthood e.g. structural and functional changes in the somatosensory cortex wiring. Thus, the early appearance of 5-HT and its receptors during foetal development, as well its effects on neurodevelopment, suggests that 5-HT acts as a developmental signal before it functions as a neurotransmitter. These findings suggest that we could consider depression a problem of neuronal circuitry disturbances due to altered monoamine levels.

The neurodevelopmental role of 5-HT and SERT

5-HT is required for normal embryonic development (Buznikov, Lambert & Lauder, 2001; Côté et al., 2007; Whitaker-Azmitia, Druse, Walker & Lauder, 1995). 5-HT is one of the key regulators of neurogenesis, differentiation, survival, and neuronal migration, dendrite formation, synaptogenesis, axon branching, morphogenesis, apoptosis and is released from developing neurons before synapses are even formed (Azmitia, 2001; Brezun & Daszuta, 1999; 2000a, 2000b; Hansson, Mezey & Hoffman, 1999; Lauder, 1990; Levin, Buznikov & Lauder, 2006; Matsukawa, Nakadate, Ishihara & Okado, 2003; Vitalis et al., 2007; Vitalis & Parnavelas, 2003).

The serotonergic regulation of all these neurodevelopmental processes suggest that disturbances in early 5-HT levels will alter the connectivity of neural circuitry and effectively alter neuroplasticity. Such neurodevelopmental changes could be underlying emotional and behavioral impairments seen in *s* allele carriers of the 5-HTTLPR and in SERT knockout models. Humans, for example, undergo a period of high brain 5-HT synthesis during early childhood and disturbances in which are linked to neurological disorders such as autism (Chugani, 2002). SERT knock out rats also display altered raphe-prefrontal network formation compared with wild-types due to altered chemotrophic interactions between origin and targets of the 5-HT projection system (Witteveen et al., 2013).

The serotonergic system is an early forming component of the CNS (Aitken & Törk, 1988; Lauder & Bloom, 1974; Lauder, 1990; Olson & Seiger, 1972). Serotonergic neurons are seen starting to develop in the foetal rat brain at embryonic day 12-13 and 5-HT fibre outgrowth begins at E13 (Aitken & Törk, 1988; Hansson et al., 1999; Lauder & Bloom, 1974; Lauder, 1990; Lidov & Molliver, 1982; Olson & Seiger, 1972; Wallace & Lauder, 1983). In humans, 5-HT is detected as early as the 7th week of gestation (Olson et al., 1973). Moreover, 5-HT is not only involved in serotonergic system development but also in most non-serotonergic circuits (D'Amato et al., 1987; Daubert and Condron, 2010; Hansson et al., 1999; Lauder & Krebs, 1978; Lidov & Molliver, 1982; Sikich, Hickok & Todd, 1990; Vitalis & Parnavelas, 2003; Vitalis et al., 2007). Transient 5-HT expression is seen throughout entire brain/ in most brain structures including in non-serotonergic neuronal cells as early as E12 and occurs through uptake into these cells by plasma membrane transporters rather than synthesis (Cases et al., 1998; De Vitry et al., 1986). 5-HT also initiates and amplifies its own synthesis in embryonic cells (De Vitry et al., 1986).

SERT expression begins at E10-E12 in rodents, preceding synapse formation (Brüning, Liangos & Baumgarten, 1997; Hansson et al., 1999; Zhou, Sari & Zhang, 2000). The transient presence of *Slc6a4* mRNA in the sensory processing, affect (limbic system), neuroendocrine (hypothalamus and pituitary) and circadian rhythm systems during embryonic development also indicates a role for 5-HT during development on cells which respond, or will respond, to 5-HT in adulthood (Hansson et al., 1999; Lebrand et al., 1998). In mice, SERT is first expressed in serotonergic Raphe neurons at mid gestation (E11) then rapidly appears in non-serotonergic neurons and ends in the non-serotonergic neurons during the second postnatal week, which coincides with the maturation of these neural circuits (Homberg et al., 2010). These studies suggest that alterations in (early) 5-HT levels may have far reaching consequences, not just for the serotonergic system but beyond that for other neurotransmitters as well.

Maternal 5-HT drives embryonic morphogenesis before the appearance of foetal serotonergic neurons and foetal tryptophan hydroxylase expression (Côté et al., 2007). This suggests that (genetic) changes in maternal 5-HT can have long-lasting consequences for the development of the offspring. Inhibition of embryonic 5-HT synthesis leads to a delayed onset of differentiation of 5-HT target cells (Lauder, 1990; Lauder & Krebs, 1978).

Axonal development overlaps with serotonergic neuron development (Lidov & Molliver, 1982; Seiger & Olson, 1973). SERT is expressed in axons, dendrites and growth tips of embryonic neurons including transient expression in non-serotonergic neurons and 5-HT regulates axon guidance cues (e.g., Netrin-1; Bonnin et al., 2007; Narboux-Nême et al., 2008; Zhou et al., 2000). Both high and low levels of 5-HT affect developmental axon growth. For example, 5-HT depletion results in a decrease in the number and density of axons in both high- and low-density serotonergic axon regions of neonatal rat somatosensory cortices (D'Amato et al., 1987), while SERT and Monoamine oxidase A inhibition produces abnormal segregation of axons in the retinogeniculate and somatosensory thalamocortical systems (Salichon et al., 2001; Upton et al., 2002). Similarly, a broad acting 5-HT receptor agonist 5-methoxytryptamine (5-MT) had a biphasic effect on serotonergic neuron terminal growth, where at low doses it was inhibitory but at high doses it stimulated terminal growth (Shemer, Azmitia & Whitaker-Azmitia, 1991; Whitaker-Azmitia, Shemer, Caruso, Molino & Azmitia, 1990b). 5-HT1A agonist treatment also prevents the loss of dendritic spines that typically follows neonatal 5-HT depletion and increases dendritic tree length and branching

of cholinergic neurons, while 5-HT1A antagonist treatment results in dendritic spine loss comparable to that of the 5-HT depletion (Riad et al., 1994; Yan, Wilson & Haring, 1997b).

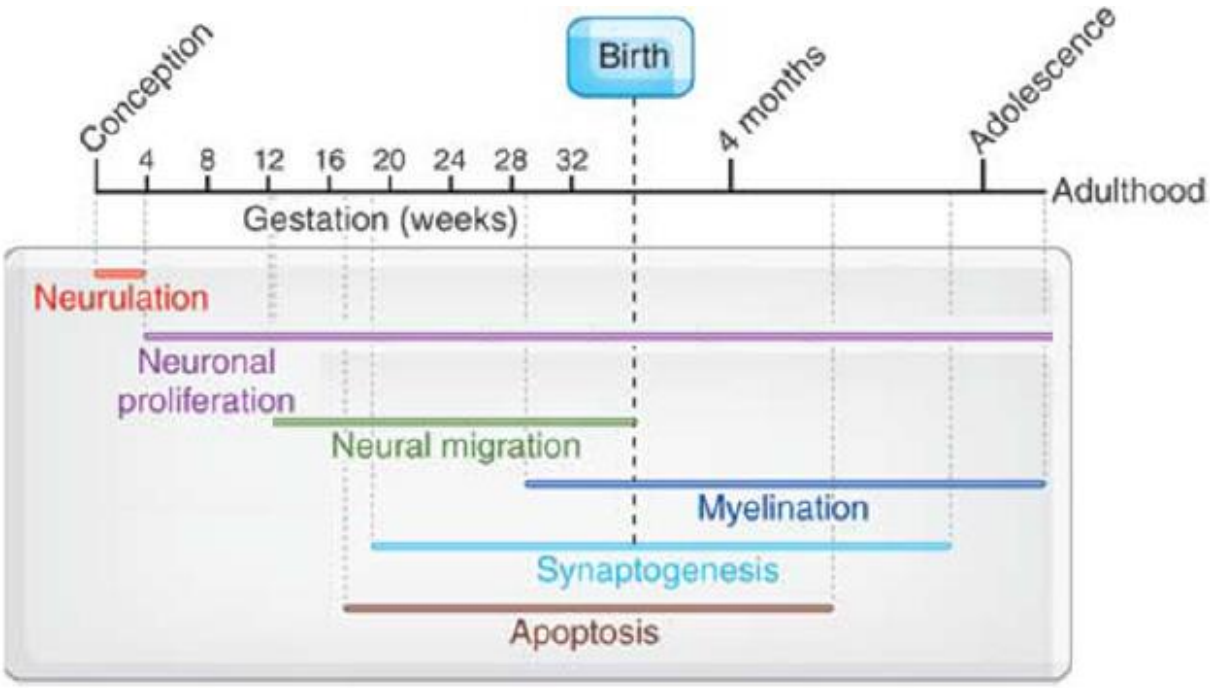
5-HT depletion leads to a decrease in the neurotrophic growth factor S100β (which typically stimulates neurite growth; Azmitia et al., 1990; Haring et al., 1993) and decreases dendritic spine density, neurite extension and synapse formation in dentate gyrus (DG) granule cells (Brezun & Daszuta, 1999; Mazer et al., 1997; Yan et al., 1997a). It also reduces Microtubule-associated protein 2 (MAP-2) immunoreactivity density, which has a role in dendritogenesis and is an immunocytochemical marker for dendrites (Azmitia, Rubinstein, Strafaci, Rios & Whitaker-Azmitia, 1995; Mazer et al., 1997). The converse also occurs with SSRI fluoxetine administration leading to increases in S100β (Haring et al., 1993).

Astrocytes are central to plasticity within the brain, since they not only make glucose available to neurons, but also provide adhesion and trophic factors for neuronal growth and migration (Azmitia, 2001). Astrocytes also express 5-HT1A receptors and 5-HT1A agonist stimulation leads to the release of S100β and maturation of astrocyte morphology (Azmitia, 2001; Azmitia et al., 1995; Whitaker-Azmitia, Murphy, Azmitia & 1990a). Moreover, S100β is transiently observed in the developing foetal raphe region suggesting a functional interaction between astrocytes and neurons during development which facilitates maturation of these cell types via the astrocytic 5-HT1A receptor (Whitaker-Azmitia et al., 1990a).

Synapses are a crucial substrate for plasticity in adult neural circuits (Holtmaat & Svoboda, 2009; Trachtenberg et al., 2002). The timing of serotonergic innervation coincides with a period of pronounced and critical synpatogenesis (D'Amato et al., 1987). Synaptogenesis of neurons in the rat begins on E18-19 in the medial raphe nuclei and is first observed at the perikarya of these neurons at E20 (Lauder & Bloom, 1975). In humans, the earliest synaptic connections form by the 5th gestational week with intense synaptogenesis occurring during the first few years of life, particularly during the first year (Tau & Peterson, 2010). The 28th gestational week in human pregnancy is a period of intense synaptogenesis and corresponds to postnatal day (PND) 7-8 in rats (Liu, Chow & Sherry, 2013a; Oberlander et al., 2009; Tau & Peterson, 2010; Uytun, 2018). Timelines depicted in figures 2 and 3.

Figure 2

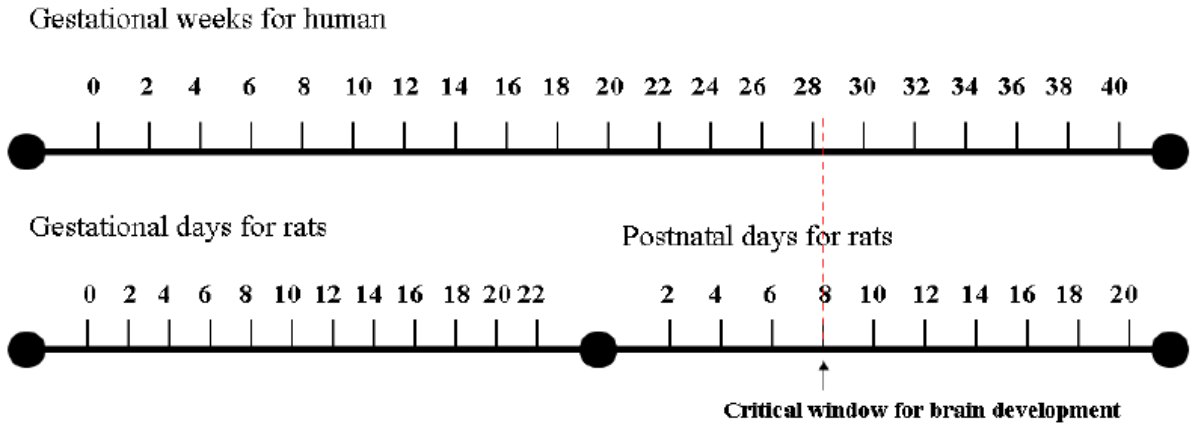
Time line of human neurodevelopmental events



Note. Modified from Tau and Peterson (2010)

Figure 3

Comparison of human and rat developmental timelines



Note. Adapted from Liu et al. (2013a)

5-HT depletion and 5-HT1A antagonism both decrease dendritic spines, and this is accompanied by a decrease in synaptic density (Faber & Haring, 1999). Similarly, new research from our own laboratory found that neurons in primary cell cultures showed a gene dosage dependent decrease in spine density, with homozygous SERT knockouts having the smallest, and WT, the largest number of spines (Chaji, Venkatesh, Shirao, Day & Ellenbroek, 2021). On the other hand, antidepressant treatment increases synapses in the hippocampal

CA1 region (Chen, Madsen, Wegener & Nyengaard, 2010). Activation of the 5-HT7 receptor promotes neurite elongation, dendritic spine formation and synaptogenesis while deactivation decreases the number of spines (Speranza et al., 2017). 5-HT2A receptors also have a role in synaptogenesis. For example, Niitsu, Hatnada, Hamaguchi, Mikuni and Okado (1995) demonstrated that treatment of chick embryos with a 5-HT2A receptor agonist (DOI) and antagonist (ketanserin) increase and decrease synaptic density in the lateral motor column of the spinal cord, respectively.

5-HT clearly plays a role in regulating neuronal connectivity. Overall, the expression of 5-HT prior to synaptogenesis and in growing serotonergic fibre terminals as well as the appearance of 5-HT receptors in cells prior to 5-HT synthesis suggests a role for 5-HT in directing this growth and in effect, the connectivity of 5-HT and several other neuronal systems (Aitken & Törk, 1988; Côté et al., 2007; Lauder, 1990). Importantly, disturbances in 5-HT homeostasis alter neuronal connectivity.

Neuroplasticity/neural circuitry

In addition to its role in development, 5-HT also shapes the brain networks and connectivity underlying a wide range of essential neuronal functions ranging from perception and cognitive appraisal to emotional responses in the mature brain (Hansson et al., 1999; Lebrand et al., 1998). Neuroplasticity refers to the adaptive changes in neuronal function which allow the brain to learn from experiences and sensory input (Feldman, 2009). This includes structural plasticity (i.e., changes in spine and dendrite morphology and wiring and neurogenesis) and Hebbian synaptic plasticity (i.e., functional changes in synapse signalling strength) which are both affected by 5-HT (Feldman, 2009; Holtmaat & Svoboda, 2009; Krishnan & Nestler, 2008; Pittenger & Duman, 2008; Teissier, Soiza-Reilly & Gaspar, 2017).

Activity at synapses is an important contributor to synaptic plasticity through different patterns of receptor activation or second messenger regulation of synaptic vesicle release machinery and can lead to long term potentiation (LTP) or long term depresson (LTD) (Leenders & Sheng, 2005; Lüscher, Nicool, Malenka & Muller, 2000; Sheng & Kim, 2002; Stepan, Dine & Eder, 2015). LTP represents the synaptic plasticity involved in learning and memory and is affected by 5-HT (Nguyen & Kandel, 1996; Pittenger & Duman, 2008; Saxe et al., 2006).

Disturbances in 5-HT-moderated synaptic signalling alters synaptic plasticity and neurocircuitry (Lesch & Waider, 2012; Niitsu et al., 1995). For example, behavioural testing of 5-HT depleted animals showed delayed extinction of behaviour and deficits in spatial learning and memory systems located predominantly within the hippocampus and are consistent with the altered synaptic morphology observed (Mazer et al., 1997). SERT ablation as well as SSRI treatment also increase prefrontal cortex glutamate synapses on 5-HT and GABA neurons in the DRN (Soiza-Reilly et al., 2019). The 5-HTTLPR is also associated with amygdala activation and *s* allele carriers show greater coupling between the amygdala and the ventromedial prefrontal cortex, this may contribute to the abnormally high activity in the amygdala and medial prefrontal cortex seen in major depression (Hariri et al., 2002; Heinz et al., 2004; Munafò et al., 2008). 5-HT also influences the postnatal remodelling of circuitry (Teissier et al., 2017).

Neurogenesis

Typically, neurogenesis (the formation of new neurons) occurs from prenatal development into adulthood (Eriksson et al., 1998; Jacobs, van Praag & Gage, 2000). In mammalian brains, most neurons are created during the pre- and perinatal period of development (Jacobs et al., 2000).

A common notion about the function of adulthood neurogenesis is that new neurons in the adult brain are beneficial as they increase neuroplasticity. However, the amount of adult neurogenesis decreases with increasing brain complexity. While some lower vertebrates can regenerate entire brain parts, mammalian adult neurogenesis is limited to the subventricular zone (SVZ) of the lateral ventricles which generate the granule cells that end up in the olfactory bulb and the subgranular zone (SGZ) of the dentate gyrus (at the border between the hilus and granule cell layer) which gives rise to granule cells from neural stem/progenitor cells in the hippocampus (Altman & Das, 1966; Alvarez-Buylla & García-Verdugo, 2002; Duan, Kang, Liu, Ming & Song, 2008; Duman, 2004; Ehninger & Kempermann, 2008; Eriksson et al., 1998; Gage, 2002; Kempermann, Wiskott & Gage, 2004a, Kempermann, Jessberger & Kronenberg, 2004b; Kuhn, Dickinson-Anson & Gage, 1996; Vadodaria & Jessberger, 2014; van Praag et al., 2002; Zaverucha-do-Valle et al., 2013; Zhao, Deng & Gage, 2008). Depicted in figure 4. This restricted neurogenesis is suggestive of a trade-off between the benefits of producing new neurons and the complications they

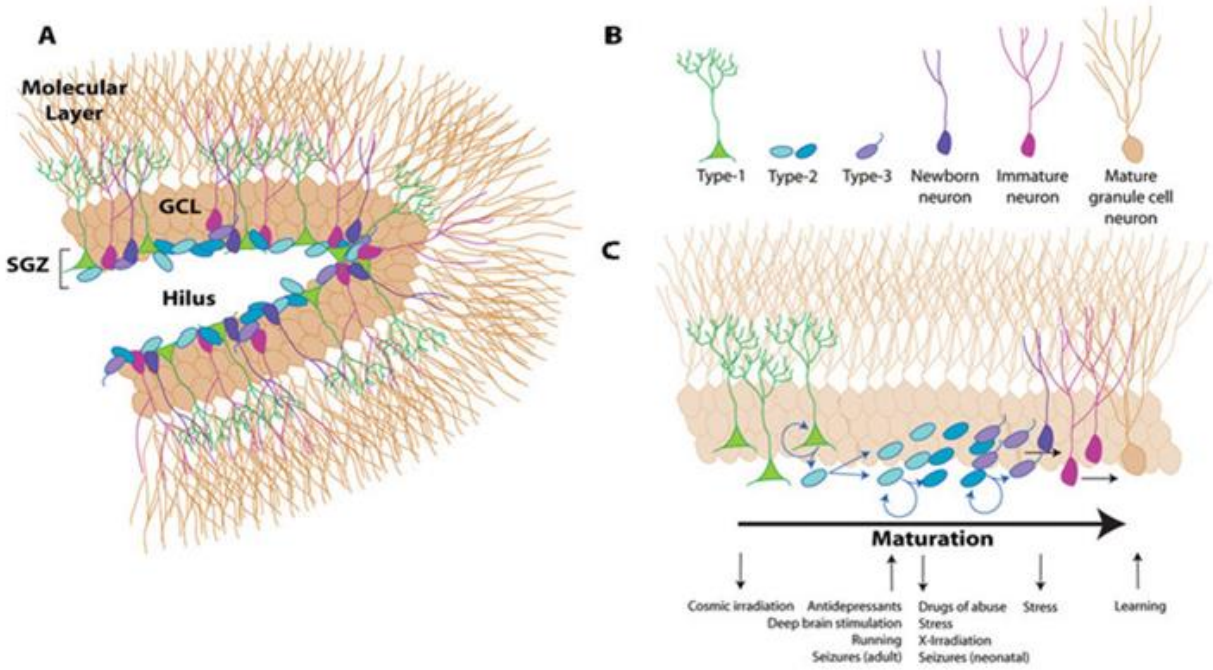
create for the network structure into which they need to integrate (Kempermann et al., 2004a).

These neurogenic niches are very important as their unique microenvironments and constituent regulatory environmental cues allow neurogenesis to occur, even transplanting neural stem/ progenitor cells from non-neurogenic brain regions allows them to differentiate into neurons in these zones (Ehninger & Kempermann, 2008; Shihabuddin, Horner, Ray & Gage, 2000; Suhonen, Peterson, Ray & Gage, 1996). In these two neurogenic regions the benefits of neurogenesis outweigh the complications and therefore, must serve an important hippocampal function (Kempermann et al., 2004a).

Hippocampal neurogenesis is the process of neural stem cells/ progenitors in the SGZ dividing and maturing into functional neurons (Ming & Song, 2011). This occurs through radial-glia-like stem cells (type-1 cell) with astrocytic properties progressing through at least two stages of amplifying lineage-determined progenitor cells (type-2 and type-3 cells) to early postmitotic and to mature neurons (See figure 4; Eisch et al., 2008; Kempermann et al., 2004b). Granule cell formation occurs during pregnancy and continues postnatally (Bayer, 1980; Seress, Ábrahám, Tornóczky & Kosztolányi, 2001). One estimate posits that up to 9000 new cells are generated daily in the adult rat brain (Cameron & Mckay, 2001).

Figure 4.

The process of neurogenesis



Note. Dentate gyrus layers depicted in A. B and C illustrate the progression through neurogenesis stages. C also notes factors effecting the maturation of cells undergoing neurogenesis. From Eisch et al. (2008).

The majority of new cells (50- 80%) in the DG differentiate into neurons while some differentiate into glial cells (Barlow & Targum, 2007; Warner-Schmidt & Duman, 2006). Once in the granule cell layer, cells that will become neurons, develop the characteristics of adult granule cells which includes dendrite extension into the molecular layer, axon elongation into the CA3 pyramidal cell layer via the mossy fibre pathway where they terminate in synapse and interneuron rich areas, receiving synaptic inputs, exhibit the classic plasticity period of developmentally generated neurons (Ge, Yang, Hsu, Ming & Song, 2007) and activity-dependant gene expression (Duman, 2004; Ehninger & Kempermann, 2008; Jessberger & Kempermann, 2003; Kempermann et al., 2004b; van Praag et al., 2002).

Early postnatal hippocampal development is a crucial period of neurogenesis (Arnold & Trojanowski, 1996; Seress et al., 2001). At birth, the dentate gyrus only contains approximately 70% of the number of cells seen in adulthood, the remaining 30% are produced postnatally (Bauer, 2004). In humans, major cortical neurogenesis takes place between gestational weeks 6-16 with further cortical development continuing postnatally (Sidman & Rakic, 1973) while several early cellular events characterising cortical embryonic/foetal neurogenesis occur by 6-7 weeks (Choi, 1988). It is only after 12-15 postnatal months that hippocampal morphology resembles the adult structure (Bauer, 2004). Studies of infant memory indicate that this early-developing hippocampus is largely responsible for early competencies (Bauer, 2004; Gómez & Edgin, 2016).

The adult brain faces a challenge in that it must not only maintain stability in neural circuitry, but also allow plasticity to be able to change and adapt to environmental challenges. Neural circuits are undeniably shaped by the environment and thus change across the lifetime (Abrous, Koehl & Le Moal, 2005; Teissier et al., 2017). Experience and learning in early critical periods, as well as in adulthood, underlie sensory plasticity and involve synaptic regulation including formation, removal and morphological remodelling of synapses and dendritic spines (Feldman, 2009; Holtmaat & Svoboda, 2009; Teissier et al., 2017; Trachtenberg et al., 2002; Wilbrecht, Holtmaat, Wright, Fox & Svoboda, 2010).

Environmental stimulion is a key driver of neuroplasticity and learning (Novkovic, Mittmann & Manahan-Vaughan, 2015). Environemental enrichment is an animal model for the environmental stimuli-based neural plasticity and circuitry modulation seen in normal brain functioning and is shown to improve LTP (Novkovic et al., 2015). Similarly, activity-dependant neurogenesis occurs when excitatory stimuli act directly on neural progenitor cells through excitatory receptors to stimulate new neuron production (Deisseroth et al., 2004; Lüscher et al., 2000).

There is emerging consensus that these newborn neurons are involved in learning and memory (Bath, Akins & Lee, 2012; Gould, Beylin, Tanapat, Reeves & Shors, 1999). Manipulations that increase adulthood neurogenesis (AHN), such as environmental enrichment, exercise and antidepressant treatment have been found to enhance performance in pattern separation, verbal declarative memory, spatial learning and LTP (Karabeg et al., 2013; Olson, Eadie, Ernst & Christie, 2006; Sahay et al., 2011; van Praag, Christie, Sejnowski & Gage, 1999; Vermetten, Vythilingam, Southwick, Charney & Bremner, 2003). On the other hand manipulations that decrease AHN, such as aging (Drapeau et al., 2003) or ablation of neurogenesis, impair performance in learning and memory such as fear conditioning (Shors et al., 2002; Winocur et al., 2006), spatial navigation learning (Drapeau et al., 2003; Hu et al., 2009; Imayoshi et al., 2008; Mohapel, Leanza, Kokaia & Lindvall, 2005; Morris et al., 1982; Zhang et al., 2008), spatial pattern separation (Clelland et al., 2009; Kim, Christian, Ming & Song, 2012), impair normal endocrine responses and increase behavioural stress responses (Snyder, Soumier, Brewer, Pickel & Cameron, 2011). Moreover, hippocampal lesion-induced memory impairments illustrate that neurogenesis is involved in memory processes (Shors et al., 2001, 2002; Imayoshi et al., 2008; Vargha-Khadem et al., 1997).

However, different AHN-reducing manipulations have produced conflicting results (Saxe et al., 2006) with some affecting spatial learning but not fear conditioning (Zhang et al., 2008) or vice versa (Shors et al., 2002). This is possibly due to the functional differences observed in the septotemporal axis, as lesion studies demonstrate that the septal pole is more involved in spatial learning and memory while the temporal pole plays a greater role in emotional behaviours (Fanselow & Dong, 2011; Kjelstrup et al., 2002; Moser, Moser, Forrest, Anderson & Morris, 1995; Moser & Moser, 1998; Sahay & Hen, 2007; Tanti & Belzung, 2013).

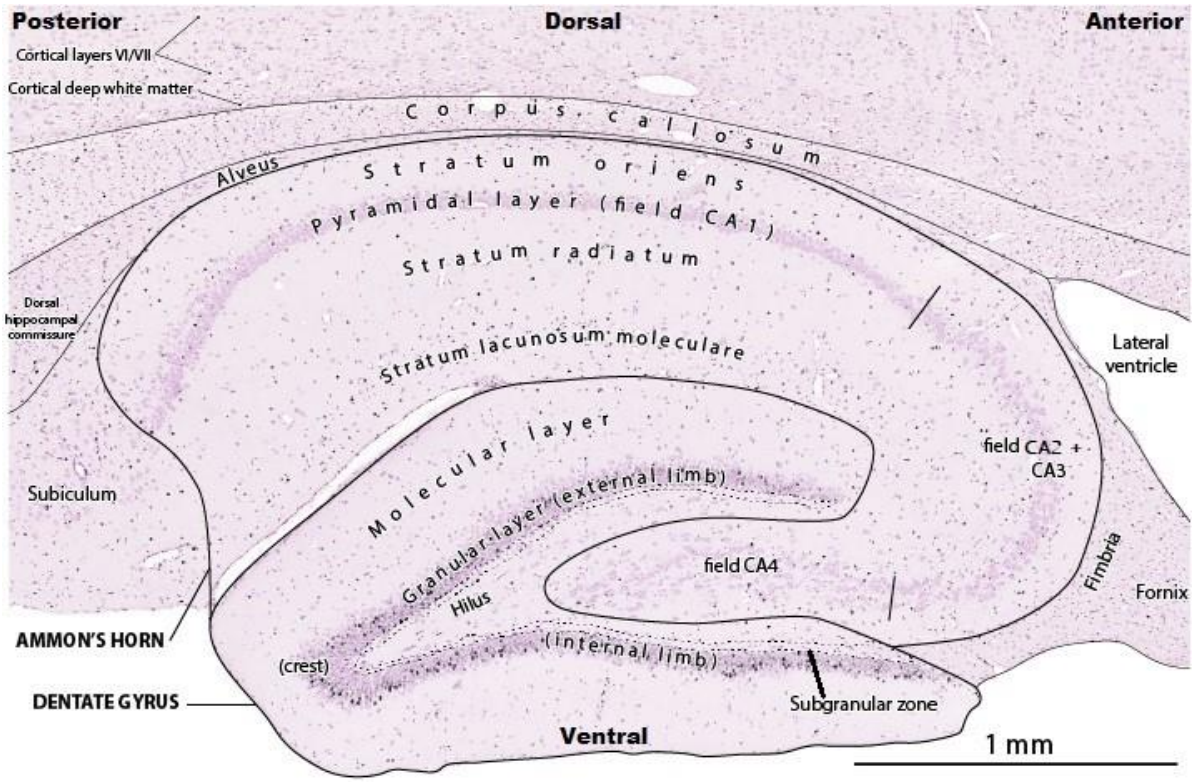
The hippocampus

The hippocampus is critical to the process of forming and recovering certain types of memory (Eichenbaum, 2010; Squire, Stark & Clark, 2004). It is a bilateral subcortical structure in the temporal lobe and has a laminar (layered) organization (Andersen, Soleng & Raastad, 2000; Sapolsky, 2000; Sloviter & Lømo, 2012).

The hippocampal formation consists of the hippocampal proper which is divided into four zones, called '*cornu ammonis*' 1-4 (CA1 - CA4) and its related parts- the dentate gyrus and subiculum (Giap et al., 2000; Mendoza & Foundas, 2008). CA1 is the outermost segment merging with the subiculum while CA3 and CA4 (sometimes referred to as CA3 together) is the innermost segment of the hippocampus proper adjacent to the dentate gyrus (Mendoza & Foundas, 2008; See figure 5).

Figure 5.

Saggital section of rat hippocampus on PND60.



Note. Modified from Brain Developmental Maps (2020).

The hippocampus proper is comprised of three distinct layers. The pyramidal cell layer (stratum pyramidal) contains pyramidal cell bodies- the principal cells of the

hippocampus. The molecular layer (divided into a more external stratum lacunosum moleculare and a more internal stratum radiatum) consists of apical dendrites from pyramidal cells and axons originating from various other regions. Lastly, the polymorphic layer (stratum oriens) contains various interneurons, including multipolar GABAergic basket cells (Giap et al., 2000; Jacobson & Marcus, 2008).

The dentate gyrus also consists of three layers (see figure 4). The molecular layer contains the dendrites of granule cells. The granule cell layer of small unipolar neurons whose dendrites emerge from the apical end of the cell and into the molecular layer. Lastly, there is a polymorphic cell layer (aka the hilus) which is continuous with the CA4 (Amaral et al., 2007; Giap et al., 2000; Jacobson & Marcus, 2008).

The subiculum and hippocampus are mainly comprised of efferent pyramidal neuronal cells, which project to several structures, while the dentate gyrus sends projections to the hippocampus alone (Giap et al., 2000). The pyramidal and granule cells are excitatory neurons utilizing the neurotransmitter glutamate, while basket cells in the polymorphic layers are inhibitory GABA-ergic interneurons (Giap et al., 2000; Jacobson & Marcus, 2008).

The trisynaptic circuit is a relay of hippocampal synaptic transmission beginning with projections from the entorhinal cortex that synapse on the DG via granule cell fibres (the perforant path), the DG then synapses on CA3 pyramidal cells via their mossy fibres, the CA3 then synapse in the CA1 via Schaffer collaterals (Insausti & Amaral, 2003; Sapolsky, 2000).

The septotemporal axis of the hippocampus influences the rate of neurogenesis as studies have found that for example the septal (dorsal) DG has a faster rate of neurogenesis than the temporal (ventral) DG, and these new neurons mature faster in the septal DG than the temporal DG (Piatti et al., 2011; Snyder, Ferrante & Cameron, 2012). Snyder et al. (2012) found different rates of neuronal maturation along the septotemporal and transverse axis of the DG and posit that the prolonged maturation in the temporal region translates to a longer duration of plasticity in this region.

5-HT is an important component of hippocampal function. The dentate gyrus receives plenty of serotonergic innervation and is enriched with 5-HT1A receptors (Azmitia et al.,

1996; Azmitia & Segal, 1978; Blier & Ward, 2003; Freund, Gulyás, Acsády, Görcs & Tóth, 1990; Gérard et al., 1994; Klempin et al., 2010). Serotonergic fibres project diffusely throughout the brain and into the dentate gyrus of the hippocampus where they form synapses with granule cells and interneurons (Alenina & Klempin, 2015). 5-HT is implicated in cell proliferation as raphe lesioning and 5-HT depletion leads to decreased granule cell proliferation in the dentate gyrus, while serotonergic raphe grafts reversed these post-lesion decreases in granule cell immunostaining and decreases the amount of the neural cell adhesion molecule PSA-NCAM which is a marker for molecular plasticity in the DG (Alvarez-Buylla & García-Verdugo, 2002; Brezun & Daszuta, 1999, 2000a; Djordjevic et al., 2012). These effects are reversed after partial 5-HT hippocampal reinnervation (Brezun & Daszuta, 2000b).

Considerable evidence implicates the serotonergic system in the stimulation of neurogenesis. Firstly, conditions associated with decreased neurogenesis such as aging (Kempermann, Kuhn & Gage, 1998; Kuhn et al., 1996), high corticosterone levels (Campbell & MacQueen, 2004), stress (Mirescu & Gould, 2006) and malnutrition (Debassio, Kemper, Tonkiss & Galler, 1996) also affect the serotonergic system by decreasing 5-HT fibre or 5-HT receptor density or decreasing extracellular 5-HT levels for example (Blatt, Jin-Chung, Rosene, Volicer & Galler, 1994; Chalmers, Kwak, Mansour, Akil & Watson, 1993; Gould, 1999; McKittrick et al., 1995; Míguez, Aldegunde, Paz-Valiñas, Recio & Sánchez-Barceló, 1999; Neumaier, Petty, Kramer, Szot & Hamblin, 1997; Watanabe et al., 1994). Conversely, conditions associated with increased neurogenesis such as exercise, environmental enrichment, BDNF and antidepressant treatment also increase 5-HT signalling (Dekeyne et al., 2008; Duman, 2004a; Kim et al., 2012; Klempin et al., 2013).

Studies show that conditions associated with exacerbating depression, such as stress (Pittenger & Duman, 2008) suppress AHN (Dranovsky & Hen, 2006; Mirescu & Gould, 2006; Nasir & Khan, 2011; Perera et al., 2007; Schoenfeld & Gould, 2012), while those that alleviate depressive symptomology such as exercise (Cooney et al., 2013; Liu et al., 2013b; Zheng et al., 2006), increase AHN (Olson et al., 2006). Moreover, several stress models of depression, such as learned helplessness and chronic mild stress, are associated with reductions in hippocampal neurogenesis (Chen et al., 2010; Lee et al., 2006; Vollmayr, Simonis, Weber, Gass & Henn, 2003). Stress-induced reduction in neurogenesis is also a causal factor in precipitating episodes of depression (Jacobs et al., 2000).

Neuroanatomical changes are also associated with depression and mood disorders. For example, studies consistently show that individuals with depression have lower hippocampal volumes (usually a larger reduction in the right hemisphere), including less granule cells, than healthy controls (Boldrini et al, 2013; Bremner et al., 2000; Campbell, Marriott, Nahmias & MacQueen, 2004; Kronmüller et al., 2009; Sheline ,Wang, Gado, Csernansky & Vannier, 1996; Videbech & Ravnkilde, 2004) with longer illness duration directly relating to the magnitude of hippocampal decrease (MacQueen et al., 2003; McKinnon, Yucel, Nazarov & MacQueen, 2009; Sheline, Sanghavi, Mintun & Gado, 1999; Stockmeier et al., 2004). The fact that hippocampal volume differences occur in depressed children lends further support to the idea that the early postnatal period is an indeed important period for hippocampal neurogenesis and that its dysregulation is associated with depression (McKinnon et al., 2009).

Depressed individuals also show impairments in hippocampal dependant functions such as impaired learning, memory, particularly including impaired performance on hippocampal dependant memory tests (Austin, Mitchell & Goodwin, 2001; Bremner, Vythilingam, Vermetten & Vaccarino & Charney, 2004; Burt, Zember & Niederehe, 1995; Ilsley, Moffoot & O'Carroll, 1995; MacQueen et al., 2003). Individuals that scored high on the Beck Depression Inventory show impairment in the hippocampus-dependant delayed match to sample task (Becker, MacQueen & Wojtowicz, 2009).

Neurogenesis is also regulated by antidepressant treatment and appears to be important for the therapeutic effects of antidepressants (Airan et al., 2007; Encinas, Vaahtokari & Enikolopov, 2006; Sahay & Hen, 2007; Santarelli et al., 2003; Surget et al., 2008).

While antidepressants have relatively rapid effects on monamine signalling, there is typically a latency of more than 3 weeks of treatment before they are clinically efficacious (Sapolsky, 2004; Wong & Licinio, 2001) suggesting that they act through slow neurochemical and structural changes, such as neurogenesis, within the brain (Duman, 2004; Duman & Monteggia, 2006; Massart et al., 2012; Santarelli et al., 2003). Antidepressant treatment upregulates hippocampal neurogenesis, both in the presence and absence of depressive symptomology/behaviours (Airan et al., 2007; Dranovsky & Hen, 2006; Duman,

2004; Encinas et al., 2006; Klempin et al., 2010; Malberg et al., 2000; Perera et al., 2007; Santarelli et al., 2003; Warner-Schmidt & Duman, 2006). Moreover, the beneficial effects of antidepressants are dependent on neurogenesis (Airan et al., 2007; Santarelli et al., 2003). These findings are in line with the studies mentioned above, that neurogenesis may be dysregulated in depression (Duman, 2004a; Manji et al., 2001).

Summary

This mounting evidence implicates neurogenesis in the neurobiological pathology of depression (Paizanis, Hamon & Lanfumey, 2007). Evidence, particularly from studies of antidepressant effects on neurogenesis, suggests that it is partially through neurogenesis that antidepressants elicit their beneficial effects (Santarelli et al., 2003; Surget et al., 2008). Moreover, it appears that 5-HT is a potent regulator of the hippocampal neurogenesis where increased 5-HT appears to be conducive to increased neurogenesis (Banasr, Hery, Printemps & Daszuta, 2004; Klempin et al., 2013). What remains to be examined are the effects of genetically increased 5-HT levels and neurodevelopmental changes that could underlie depression.

Given that 5-HT has such a crucial role in embryonic brain development, the role of the maternal influence over these processes needs to be determined. The placenta is the primary source of 5-HT for the developing feotal brain (Côté et al., 2007; Huang, Zhang, Di & Zhang, 1998; Kliman et al., 2018; Rosenfeld, 2020). Alterations in maternal 5-HT produce long term morphological brain changes in foetal rats (Côté et al., 2007; Matsukawa et al., 2003; Vitalis et al., 2007) and leads to maladaptive depressive- and anxiety-like behavioural phenotypes in offspring (Kesper & Homberg, 2015; Shah, Courttiol, Castellanos & Teixeira, 2018). The maternal SERT genotype impacts the placental 5-HT levels in the foetal forebrain, thus effecting foetal neurodevelopment (Muller et al., 2017). For example, the maternal SERT Ala56 genotype is associated with decreased placental and embryonic forebrain 5-HT levels (Muller et al., 2017).

As 5-HT participates in so many crucial neurodevelopmental processes, it is important to examine more global changes in SERT knockout neurochemistry. Matrix assisted laser desorption ionization imaging mass spectrometry (MALDI- IMS) is a valuable technique which allows for the detection, identification and distribution mapping of chemical compounds within tissue (Schubert, Weiland, Baune & Hoffmann, 2016). Thus, further

analysis of not only 5-HT but other neurotransmitter, receptor, and metabolite changes can be accomplished which would allow us to draw conclusions on larger-scale pathways involved in depression.

This study

The monoamine hypothesis of depression proposes that because antidepressant alleviate depression, it must arise from a lack of monoamines, however it does not account for the serotonin paradox where individuals with greater (genetically determined) extracellular 5-HT levels also experience depressive symptomology. The neurogenic hypothesis on the other hand, proposes that it is a reduction in neurogenesis and neuroplasticity that underlies depression. However, given that 5-HT and neurogenesis are tightly related, a genetic increase in 5-HT would likely lead to increased neurogenesis (at least early in life) rather than the decreased neurogenesis typically associated with depression. See figure 6.

Therefore, in this study, we will first examine whether rats with a genetically compromised SERT system have altered neurogenesis early in life (PND7). Moreover, as highlighted above, since neuronal development critically depends on maternal 5-HT levels, we will investigate whether the maternal SERT genotype affects neurogenesis. As BrdU immunohistochemistry has been instrumental in confirming that neurogenesis occurs in adult mammalian brains and in examining the effects of antidepressant treatments on neurogenesis (Eriksson et al., 1998; Kuhn et al., 1996; Malberg et al., 2000; Radley & Jacobs, 2002; Taupin, 2007), this technique will be used to stain new cells in different hippocampal sections (CA1, CA2+CA3 and the DG) to determine the average levels of neurogenesis across genotypes.

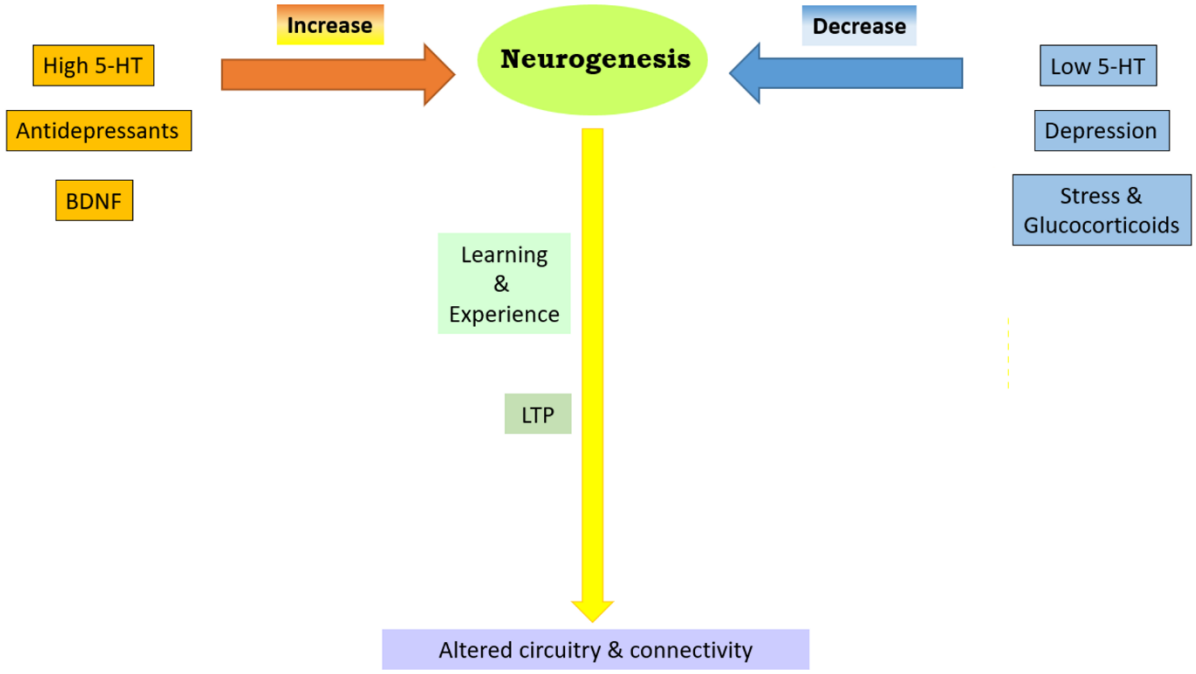
Firstly, we hypothesise that the dentate gyrus (DG) will have larger amount of neurogenesis than the CA1, and CA2+3 regions as it contains the neural progenitor cells within the neurogenic niche of the SGZ (Altman & Das, 1966; Zhao et al., 2008).

Secondly, we hypothesis that there will be a gene-dosage dependent increase in neurogenesis, with wildtype (WT, SERT+/+) have the lowest and homozygous (HOM, SERT-/-) rats having the highest levels of newly formed cells.

Lastly, we hypothesis that the maternal genotype also affects neurogenesis, with HOM mother leading to higher levels of neurogenesis than HET (SERT+/-) or WT mothers.

Figure 6

Factors effecting neurogenesis



Note. Yellow arrow indicates the hypothesised mechanism underlying the neurogenic and monoamine hypotheses of depression, which is affected by the level of extracellular 5-HT.

In the second part of this study, we planned to investigate more global changes in brain neurochemistry in rats with a genetically compromised SERT system, given the fact that serotonin (and the SERT) has such wide-spread effects on neuronal development early in life. For that, we aim to develop the MALDI protocol which would allow us to visualize and quantify the neuronal distribution of multiple neurochemical compounds simultaneously. This optimization largely focuses on the derivatization of neurotransmitters. Unfortunately, while we made significant progress, we were only able to partially develop the technology as we will discuss in the next chapter.

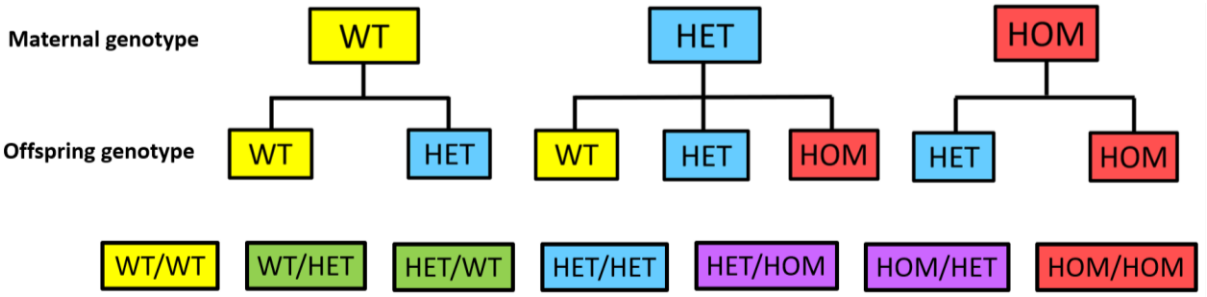
Method

Animals

This study used Wistar rats which were bred and housed in the Victoria University of Wellington vivarium in accordance with the Animal Welfare Act 1999, with all procedures being pre-approved by the VUW Animal Ethics Committee (AEC number 22375). All animals were housed in polycarbonate cages with one dam and her offspring per cage in a temperature and humidity-controlled environment at 21°C ± 2°C and 55-60% respectively. A reverse 12-hour light cycle was used with lights on at 1900 - 0700 hours. Food and water were available to the animals ad libitum. To obtain all possible maternal and offspring genotypes, a separate cohort of animals, which consisted of 13 litters of pups bred from seven different genotype pairing combinations, was used. These seven pairings were accomplished by breeding heterozygous (HET; SERT+/-) males with wild type (WT, SERT+/+) homozygous (HOM; SERT-/-) and HET females (genotypes shown in figure 9). Due to time constraints, only male pups were examined (litters: 6, pups: 46).

Figure 9

Offspring genotype combinations



Note. Last row lists 7 possible SERT genotype combinations examined.

Tissue Preparation

On postnatal day 7 (PND7), pups were removed from cages in pairs and taken into a different room to avoid stressing the remaining animals. Pups were weighed then injected with 25mg/kg of BrdU (based on previous pilot work in the VUW Behavioural Neurogenetics lab) intraperitoneally, marked for identification and returned to dams within five minutes. Following a two-hour integration phase, the animals were taken into another room and were deeply anaesthetized with 50mg/ml pentobarbital. Once they demonstrated no physical response to a foot pinch, they were rapidly decapitated, and their brains were

submerged in a 4% paraformaldehyde solution for 24 hours at +4°C for protein fixing. Tail clippings were taken from each animal to establish their genotypes (Transnetyx, Inc.). Following this, brains were immersed in a 30% sucrose solution at +4°C for cryoprotection. Once the brains showed no buoyancy (indicating the complete transfer of water for sucrose solution had occurred), the brains were snap frozen at -40°C in isopentane with dry ice. The brains were subsequently stored at -80°C until they were sectioned. All brains were coded to keep the experimenter blind for the remainder of the experiment. 30µm thick coronal sections were sliced using a cryostat with every sixth section used in the analysis to ensure a 180µm distance between sections to avoid counting the same cell multiple times. Slices used in this study were between -1.20mm and -2.00mm from Bregma (Khazipov et al., 2015). Slices were stored in phosphate buffered saline (PBS, 10 mM, pH 7.4) with 0.1% azide at + 4°C until processed.

BrdU immunohistochemistry technique principles for new cell labelling

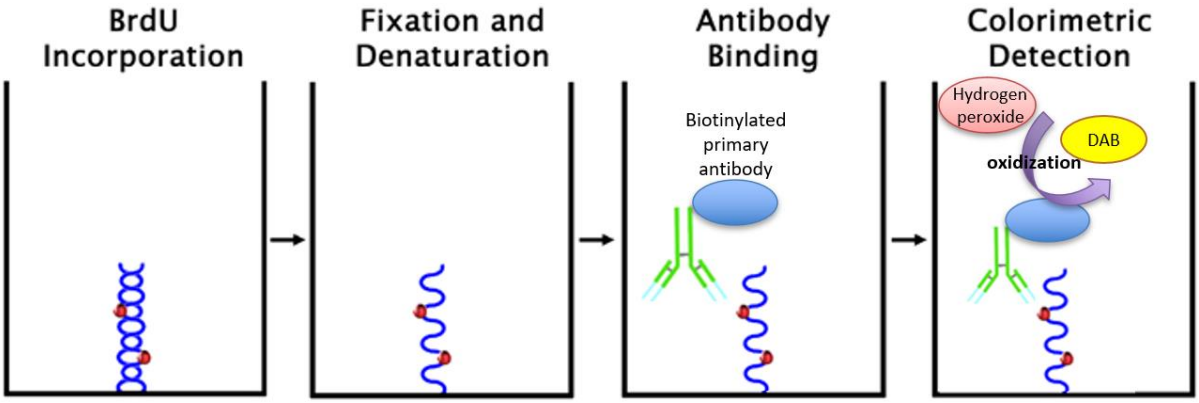
Bromodeoxyuridine (aka 5-bromo-2'-deoxyuridine, BrdU) is a synthetic thymidine analogue that incorporates into the DNA of dividing cells during the S-phase of the cell cycle (Eriksson et al., 1998; Taupin, 2007). BrdU can be immunohistochemically detected (Gratzner, 1982; Nagashima, DeArmond, Murovic & Hoshino, 1985) in vitro and in vivo, and as BrdU is incorporated into newly synthesised DNA, an antibody can then be used to visualise newborn cells that were dividing during this BrdU exposure (Duman, 2004; Magavi & Macklis, 2008). BrdU is therefore utilized to monitor birth dating and cell proliferation (Taupin, 2007). BrdU immunohistochemistry is advantageous as it is highly reproducible and quickly detected while the previously used tritiated thymidine autoradiography method is more variable and typically requires at least weeks of exposure (Magavi & Macklis, 2008). BrdU exposure as short as 6 minutes is detectable (Graztner, 1982). BrdU labelling can also be used for thicker slices 40-50µm thick while tritiated thymidine can only detect labelled cells in the topmost 3–5µm of sections (Magavi & Macklis, 2008). BrdU provides very low background intranuclear labelling and immunohistochemical detection up in sections up to 35µm thick (Markakis & Gage, 1999). After a short period (approximately two hours after BrdU administration) BrdU-immunopositive cells are found in clusters in the SGZ between the granule cell layer and the hilus (Duman, 2004). Typically, two hours is sufficient for BrdU uptake but not mitosis or migration (Gould et al., 1998) and therefore labels cells in the proliferation stage of neurogenesis, however depending on when BrdU is administered it can

also illustrate cell differentiation, migration and survival (Abrous et al., 2005). Thus, BrdU immunohistochemistry is the method of choice for labelling newly generated neurons (Ngwenya, Peters & Rosene. 2005).

BrdU immunohistochemistry is performed with the Avidine-Biotine Complex (ABC) method (shown in figures 7 and 8). The ABC method utilizes the high affinity of Avidin, a glycoprotein, for Biotin, a low molecular weight vitamin (Bratthauer, 2010). Large amounts of biotin can be easily conjugated to antibodies and enzymes via covalent attachment (Bratthauer, 2010). While the common ABC method uses a primary and secondary antibody (the three step ABC method), some primary antibodies such as the one used in this study is biotinylated itself and reacts with an avidin-biotin-peroxidase complex of avidin bound to biotinylated peroxidase to amplify this signal, which eliminates the need for a secondary biotinylated antibody (the two step ABC method; Bratthauer, 2010; Hsu, 1990). Peroxidase has an oxidative function which allows for the use of chromogens that change colour when oxidized (Bratthauer, 2010). Thus, when hydrogen peroxide is added, it is degraded by the peroxidase and releases oxygen that goes on to react with (oxidise) 3,3'-diaminobenzidine (DAB) (Bratthauer, 2010) and nickel chloride which changes its colour from a light pink in solution to a purple-black that allows the visualisation of the antibody-antigen reaction. However, as commercial antibodies only recognise BrdU in single-stranded DNA, the DNA need to be unwound into single strands with hydrochloric acid (HCl) (Ngwenya et al., 2005). The tissue is therefore preserved in paraformaldehyde, which provides enough fixation of brain tissue and proteins that the HCl treatment does not compromise tissue integrity (Ngwenya et al., 2005). This method is one of the most widely used immunocytochemistry methods used today (Bratthauer, 2010).

Figure 7

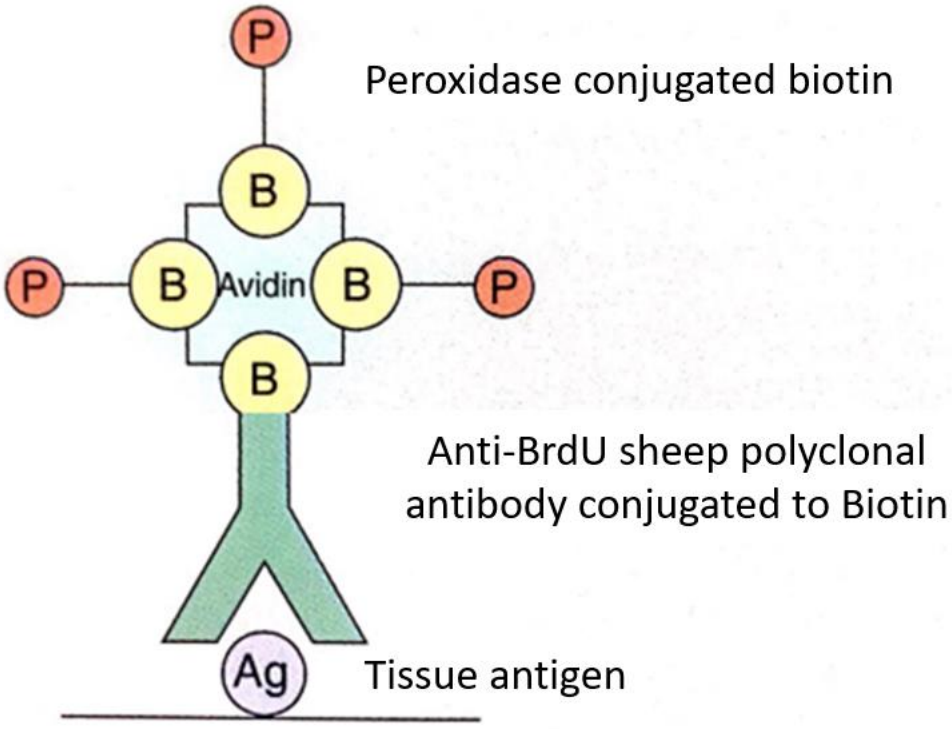
BrdU and two-step ABC immunohistochemistry



Note. Modified from Creative Bioarray. (n.d.).

Figure 8

The two-step Avidin-Biotin labelling technique



Note. Modified from Slap (2002).

BrdU Labelling Procedure

The ABC peroxidase method for labelling BrdU-positive cells in free floating sections, a procedure previously established within the VUW behavioural neurogenetics lab, was used in this experiment. All steps were carried out under mild agitation and at room temperature. Sections were removed from storage and washed for five minutes, three times in PBS which was replaced between each wash. An antigen retrieval process was used to denature the DNA strands to allow access for BrdU. This was achieved by incubating the sections for 90 minutes in 5 mL of 2 N HCl. Following this incubation, sections were washed three times for five minutes as stated above. Sections were then incubated in 1% Bovine Serum Albumin (BSA; Sigma-Aldrich) in a PBST solution (created by adding Triton X-100 [ThermoFisher] to PBS) for 60 minutes to increase membrane permeability. Sections were then incubated in 3% hydrogen peroxide (H_2O_2) in a PBS solution for 30 minutes (to quench endogenous peroxidase and prevent non-specific peroxidase-based background staining) then washed in PBS three times for five minutes as above. Sections were then incubated overnight in the primary anti-BrdU sheep polyclonal antibody, conjugated to Biotin, ab2284 (Abcam) diluted in PBST and 1% BSA. Following this, sections were washed once again in PBST as above then incubated in Avidin-biotin peroxidase complex (ABC; Vectastain) diluted 1:1000 in PBST (prepared 30 minutes beforehand) for 60 minutes. Following another washing in PBST as above, sections were incubated in a DAB (0.2 mg/mL) and Nickel chloride ($NiCl_2$, 0.4mg/mL) solution prepared in Tris HCl buffer (50 mM, pH 7.4) with 0.3% H_2O_2, to induce oxidization of the DAB. Thus, peroxidase breaks down H_2O_2, which releases oxygen, which then oxidises with DAB to give a brown (DAB alone) or blackish (DAB+$NiCl_2$) precipitate, producing coloured staining of the BrdU sites. Sections were washed once more in PBST as above.

Following staining, sections were stored at 4°C overnight until mounting within 48 hours. Stained sections were extracted from their baskets and immersed in PBST and gently mounted onto gelatinised slides and allowed to dry for at least 30 minutes. After mounting, counterstaining was undertaken to provide a stronger contrast between BrdU positive cells and the surrounding tissue. This involved immersion of the mounted sections in 1% neutral red (pH 4.8) solution (Biovision) for two minutes, followed by a brief immersion in a progressively increasing strength of ethanol: 70%, 90% twice, then 100% twice. Following this, sections were immersed in two baths of Histo-Clear (National Diagnostics). Lastly, a coverslip was applied with DPX (Sigma-Aldrich) and allowed to dry for at least 48 hours.

Manual counts of BrdU-labelled cells were performed blind using the software Neurolucida (MBF Bioscience; Williston, VT, USA) and Olympus BX51 microscope (Olympus; Wellington, New Zealand) with a motorized x-y stage and a Lumenera_camera (Teledyne Lumenera, Ottawa, Canada) at a 20x magnification. The DG, CA1 and CA2+CA3 regions were manually traced first at 10x magnification using a mouse-controlled cursor on a digital image of the slice to obtain the areas for each region. Cell densities were calculated by dividing the total number of cells counted in each of the three hippocampal regions by the total area of that region for each slice (bilaterally).

Results

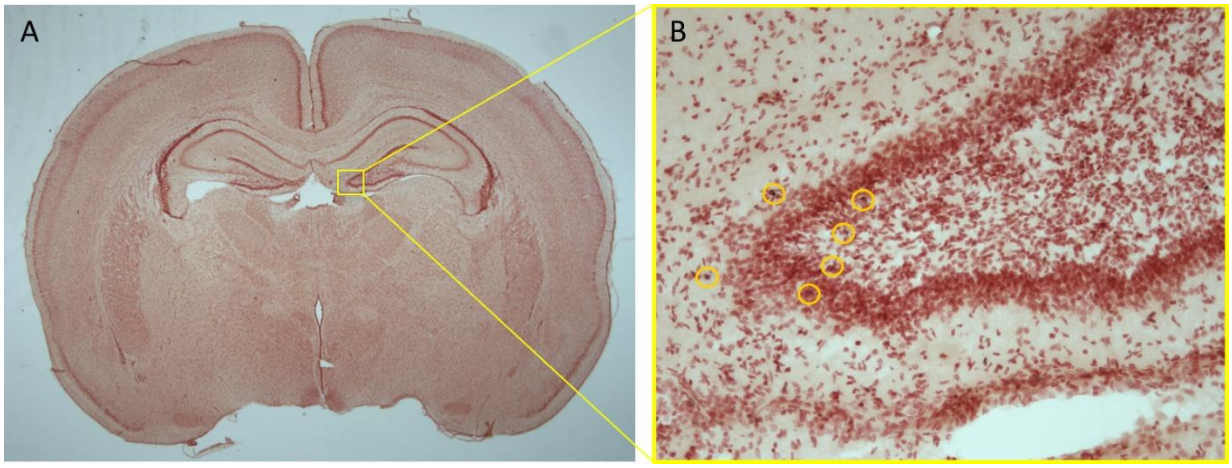
The data were analysed with IBM SPSS Statistics (version 25). Data was approximately normally distributed, assessed visually using a Q-Q plot. Levene's *F* tests were significant therefore we could not assume equal variances, so estimated marginal means and robust standard errors bars are used when presenting the results. Outliers from three brains (2 offspring HET/maternal WT, 1 offspring WT/maternal HET) were removed where little to no cells were detected.

As the homogeneity of variances was not met and as not every offspring genotype and maternal genotype combination was observed, data was initially analysed using three two-way factorial ANOVAs with Type IV sums of squares, after discussion with a statistician. These ANOVAs were conducted to assess the influence of the independent variables, offspring SERT genotype and maternal SERT genotype, on cell density for each of the three hippocampal regions to assess whether there were any mean cell density differences between offspring and maternal genotypes. The offspring genotypes had three levels (WT, HET, HOM) as did maternal genotype (WT, HET, HOM) for each region.

If the differences between offspring genotypes were found to differ between maternal genotypes (i.e., a significant interaction term), then this analysis was followed up with planned comparisons to compare maternal genotype differences within each offspring genotype. This was done using either an independent-samples t-test or one-way ANOVA as appropriate, and the Bonferroni correction was applied to correct for multiple comparisons such that *p-adj* denotes Bonferroni-adjusted *p*-values. If the differences between offspring genotypes were not found to differ between maternal genotypes, Bonferroni-adjusted pairwise comparisons of the estimated marginal means (*M**) were used to determine differences between genotypes and maternal genotypes.

Figure 10

Coronal section from a BrdU-stained HET x HOM brain at PND7



Note. Coronal section in at Bregma -1.60mm to -1.80mm in A (Khazipov et al., 2015). BrdU-labelled cells in the DG in B.

Means and standard deviations of the total area (mm^2) of all three hippocampal sections were computed for DG, CA1 and CA2 + CA3 to determine if there was equivalence of the hippocampal sizes between offspring and maternal genotype groups (see Appendix A).

Equivalence of the size of the mean area for each hippocampal region across offspring and maternal genotypes was determined using two-way analyses of variance (ANOVAs) which revealed no offspring or maternal genotype differences for DG, CA1 or CA2+CA3 mean area (See appendix).

After confirmation of size equivalence, means and SDs for BrdU-positive cell density were calculated (table 1).

Table 1

Density of BrdU-positive cells (number of per mm²) for the three hippocampal

Offspring genotype	Maternal genotype	DG		CA1		CA2 + CA3		
		M	*SD*	*M*	*SD*	*M*	*SD*	*n*
WT	WT	.55	.08	.21	.07	.26	.06	4
	HET	.32	.21	.11	.08	.16	.14	8
HET	WT	.43	.21	.15	.09	.19	.12	9
	HET	.44	.17	.16	.08	.18	.08	9
	HOM	.43	.21	.16	.07	.20	.06	4
HOM	HET	.53	.14	.21	.09	.25	.08	3
	HOM	.59	.11	.24	.05	.28	.07	9

Next a one-way Welch's ANOVA was conducted to assess whether BrdU-positive cell density differed by hippocampal region (Delacre, Leys, Mora & Lakens, 2018) and showed a significant main effect [F (2, 213.08) = 104.93, $p < .001$] indicating that cell density did differ across hippocampal regions. Post hoc comparisons using the Games-Howell test were conducted to identify the origin of the differences. All three areas differed significantly to each other with the DG having the highest cell density followed by the CA1 and lastly the CA2+CA3 (See table 1 for means and SDs).

Neurogenesis in the DG

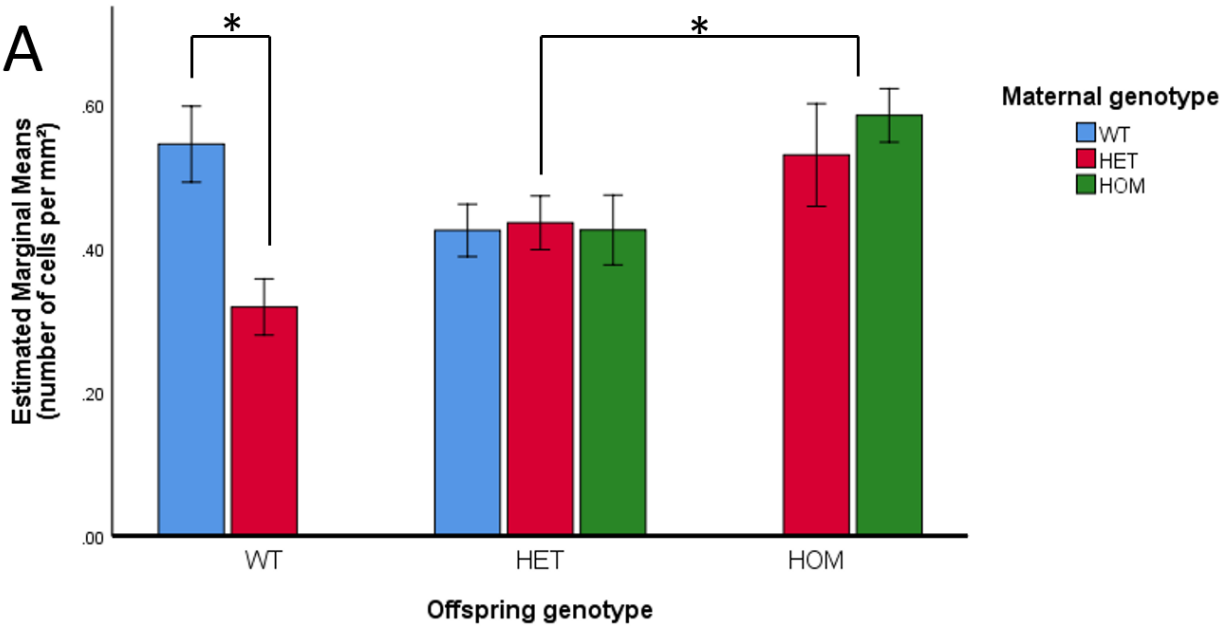
A two-way ANOVA was conducted to assess the influence of maternal SERT genotype and offspring SERT genotype on cell density for the DG. We found a significant interaction between maternal and offspring genotype for mean cell density in the DG [F (2, 110) = 4.01, p = .021]. This indicates that the genotype difference in cell density depends on the maternal genotype. There was a significant genotype effect [F (2, 110) = 4.027, p = .021],

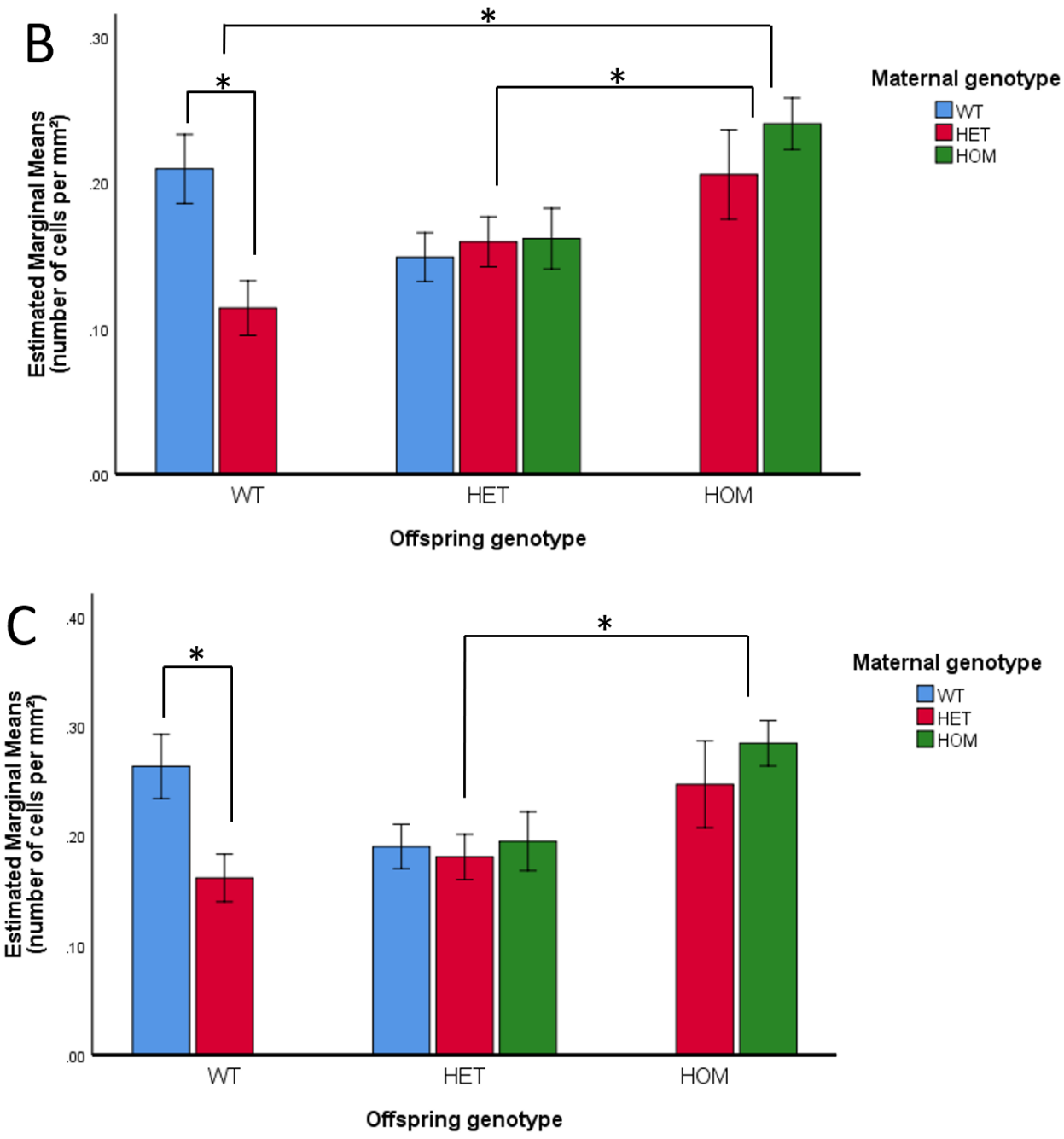
with a significant difference between HET (*M** = .429, *SE* = .024) and HOM (*M** = .558, *SE* = .040, *p* = .021), indicating HOMs had higher mean cell density within the DG. The difference between HOM and WT (*M** = .432, *SE* = .033) was approaching significance (*p* = .052) while there was no significant difference between WT and HET (*p* = 1.00). The effect of maternal genotype was not significant [*F* (2, 110) = .115, *p* = .891]. See figure 11A.

Planned comparisons revealed that the mean difference in cell density between maternal WT and HET genotypes within WT offspring was significant in the DG [*t*(26.152) = 4.248, *p-adj* < .001], but was not significant between HET and HOM genotypes within HOM offspring [*t*(6.710) = -.904, *p-adj* = 1.191], or between WT, HET and HOM genotypes within HET offspring [*F*(2, 55) = .019, *p-adj* = .2.943].

Figure 11

Bar charts of mean new cell density for hippocampal DG, CA1 and CA2+CA3 regions.





Note. A. B and C correspond to DG, CA1 and CA2+CA3 respectively. Error bars represent ± 1 standard error of the means. * Denotes significant differences.

Neurogenesis in the CA1

Next, a two-way ANOVA was conducted to assess the influence of maternal SERT genotype and offspring SERT genotype, on cell density for the CA1. There was also a significant interaction between maternal and offspring genotype in CA1 mean cell density [F (2, 95) = 3.808, p = .026], indicating that in the CA1, maternal genotype affects the

difference in mean cell density in offspring genotype (figure 11B). There was a significant genotype effect [F (2, 95) = 4.579, p = .013], with a significant difference between WT (M* = .162, SE = 0.015) and HOM (M* = 0.223, SE = .018, p = .029) with HOMs having a higher mean cell density than WT in the CA1. There was also a significant difference between HET (M* = .157, SE = .011) and HOM (p = .005) with HOMs having higher cell density again, but no significant difference between WT and HET (p = 1.00). The effect of maternal genotype was not significant [F (2, 95) = .362, p = .697].

The mean difference in cell density between maternal WT and HET genotypes within WT offspring was significant in the CA1 [t(20.663) = 3.199, p-adj = .012] but not significant between maternal HET and HOM genotypes within HOM offspring [t(6.069) = -.922, p-adj = 1.176], or between maternal WT, HET and HOM genotypes within HET offspring CA1 F(2, 49) = .126, p-adj = 2.646].

Neurogenesis in the CA2+CA3

Lastly, a two-way ANOVA was conducted to assess the influence of maternal SERT genotype and offspring SERT genotype, on cell density for the CA2+CA3 region. For the CA2+CA3 region the interaction was not significant [F (2, 95) = 1.973, p = .144]. There was a significant genotype effect within the CA2+CA3 region [F (2, 95) = 3.868, p = .024], with a significant difference between HET (M* = .189, SE = .013) and HOM (M* = .266, SE = .022, p = .011) where HOMs had a higher mean cell density, but no significant difference between WT (M* = .212, SE = .018) and HET (p = .881) or WT and HOM (p = .201). The effect of maternal genotype was not significant [F (2, 95) = .454, p = .636]. See figure 11C.

The mean difference in cell density between maternal WT and HET genotypes within WT offspring was significant in CA2+CA3 [t(28.269) = 2.854, p-adj = .024] but not significant between maternal HET and HOM genotypes [t(7.293) = -1.064, p-adj = .963] or between maternal WT, HET CA2+CA3 [F(2, 35.792) = .185, p-adj = 2.496].

Discussion

This experiment aimed to examine the effects of both offspring and maternal SERT genotype on early postnatal neurogenesis in the hippocampus. While most studies have focused on the genotype of the individual, the maternal SERT genotype's effect on early neurogenesis has been less studied. It was hypothesised that the DG would have the highest cell density of the hippocampal regions. Second, it was hypothesised that the HOM offspring group would have the greatest density of newly formed cells, followed by the HET then the WT groups. Third, it was hypothesised that the maternal HOM would have higher cell density than the maternal HET and WT groups across the offspring genotypes.

Firstly, we found that the DG had the highest newborn cell density of the three hippocampal subfields which supported the first hypothesis. This is consistent with studies showing that the DG contains the neurogenic SGZ (Altman & Das, 1966; Alvarez-Buylla & García-Verdugo, 2002; Duan et al., 2008; Duman, 2004; Ehninger & Kempermann, 2008; Eriksson et al., 1998; Gage, 2002; Kempermann et al., 2004a, 2004b; Kuhn et al., 1996; Vadodaria & Jessberger, 2014; van Praag et al., 2002; Zaverucha-do-Valle et al., 2013; Zhao et al., 2008).

Secondly, our findings indicate that offspring SERT genotype influences neurogenesis in early life, with HOMs having higher newborn cell densities than HETs and WT. This partly supports our second hypothesis as HOMs consistently had the highest newborn cell density in all three regions. However, the differences were only between HOM and HETs in all regions and between HOM and WTs in the DG and CA1. WTs were not significantly different than HETs in the CA1 and CA2+CA3 regions or from HOMs in the CA2+CA3. Nonetheless, WTs had lower levels of newborn cell density than HOMs in all regions.

This is consistent with previous findings that altered amounts of 5-HT during development affects neurodevelopment (e.g., Faber & Haring, 1999; Matsukawa et al., 2003; Massart et al., 2012; Santarelli et al., 2003; Vitalis et al., 2007). As HOM offspring showed higher cell density than HETs and WTs the findings are partially consistent with previous research illustrating that a genetic inactivation of SERT and its resulting increased 5-HT levels, leads to increased neurogenesis (Karabeg et al., 2013).

While our hypothesis was not fully supported, it should be noted that virtually all research (behavioural and otherwise) are typically performed in offspring of HET/HET breeding. Inspection of the results show that in these animals we do find the gene-dosage dependent increase in neurogenesis in all three hippocampal subregions. The non-significant result may be a result of the unequal variances and a lack of statistical power as some genotype groups had very small numbers.

Lastly, we found significant interactions between the maternal and offspring genotype in the DG and CA1 regions, indicating that in these regions the maternal genotype influences newborn cell density. Further analysis revealed maternal genotypes only differed in WT, where WT offspring from maternal WTs had greater newborn cells than WT offspring of maternal HET. On the other hand, there were no maternal genotype effects for HET or HOM offspring. Thus, these interactions were driven by maternal genotype differences within WT offspring, leading to WT animals with HET mothers have lower neurogenesis than WT animals with WT mothers.

To the best of our knowledge, this is the first time a maternal genotype effect on neurogenesis has been described and, as such, it is very difficult to interpret, particularly as this effect was only seen in WT offspring rats. It can be speculated, however, that the maternal HETs have (albeit slightly) higher extracellular serotonin levels which may have affected foetal development. As discussed in the introduction, early in development, 5-HT levels in the foetus are mainly driven by maternal supply. This excess maternal 5-HT during development may 'program' WT offspring neurodevelopment to prepare them for their environment, as seen in Heiming et al. (2009). Excess maternally supplied 5-HT may, for example, activate or even upregulate the offspring's 5-HT1A autoreceptors to decrease serotonergic cell firing and decrease extracellular 5-HT. This effect is certainly seen in SERT knockout mice and after SSRI treatment (Fabre et al., 2000; Le Poul et al., 2000). Given that HET and HOM offspring are likely to have downregulated 5-HT1A receptors, the influence of the maternal genotype (and the resulting changes in 5-HT levels) may have been blunted in these animals.

In addition to the maternal genotype dependent differences in perinatal 5-HT levels, it is important to realize that all brains were taken one week after birth, suggesting that

differences in maternal care may also have played a role in the maternal genotype dependent changes in neurogenesis.

Poor maternal care is a stressful early life experience which influences the emotional, behavioural and cognitive outcomes of the offspring. Overall, insufficient maternal care exacerbates stress responses, leads to more depressive and anxious behaviours and alters serotonergic signalling (Benekareddy, Goodfellow, Lambe & Vaidya, 2010, Benekareddy, Vadodaria, Nair & Vaidya, 2011; Bravo, Dinan & Cryan, 2014; Houwing, Ramsteijn, Riemersma & Olivier, 2019). For example, maternal separation in rats reduced raphe 5-HT concentration and SERT expression and alters 5-HT1A and 5-HT2 receptor expression (Benekareddy et al., 2010, 2011; Bravo et al., 2014; Lee et al., 2007; Vázquez, Eskandari, Zimmer, Levine & López, 2002; Vicentic et al., 2006).

Studies have also shown that there is a link between maternal SERT genotype and maternal care behaviour as human *s* allele carriers and SERT knockout rodents demonstrate altered maternal caregiving (Bakermans-Kranenburg & van Ijzendoorn, 2008; Heiming et al., 2013; Johns et al., 2005; Mileva-Seitz et al., 2011; Winokur, Lopes, Moparthi & Pereira, 2019). Altered maternal caregiving might arise in genetically deficient SERT knockout models of depression through altered hippocampal function. For example, in the hippocampus, 5-HT affects the regulation of oxytocin which plays a crucial role in parenting and maternal care (Bakermans-Kranenburg & van Ijzendoorn, 2008; Feldman & Bakermans-Kranenbur, 2017; Jørgensen, Riis, Knigge, Kjær & Warberg, 2003; Johns et al., 2005). In line with this, Kikusui, Ichikawa and Mori (2009) found that a lack in mother-pup interaction during late lactation leads to increases corticosterone synthesis, reduced BDNF and reduced neurogenesis while Liu, Diorio, Day, Francis and Meaney (2000) find greater maternal care to be associated with increases synaptogenesis or synaptic survival. Maternal care-induced offspring outcomes are also affected by offspring's genotype which may confer a sensitivity to (early) environmental stress for a genetically vulnerable group (Drury et al., 2012; Madrid et al., 2018).

We can therefore speculate that maternal care may be a factor that affected neurogenesis in our study. Based on our findings that WT/WT offspring had neurogenesis levels higher than WT/HETs while no other maternal genotype influences were detected, we would hypothesise that a WT mother may provide better maternal care than HET or HOM

mothers. It could be that the lower neurogenesis observed in WT offspring from HET mothers compared to WT/WTs is due to poorer maternal care by HET mothers. If so, this may mean the WT animals may have a neurodevelopmental sensitivity for early life experiences as seen in previous research (Drury et al., 2012; Madrid et al., 2018). The lack of maternal differences in HET and HOM offspring would, conversely, suggest a blunted response to early life experiences in these animals.

Research also needs to be done examining interactions between maternal care behaviour and offspring genotypes, to determine whether a WT mother displays differences in care behaviour for a WT versus a HET offspring. If so, further studies need to examine the effects of cross-fostering HET and HOM offspring with WT mothers to determine how this effects offspring neurogenesis.

Theoretical implications

Although the maternal genotype/offspring genotype interaction are difficult to explain at this point in time, it is important to note that virtually all previous research on SERT knock-out rats and mice have been done using HET/HET breeding pairings. This includes the research on the biochemical changes observed, as well as the increase in depressive and anxiety-like behaviours in these offspring (Homberg et al., 2007; Olivier et al., 2008). In the current study we found that, for this pairing, there was a gene-dosage dependent increase in offspring neurogenesis and therefore, the following implications are discussed for the main effect of an offspring genotype-dependant increase in neurogenesis.

These results demonstrate that through genetically increasing extracellular 5-HT in development, neurogenesis is increased. The monoamine hypothesis postulates that lower adult levels of monoamines, particularly serotonin, underlie the neurobiology of depression and anxiety, and this is associated with lower levels of adult neurogenesis (Boldrini et al, 2013; Bremner et al., 2000; Brezun & Daszuta, 1999, 2000a; Campbell et al., 2004; Campbell & MacQueen, 2004; Kronmüller et al., 2009; Parihar, Hattiangady, Shui & Shetty, 2013; Sheline et al., 1996; Stockmeier et al., 2004; Videbech & Ravnkilde, 2004). In line with this hypothesis, conditions associated with increased neurogenesis are associated with increases in 5-HT signalling (Dekeyne et al., 2008; Kim et al., 2012; Klempin et al., 2013), and

conversely, conditions associated with decreased neurogenesis such as aging, high corticosterone levels, stress and malnutrition also alter the serotonergic system (Blatt et al., 1994; Chalmers et al., 1993; Gould, 1999; McKittrick et al., 1995; Míguez et al., 1999; Neumaier et al., 1997; Watanabe et al., 1993). Thus, serotonergic signalling appears to be critically involved in neurogenesis, and our data are consistent with the stimulatory role of 5-HT on neurogenesis. This effect is likely mediated (at least in part) by BDNF as this protein regulates neurodevelopment and plasticity in neural circuits (Mattson, Maudsley & Martin, 2004; Pascual-Brazo et al., 2012; Scharfman et al., 2005) and is also enhanced by 5-HT (Kronenberg et al., 2016). Studies showing that SSRIs increase BDNF expression also suggest a role for BDNF in antidepressant action (Brunoni, Lopes & Fregni, 2008; Duman & Monteggia, 2006; Martinowich & Lu, 2008).

Our findings are also consistent with the paradoxical effects of serotonin. While in adulthood depression seems to be associated with decreased 5-HT and decreased neurogenesis, our data show that early life increases in 5-HT are associated with increased neurogenesis in rats that in adulthood develop symptoms of depression and anxiety. These data are in line with other animal research emphasizing the 5-HT paradox. Overall, stress models of depression find reductions in adult hippocampal neurogenesis and neuroplasticity (Chen et al., 2010; Djordjevic et al., 2012; Frodl et al., 2010; Jacobs et al., 2000; Jayatissa, Bisgaard, Tingström , Papp & Wiborg, 2006; Lee et al., 2006; Luo et al., 2005; Perlman et al., 2012; Vollmayr et al., 2003; Zeng et al., 2012), while SERT knock out models exhibit an increase in neurogenesis (Ferrés-Coy et al., 2013; Karabeg et al., 2013; Schipper, Kiliaan & Homber, 2011).

The difference between the two types of depression models studied, however, is the fact that SERT knock out affects hippocampal connectivity starting at development while stress models of depression induce depression without this early alteration of neural connectivity taking place. We propose that this difference is the basis for the 5-HT paradox, where depression in genetically SERT compromised individuals versus non-compromised individuals arises via different underlying mechanims, ultimately leading to two (or more) different types of depression. In this respect, it is important to realize that neurogenesis during development and in adulthood are fundamentally different processes, as alluded to in the introduction. It is therefore paramount to investigate whether HOMs and HETs also have

altered neurogenesis in adulthood, and, if so, whether adult neurogenesis is increased or decreased, as well as the associated depressive outcomes.

Irrespective of this, neurogenesis is only the first step in neuronal plasticity. 5-HT regulates other developmental processes, such as synaptogenesis, spine genesis and axon guidance, where alterations in 5-HT signalling result in aberrant growth, connectivity and circuitry (Chen et al., 2010; Chugani, 2002; Côté et al., 2007; Faber & Haring, 1999). In addition, we do not know whether these new cells develop into neurons or glial cells and whether or how these are integrated in the hippocampal circuitry remains to be investigated.

Several studies do show that newly generated neurons are functional additions to hippocampal circuitry as they form axonal projections, make synaptic connections and have the same electrophysiological characteristics of neighbouring mature cells in neurotypical animals (Markakis & Gage, 1999; van Praag et al., 2002). However, at least in cell cultures, HOM rats have reduced spine formation (Chaji et al. 2021). Thus, it is conceivable that even if more newborn cells develop into more neurons in HET and HOM offspring, they may not correctly synapse onto their postsynaptic targets. The neurogenesis seen in HOM animals could thus result in aberrant neural connectivity. We suggest that the connections their new neurons might make are likely to be altered from normal development because they are a result of dysregulated 5-HT levels. Indeed, proper synaptogenesis and the development of neurocircuitry requires a critical concentration of 5-HT while many aspects of neurodevelopment are altered in SERT knockout and SSRI treated rodents (Azmitia, 2001; Brezun & Daszuta, 1999; 2000a, 2000b; Chen et al., 2010; Chugani, 2002; Vitalis et al., 2007; Vitalis & Parnavelas, 2003).

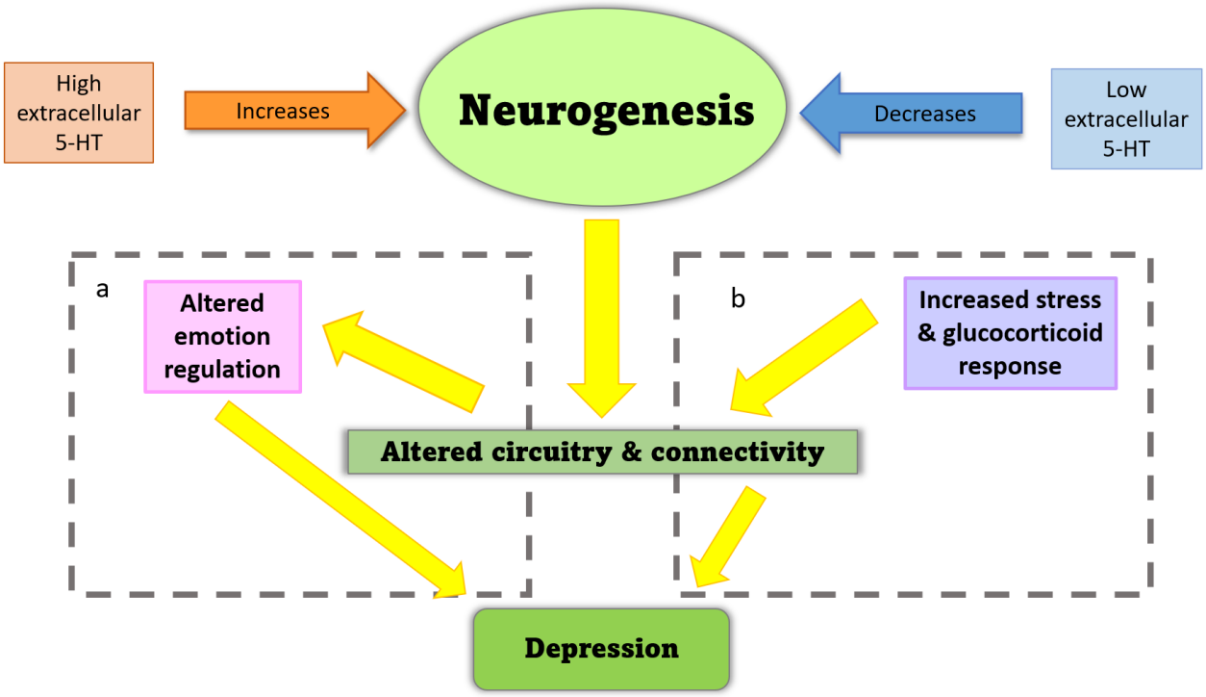
As the hippocampus plays an esssential role in emotion regulation (Kirkby et al., 2018), alterations in the connectivity of the hippocampus is likely to alter emotion regulation. Indeed, mood dysregulation, including depressive behaviour, is associated with hippocampal dysregulation including reductions in neurogenesis, neuron loss and hippocampal volume loss (Boldrini et al, 2013; Bremner et al., 2000; Campbell & MacQueen, 2004; Kronmüller et al., 2009; Parihar et al., 2013; Sheline et al., 1996; Stockmeier et al., 2004; Videbech & Ravnkilde, 2004). Animal studies also find dramatic alterations in the neurocircuitry of SERT knockout animals, such as that of the limbic cortical–ventral striatopallidal reward pathway

(Bearer, Zhang, Janvelyan, Boulat & Jacobs, 2009; Daubert & Condron, 2010). Depressed individuals clearly have altered/disrupted neural connectivity, especially within the hippocampus (Chase et al., 2014; Cullen et al., 2009) and show impairments in other hippocampal dependent functions (Austin et al., 2001; Becker et al., 2009; Bremner et al., 2004; Burt et al., 1995; Ilsley et al., 1995; MacQueen et al., 2003). Therefore, we suggest that the increased neurogenesis in HOM animals in this study is likely to alter their hippocampal regualtion of emotional behaviours which would align with the previous findings that SERT knockout rats have demonstrated altered emotonality and behaviour previously (Kalueff et al., 2010; Olivier et al., 2008).

We therefore propose that the differential effects of 5-HT in the 5-HT paradox are brought about through early-life increases in 5-HT in both animal models and *s* allele carriers, altering their hippocampal neurocircuitry during development. We propose that it is this early altered hippocampal connectivity that leads to altered emotion regulation in genetically vulnerable depressed models and individuals. On the other hand, we hypothesise that in individuals without this genetic reduction in SERT, the induction of depression in later life by stressful life experiences alters their neural circuitry, but does so through a stress-induced loss of connectivity which is restored via antidepressant treatment (see figure 12).

Figure 12

The proposed neurobiological etiology of neurogenic vs stress-induced depression



Note. A shows the neurogenic model of depression which occurs due to developmental serotinergic changes and B shows the proposed mechanism of stress induced depression.

Experience and learning throughout the lifespan underlie sensory plasticity and involve the regulation of synaptic formation, removal and morphological remodelling of synapses and dendritic spines (Feldman, 2009; Holtmaat & Svoboda, 2009; Teissier et al., 2017; Trachtenberg et al., 2002; Wilbrecht et al., 2010). An important aspect of the neurogenic hypothesis that needs further study is the effect increasing neurogenesis has on learning and the response to stressful experiences. Theories regarding the role of adult neurogenesis in hippocampal function suggest a role in learning, or to allow the DG to accommodate continual modulation and information-processing demands (Gould et al., 1999a; Kempermann, 2002). Indeed, increasing neurogenesis benefits learning (Mattson et al., 2004; Sahay et al., 2011) while reducing neurogenesis impairs learning (Hu et al., 2009; Kim et al., 2012; Mohapel et al., 2005; Saxe et al., 2006; Shors et al., 2002; Winocur et al., 2006; Zhang et al., 2008). Supporting this notion, studies have shown that disruption of neurogenesis also leads to significant memory and learning impairments and that altered hippocamapal functioning in mood disorders is also associated with cognitive and learning impairments (Becker et al., 2009; Bremner et al., 2004; Deckersbach et al., 2006; Gourevitch, Rocher, Pen, Krebs & Jay, 2004; Moser et al., 1995; Moser & Moser, 1998; Snyder, Hong, McDonald & Wojtowicz, 2005).

The neurogenic hypothesis postulates that it is the structural plasticity associated with enhanced neurogenesis that is compromised in depression. Indeed, structural plasticity, particularly in synapses, connectivity and receptors, is involved in depression (Albert & Lemonde, 2004; Cai et al., 2013; Ferrés-Coy et al., 2013; Fuchs et al., 2004; Kaiser et al., 2015; Lesch & Waider, 2012; Manji et al., 2001; McEwen & Magarinos, 2001; Pittenger & Duman, 2008; Richardson-Jones et al., 2011; Zhuang et al., 1999).

Given the interaction of the 5-HTTLPR *s* allele and stress on offspring emotionality, behaviours and learning and the effect of experience on structural plasticity (Karg Burmeister, Shedden & Sen, 2011; Lemaire, Koehl, Moal & Abrous, 2000; Furlong et al., 1998), we speculate that the enhanced neurogenesis in animals with a genetic inactivation of SERT (compared with HET animals) could enhance their neuroplasticity within the hippocampal circuitry involved in learning and stress responses. Stress has been shown to

alter neuronal circuitry and connectivity in the amygdala and enhance molecular plasticity in the hippocampus (Djordjevic et al., 2012; Figueiredo, Bodie, Tauchi, Dolgas & Herman, 2003; Herman, Adams & Prewitt, 1995; Sripada et al., 2012; Vyas, Jadhav & Chattarji, 2006). The hippocampus typically exerts inhibitory control over the HPA axis, an effect that is reduced in depression (Duman & Monteggia, 2006; Jankord & Herman, 2008; Massart et al., 2012; Sapolsky et al., 1984; Winocur et al., 2006). As this neuroplasticity in HOM animals would be achieved through dysregulated 5-HT systems, it is likely to lead to altered connectivity and activity. This may be a mechanism for the altered stress responses seen in depression (Gold & Chrousos, 2002), *s* allele carriers and in SERT compromised rats (Jiang, Wang Luo & Li, 2009; Miller, Wankerl, Stalder, Kirschbaum & Alexander, 2013). Thus, animals with enhanced neurogenesis may effectively have an enhanced form of 'learning' in stressful contexts which enhances their stress responses and subsequently enhances the anxiety and depressive response to otherwise less salient stimuli (Heiming et al., 2009).

The enhanced neurogenesis seen in HOMs in the present study are, as discussed above, like due to an increase in extracellular 5-HT levels, leading to an enhanced stimulation of 5-HT receptors. We must, therefore, consider the role of the many different 5-HT receptors in the regulation of neurogenesis in particular. While 5-HT interacts with about 15 different receptors, the 5-HT1A, 5-HT1B/1D, 5-HT2A, 5-HT2C and 5-HT4 receptors have been implicated in neurogenesis (Cryan et al., 2005; Ferrés-Coy et al., 2013; Karabeg et al., 2013; Kondo, Nakamura, Ishida & Shimada, 2015; Mendez-David et al., 2014).

5-HT1A receptor dysregulation has been implicted in the pathophysiology of depression (Blier & Ward, 2003; Le François, Czesak, Stebul & Albert, 2008; Lemonde et al., 2003). The effects of 5-HT1A receptor stimulation is complex as it is highly abundant in the brain, with differential effects of pre- and postsynaptic and auto- and heteroreceptor activation (Celada, Bortolozzi & Artigas, 2013).

Many studies suggests that hippocampal 5-HT1A heteroreceptor activation is important for increasing neurogenesis and antidepressant effects (Barnes & Sharp, 1999; Bockaert, Claysen, Bécamel, Dumuis & Marin, et al., 2006; Pompeiano, Palacios & Mengod, 1992; Riad et al., 2000; Celada et al., 2013; Haddjeri, Blier & Montigny, 1998; Hayakawa, Shimizu, Nishida, Motohashi & Yamawaki, 2008). 5-HT1A heteroreceptors are abundant in limbic and cortical forebrain areas including the hippocampus, amygdala and PFC which are implicated in the regulation of mood and postsynaptic 5-HT1A receptor activation in

corticolimbic areas is typically antidepressive/anxiolytic (Barnes & Sharp, 1999; Bockaert et al., 2006; Pompeiano et al., 1992; Riad et al., 2000; Celada et al., 2013; Haddjeri et al., 1998). Moreover, 5-HT1A receptors on granule cells directly contribute to the neurogenic and behavioural response to SSRIs (Samuels et al., 2015). 5-HT1A autoreceptors, in contrast, induce antidepressant effects through their desensitization which increases downstream 5-HT (Artigas, Romero, de Montigny & Glier, 1996; Blier & Ward, 2003; Celada et al., 2013; Haddjeri et al., 1998; Hervás et al., 2001). The latter is also likely for 5-HT1B autoreceptors which also desensitize after chronic SSRI treatment (Fabre et al., 2000; Moret & Briley, 2000).

Thus, 5-HT1A auto- and heteroreceptor stimulation may function to increase neurogenesis via different methods: the effects of increased 5-HT after 5-HT1A autoreceptor desensitization and the activation of downstream neurogenic pathways of 5-HT1A heteroreceptors, such as CREB-mediated neurogenic pathways (Zhang et al., 2016) or astroglial S100 pathways (Amitia, 2001; Azmitia et al., 1995; Whitaker-Azmitia et al., 1990a), respectively. This fits with the finding that HOMs have the highest levels of neurogenesis as increased 5-HT in these animals would increase both 5-HT1A autoreceptor desensitization-mediated and heteroreceptor activated neurogenesis. Indeed, 5-HT1A receptor desensitization occurs in mice (Fabre et al., 2000) and rats (Homberg et al., 2007) lacking SERT. However, this has only been observed in adult rats so far. Whether these receptors are also downregulated at postnatal day 7 remains to be investigated. Neurogenesis may be lower in HETs, as their 5-HT1A autoreceptors may not desensitize since the level of extracellular 5-HT typically in HETs is similar to that of WTs (Kim et al., 2005), this would also lead to less 5-HT1A heteroreceptors activation. HOM animals and humans with two *s* alleles may thus acquire altered neurocircuitry through 5-HT1A autoreceptor desensitization and increased stimulation of postsynaptic 5-HT1A receptors on granule cells. This is a possible pathway for anxious and depressive outcomes in genetically vulnerable individuals lacking normal SERT levels. In line with this, 5-HT1A autoreceptors have been shown to be involved in anxiety-like behaviours (Gross et al., 2002; Leonardo & Hen, 2008) and their dysregulation during development results in an anxious and depressive phenotype (Garcia-Garcia et al., 2014; Le François et al., 2008; Richardson-Jones et al. 2011).

Individuals with greater extracellular 5-HT since development would also likely undergo increased 5-HT2A receptor stimulation, while non-genetically vulnerable individuals

who develop depression may have a downregulation of their 5-HT2A receptors later in life which has been observed in depressed individuals (Owens & Nemroff, 1994). As 5-HT2A receptor activation enhances associative learning and cell proliferation, increased synaptic density and cell survival (Azmitia, 2001, Banasr et al., 2003; Harvey, 2003; Klempin et al., 2010; Niitsu et al., 1995; Vitalis & Panavelas, 2003), 5-HT2A-stimulated associations may lead to aberrant hippocampal circuitry and contribute to mood dysregulation in HOMs and *s* allele carriers. As it has been suggested that 5-HT2A receptors are involved in active coping with stress and are associated with enhanced plasticty (Carhart-Harris & Nutt, 2017), an increase in 5-HT2A receptors later in life may be an adaptive response to stress-induced depression, where increasing learning and neurogenesis could help alleviate depressive symptoms.

Stimulation of 5-HT2C can also enhance neurogenesis in animals (Soumier, Banasr, Goff & Daszuta, 2010). However, the blockade of 5-HT2C receptors also potentiates SSRI-induced extracellular 5-HT increase (Cremers et al., 2004). Thus, if 5-HT2C receptors were to desensitize after overstimulation in HOM animals this may further increase extracellular 5-HT and downstream neurogenesis. Again, we have some (unpublished) evidence for a downregulation of 5-HT2 receptors in HOM rats in adulthood, but comparable results for early in life are still lacking.

As demonstrated previously, increased stimulation of hippocampal 5-HT3 and 5-HT4 receptors may be partly responsible for enhanced neurogenesis in the HOM animals, possibly through increasing p-CREB and/or BDNF for the latter (Kondo et al., 2015; Mendez-David et al., 2014; Pascual-Brazo et al., 2012; Tamburella, Micale, Navarria & Drago, 2009). 5-HT3 receptors are involved in the regulation of neurodevelopment by 5-HT, e.g. fine-tuning of microcircuitry (Engel et al., 2013). Stimulation of the 5-HT4 receptor may also reverse granule cell maturity like the effects of SSRIs (Imoto et al., 2015; Kobayashi et al., 2010).

Thus, we speculate that overall the increase in 5-HT in HOMs is likely to effect neural activity through various 5-HT receptor pathways.

The current findings inform our understanding of depression especially in the context of the monoamine and neurogenic/neuroplasticity hypotheses. The findings also illustrate that

maternal SERT genotypes, and their corresponding 5-HT levels may influence the developing offspring.

An increasing number of pregnant women with depression are being treated with SSRIs, this is important as in vitro and in vivo findings with SSRI show that disruptions of 5-HT signalling within the placenta can affect feotal cell proliferation and neurodevelopment (Rosenfeld, 2020). SSRIs readily cross the placental barrier (Kim et al., 2005; Lattimore et al., 2005; Mulder, Ververs, de Heus & Visser, 2011; Noorlander et al., 2008) and feotal exposure to SSRIs can dramatically increase the risk of mortality, long-term alterations in SERT levels in the raphe nucleus and depressive- and anxiety-related behaviours in adult mice which are similar to the behavioural phenotype seen in mice that are genetically deficient in SERT expresssion (Ansorge et al., 2004; Noorlander et al., 2008; Oberlander et al., 2009). Perinatal SSRI treatment and genetic inactivation of SERT both result in developmental delays for rats (Kroeze et al., 2016) while maternal SSRI use and depression in humans is associated with clinically significant emotional and behavioral problems for offspring (Nulman et al., 2012; Oberlander et al., 2010; Pearson et al., 2013). Our findings contribute to our understanding of the consequences of altering maternal and feotal 5-HT on brain plasticity by providing new evidence that maternal 5-HT levels determined by the maternal SERT geneotype affect early offspring neurodevelopment by affecting the level of neurogenesis taking place.

As hippocampal neurogenesis influences learning and memory, the maternal SERT genotype may, through affecting neurogenesis, influence learning and memory performance in offspring. Laplante, Brunet, Schmitz, Ciampi and King (2008) for example, found prenatal maternal stress to be associated with lower intellectual and language performance. WT animals from HET mothers showed a reduction in neurogenesis compared to WT/WT animals, thus, the finding that altered maternal SERT levels affects neurogenesis warrants further investigation of whether and how maternal SERT genotype affects offsping learning capabilities.

Neurogenesis underlies the therapeutic response to SSRIs (Santarelli et al., 2003) and is therefore though to be therapeutic. If increasing neurogenesis in later life is therapeutic (e.g., after stress-induced depressive episodes) then research into neurogenic pathways is an avenue for treatment research. Nakano-Kobayashi et al. (2017) found that inducing neurogenesis prenatally can rescue aberrant cortical formation in down syndrome mouse embryos and prevent the subsequent development of abnormal behaviours (e.g., decreased exploration, spatial learning, contextual fear learning) in these offspring, as well as rescuing proliferative deficits in human neural stem cells from individuals with down syndrome. SERT KO models may be useful in further studying this effect as the proliferative effects of 5-HT may also facilitate this restorative induction of neurogenesis.

We note that the homogeneity of variance assumption of the ANOVA was not met due to unequal sample sizes, so these results should be interpreted with caution. In addition, some of the groups were relatively small which may have led to a reduction in statistical power. However, the type IV sum of squares analysis used took the unequal variances and missing cells (as WT/HOM or HOM/WT animals are not possible) into account. Moreover the interaction was significant for two regions and all three regions combined and the genotype effect was significant and identical for all three regions, indicating that these results are indeed indicative of real differences and interaction effects of the maternal and offspring genotype.

Another factor to consider is with BrdU labelling as BrdU's bromine side group alters DNA stability increasing the risk of sister chromatid exchanges, mutations, DNA strand breaks and lengthens the cell cycles of the cells it is incorporated within (Taupin, 2007). BrdU is more a marker of DNA synthesis which is used as a proxy signal for the S-phase of the cell cycle and cell proliferation, however, BrdU will also label cells undergoing DNA repair, abortive cell cycle re-entry, as a prelude to apoptosis, and gene duplication without cell division, leading to polyploidy (Taupin, 2007). Thus, BrdU has mitogenic effects and is potentially more toxic to cells than tritiated thymidine (Magavi & Macklis, 2008; Taupin, 2007). Hancock, Priester, Kidder and Keith (2009), however, did not find this to be the case in vivo.

Cameron and Mckay (2001) found that commonly used BrdU doses of 50mg/kg only labelled a fraction of S phase cells in adult rats and the number of labelled cells did not plateau until a dose of 300mg/kg was used. Moreover, doses as high as 600mg/kg did not show noticeable detrimental effects (Cameron & Mckay, 2001). Thus, the BrdU dosage used of 25mg/kg may not have detected all the newborn cells present. Other neurogenesis detection methods may provide more accurate results. Endogenous cell-cycle proteins, for example, are increasingly being used detect cells during different phases of cell-cycle progression (Kuhn, Eisch, Spalding & Peterson, 2016). Ki67 is one such marker, expressed both during S phase and through the remainder of the cell cycle (Kuhn et al., 2016).

Maternal care behaviour was not measured in this study but further investigation would serve to examine the contribution (if any) of maternal care on offspring neurogenesis. Alternatively, cross fostering animals immediately after birth could control for genotype changes in maternal care. An important assumption of cross fostering and maternal care studies is that a mother would spend equal time and care for each pup, regardless of its genotype. However, this has not been invesitgated in any detail. Thus, subsequent studies would benefit from rapid (i.e. postnatal day 1) genotyping of the offspring and subsequent analysis of maternal care, for example, using the classical pup retrieval experiment and placing pups from different genotypes in different arms of a maze. This would allow us to invesitgate whether the latency to retrieve pups is dependent on the pup's genotype.

Future studies should further investigate the relationship between 5-HT and neurogenesis in SERT KO rats. This would include investigating whether neurogenesis is also altered in adulthood in HOM and HETs compared to WT. Moreover, while downregulation of 5-HT1A and 5-HT2 receptors have been identified in HOMs in adulthood, it is unclear whether such downregulations already occur early in life (i.e. at postnatal day 7). In addition to the SERTs there are other genetic models for depression, such as the Flinders Sensitive and Fawn Hooded rats (Overstreet, Friedman, Aleksander, Mathé & Yadid, 2005; Rezvani, Parsian & Overstreet, 2002). It would be interesting to see whether these animals also demonstrate increased hippocampal neurogenesis early in life.

Given the fact that non-genetic factors are also important in the etiology of depression and, especially early stressful life events, strongly interact with the SERT genotype, future

research should examine stress-induced changes in serotonergic receptor and neurogenesis in the brains of genetically vulnerable and genetically resilient animals.

Future research should also be directed at investigating the fate of these new cells. It should be examined whether they develop into neurons or glial cells, where in the hippocampal formation they migrate too and ideally, whether they form synaptic contacts with other cells. This could be extended into examining whether experience of stressed or depressed mothers enhances neurogenesis for offspring in a way that serves as adaptive for dealing with future stressors. Any neurogenic or emotional and behavioural differences found between genotypes during early development would need follow up in adulthood to see if they are retained.

The study of neurological diseases/disorders requires the ability to not only determine what molecules are implicated, but their concentrations and their spatial distributions (Schubert et al., 2016; Shariatgorji et al., 2014). Matrix assisted laser desorption ionization is an imaging mass spectrometry technique (MALDI- IMS) that allows for the detection, identification and distribution mapping of hundreds of molecules simultaneously (Hanrieder, Phan, Kurczy & Ewing, 2013; Shariatgorji et al., 2014). In a next step, MALDI-IMS would be used to identify global brain changes among the SERT-/- genotype, and in particular, changes neurotransmitter quantities and distributions.

Conclusion

This study examined the effects of offspring and maternal genotype on early postnatal neurogenesis to determine whether early neuroplastic changes occur in SERT knockout rats compared to wild types. We found that offspring genotype increased neurogenesis in a genotype-dependent manner from HET to HOMs but in WT offspring we found that the maternal genotype influenced neurogenesis. WT animals had lower neurogenesis when they had a HET mother while the WT/WT animals showed neurogenesis levels between HET and HOMs. Altogether these results suggest that altered 5-HT during development does indeed affect neuroplasticity in the form of altered levels of neurogenesis which support the notion of a linked monoamine signalling and neurogenic theory of depression.

CHAPTER TWO

MALDI BRAIN IMAGING

Introduction

The study of neurological diseases/disorders requires the ability to not only determine what molecules are implicated, but their concentrations and their spatial distributions. Indeed, the concentrations of chemical compounds/ molecules in the brain are crucial to normal as well as pathological processes, we therefore need this information as well as spatio-temporal distribution information to fully uncover what neurochemical changes occur in disease states (Schubert et al., 2016; Shariatgorji et al., 2014).

Matrix assisted laser desorption ionization imaging mass spectrometry allows for the detection, identification and distribution mapping of hundreds of molecules simultaneously without a great deal of a priori knowledge of potential targets of interest (Hanrieder et al., 2013; Shariatgorji et al., 2014). This mass spectrometry technique involves sample analytes being embedded in a matrix that absorbs energy from an ultraviolet laser leading to the desorption and ionization of the constituent chemicals and the acquisition of a mass spectra of these chemicals (Hanrieder et al., 2013; Schubert et al., 2016). Thus, this technique allows for the identification of compounds that are dysregulated between samples.

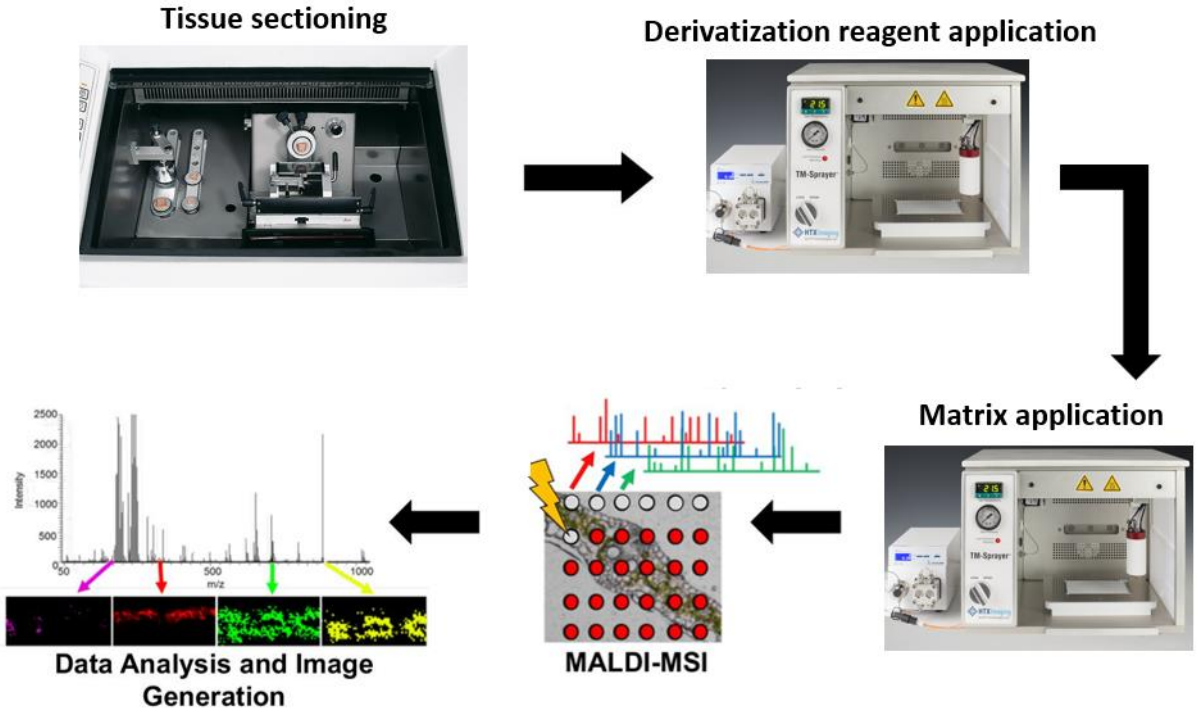
MALDI principles

MALDI-IMS is an imaging mass spectrometry technique effective for molecular studies of complex biological samples, such as tissue sections, directly (Schwamborn & Caprioli, 2010; Setou, 2010). MALDI-IMS is theoretically capable of analysing high molecular mass biomolecules of 200 kDa or more, as well as small molecules of less than 1 kDa molecular mass (Chaurand, Cornett & Caprioli., 2006; Cornett Reyzer, Chaurand & Caprioli, 2007; Karas, Bahr, Ingendoh & Hillenkamp, 1989; Schwamborn & Caprioli, 2010; Tanaka et al., 1988). MALDI-IMS allows for the identification of not only complex normal biological processes, but also pathological processes through the direct detection of biomolecules such as proteins, peptides, lipids as well as their spatial distribution and intensity profiles within tissues and single cells (Cornett et al., 2007; Hanrieder et al., 2013; Zimmerman, Monroe, Tucker, Rubakhin & Sweedler, 2008). Essentially, mass spectrometry measures the masses of isolated gas-phase ionised molecules directly by their behaviour in electromagnetic fields (Beavis & Chait, 1990) while the topical addition of an organic matrix over the sample before laser irradiation assists in laser ablation of the molecules and lessens

fragmentation to stabilise molecules (Medzihradszky et al., 2000; Zenobi & Knockenmuss, 1998; Zimmerman et al., 2008). See figure 13.

Figure 13

Simplified MALDI-IMS workflow



Note. Modified from Dueñas, Larson and Lee (2019).

Thus, MALDI-IMS generates two-dimensional ion images for each of the mass-to charge (m/z) ratio values detected at each pixel (microscopic area) of tissue and therefore provides the spatial distribution the constituent compounds within that sample (Caprioli, Farmer & Gile, 1997; Chaurand, Schwartz & Caprioli, 2002) MALDI is also amenable to a broad range of analytes including proteins, peptides, cell metabolites, lipids, hormones, glycans, toxins, drugs and drug metabolites (Aichler & Walch, 2015).

Chemical stains, immunohistochemical tags and radiolabels are common methods for visualizing and identifying molecular targets. However, they are limited in their specificity and the number of target compounds which can be simultaneously investigated. Moreover, use of specific molecular tags requires a priori knowledge of the target species, which limits their use for molecular discovery (Cornett et al., 2007; Hanrieder et al., 2013). MALDI-IMS

does not require a priori knowledge of target species nor is it dependent on antibody or primer availability and specificity which allows for identification of new compounds (Hanreider et al., 2013; Schubert et al., 2016).

Classical proteomic, metabolomic and lipidomic techniques involve homogenization steps and therefore cannot provide spatial distribution information (Schubert et al., 2016), unless repeated over many different brain regions. Sample preparation for these techniques also involve multiple time-consuming steps that can lead to analyte material loss while MALDI-IMS involves minimal sample preparation and sample handling (Schwartz, Reyzer & Caprioli, 2003; Setou, 2010). Thus, MALDI's main advantages include detection, identification and spatial mapping of hundreds of compounds simultaneously without a priori knowledge of targets (Caprioli et al., 1997; Cornett et al., 2007; Hanrieder et al., 2013; Schubert et al., 2016; Schwartz et al., 2003; Setou, 2010). Additionally, MALDI-IMS is also advantageous as it offers high tolerance against contaminants and buffers (Calvano, Monopoli, Cataldi & Palmisano, 2018; Yamaguchi, Fujita, Fujino & Korenaga, 2008), its high sensitivity and compatibility with diverse mass analysers (Palanisamy, Huang, Zhao, Zhu & Zhang, 2019).

MALDI-IMS workflow

Derivatization

Small molecules such as neurotransmitters which we are interested in, tend to have poor ionization efficiencies, and can be masked by the overlapping signals of isobaric compounds (Esteve, Tolner, Shyti, van den Maagdenberg & McDonnell, 2016; Guo & He, 2007; Hanrieder et al., 2013; Shariatgorji et al., 2014; Ye, Wang, Greer, Strupat & Li, 2013). Chemical derivatization improves the ionization and detection of small molecules, makes them less volatile and increases their mass by a known amount (Chacon et al., 2011; Esteve et al., 2016; Zaikin & Halket, 2006). Derivatization typically introduces an ionizable or ionic group to the analyte being derivatized (Zaikin & Halket, 2006). Shariatgorji and colleagues (2014, 2015) developed a method for chemical derivatization of smaller molecules such as neurotransmitters by reaction with pyrylium salts that facilitates their detection by MALDI-IMS. Pyrylium salts such as 2,4-diphenyl-pyranylium tetrafluoroborate (DPP TFB) react with the amino group (NH2) of amines to convert the amine into a pyrylium salt structure and

produce N-alkyl- or N-aryl- pyridinium derivatives (Shariatgorji et al., 2014, 2015). However, there are other reagents that can be used to derivatize amine metabolites in tissue (e.g. p-N,N,Ntrimethylammonioanilyl N-hydroxysuccinimidyl carbamate iodide [TAHS] and 4-hydroxy-3-methoxycinnamaldehyde [CA]) each with its own optimal reaction temperature and concentration (Esteve et al., 2016; Manier, Spraggins, Reyzer, Norris & Caprioli, 2014).

Matrix application

The defining step of MALDI-IMS process involves coating the sample with an energy absorbing matrix that facilitates ablation and ionization of the sample compounds Caprioli et al., 1997; Ehring et al., 1992; Hanrieder et al., 2013). The matrix facilitates soft desorption of analyte molecules upon laser irradiation, by absorbing some of the laser's energy, and reduces analyte fragmentation making them more stable molecules during analysis (Caprioli et al., 1997; Hillenkamp, Karas, Beavis & Chait, 1991; Karas, Bachmann, Bahr & Hillenkamp, 1987; Medzihradszky et al., 2000; Tanaka et al., 1988; Zaikin & Halket, 2006). The matrix usually consists of small organic aromatic molecules with high absorbance at the wavelength of the laser used, it is typically a crystalline UV absorbing matrix, although other matrices can be used which differ in the amount of energy they impart to the sample biomolecules during desorption and ionisation and the resulting degree of molecule fragmentation (Calvano et al., 2018; Leopold, Popkova, Engel & Schiller, 2018; Mann, Hendrickson & Pandey, 2001; Wang, Zhao, Guo, Cai & Liu 2013).

MALDI-IMS requires that analytes within tissue samples be embedded within the crystallised matrix or they may delocalise (Zimmerman et al., 2008). Typically, the analyte is co-crystallized with a surrounding excess of the solid matrix that crystallizes as it dries, essentially embedding these analytes within matrix crystals (Calvano et al., 2018; Dreisewerd, 2003; Leopold et al., 2018; Zenobi & Knockenmuss, 1998). The matrix typically consists of a solution of water, organic solvents containing ethanol/methanol or acetonitrile as well as a strong acid such as trifluroacetic acid (TFA) in which the matrix is dissolved (Palanisamy et al., 2019; Schwartz et al., 2003).

The choice of matrix used determines which molecules can be analysed and the level of sensitivity in their detection (Esteve et al., 2016). Similarly, the matrix deposition

conditions, composition, concentration, application method and thickness of coating are all factors contributing to the quality of the mass spectra attained (Zimmerman et al., 2008). An appropriate matrix should result in a high signal-to-noise (S/N) ratio, high spectra resolution, little analyte fragmentation and moderate matrix background to minimize the interference between matrix and analyte signals, although this is challenging for small molecules (Leopold et al., 2018). Suitable matrices must fulfil the five following criteria (Leopold et al., 2018). First, matrices require strong absorption of the wavelength of the laser as ionization efficiency and ion yield effectively increase with an increasing absorption coefficient of the matrix. Second, matrices should ensure analyte ion formation. In cinnamic and benzoic acid derivatives the carboxylic acid structure being polar and acidic ensures matrix solubility in polar solvents and analyte protonation (Leopold et al., 2018). Third, the matrix needs to remain stable under high vacuum conditions. Fourth, ideal matrices should isolate the ions generated and prevent the formation of analyte clusters such as dimer formations; this is a primary reason for the large excess of matrix over the analyte. Lastly, crystals should be as homogenous as possible to ensure high 'shot-to-shot' reproducibility (Leopold et al., 2018).

There are three classes of matrices: classic organic matrices such as cinnamic acid or benzoic acid derivatives which are most commonly used, liquid crystalline matrices for even softer ionization and inorganic matrices such as graphite (Esteve et al., 2016; Lemaire et al., 2006; Leopold et al., 2018). Cinnamic acid derivatives have an advantage of having high tolerance of high concentrations of ionic contaminants such as salts and lipids (Beavis & Chait, 1990). Other desorption and ionization techniques, such as fast atom bombardment and electrospray atmospheric pressure ionization, can get saturated with these contaminating signals (Beavis & Chait, 1990).

The most commonly used organic matrices are α-cyano-4 hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB) and Sinapinic acid (SA), with the first two used for lower weight analyte species and the latter peforming better for higher mass analytes (Norris & Caprioli, 2016; Schwartz et al., 2003; Zimmerman et al., 2008). CHCA crystals are smaller than those of DHB (Altelaar et al., 2007). CHCA generally results in high sensitivity and is a commonly used matrix for smaller low weight (LW) compounds and produces peptide signals substantially higher than other cinnamic acid derivatives indicating more efficient ionization (Beavis & Chait, 1992; Lidgard & Duncan, 1995; Mann et al., 2001; Schwartz et al., 2003; Zimmerman et al., 2008).

Matrix deposition can be either as individual droplets (spotted) on specific areas of interest on the sample tissue, or as a homogenous layer (spray coated) over the entire tissue depending on the spatial resolution desired (Chaurand et al., 2006; Chaurand, Norris, Cornett, Mobley & Caprioli 2006a; Norris & Caprioli, 2013; Schwartz et al., 2003). Spotting allows for faster analysis but tends to produce more irregular crystals and lower resolution profiles of the signals in the chosen sample regions (Schwartz et al., 2003). Spray coating is done either via pneumatic-assisted or electrospray deposition where controlled amounts of matrix dissolved in organic solvents (to achieve stable spraying conditions, e.g., ethanol. acetone, acetonitrile) are sprayed homogenously on the sample tissue in multiple layers (Schwartz et al., 2003). The advantage of automatic spraying is that the optimized spraying conditions can be replicated and applied to large sample sets to ensure reproducibility (Norris & Caprioli, 2016). Spray coating allows for mass spectra to be acquired for the entirety of the sample; therefore, it produces higher resolution images (Schwartz et al., 2003).

Spatial resolution increases as matrix crystal size decreases (Li et al., 2016). Crystal size can be reduced by using a higher solvent percentage (Norris & Caprioli, 2016). The matrix solvent solution evaporates leaving the matrix crystallized on the sample tissue. Matrix concentration affects the crystal coverage and ultimately the mass spectral quality (Schwartz et al., 2003). Higher matrix concentrations result in greater crystal coverage over the sample and higher quality spectra (Lemaire et al., 2006; Schwartz et al., 2003). Schwartz et al. (2003) found that 50:50 ratios of solvents to water produced the best quality spectra, however, this ratio typically requires optimization.

Laser irradiation

A UV-absorbing wavelength laser (there are several UV laser wavelengths and other lasers such as nitrogen and infrared; Zimmerman et al., 2008) then scans across the sample leading to molecules (analytes) to desorb from the sample as multiple laser pulses resonantly excite the matrix, causing rapid localized heating and subsequent co-ablation of analyte and matrix molecules (Dreisewerd, 2003; Hanrieder et al., 2013; Karas & Kruger, 2003; Schubert et al., 2016; Zaikin & Halket, 2006; Zimmerman et al., 2008).

Ionization

Upon laser irradiation the matrix vaporizes and carries with it the co-crystallised analyte molecules into the vapor/ gas phase (Leopold et al., 2018). Ionization occurs through this desorption, where the energy applied from the laser directly produces gaseous ions from the solid- (or liquid-) state sample (Dreisewerd, Schürenberg, Karas & Hillenkamp, 1995; Wang et al., 2013; Zimmerman et al., 2008). In the gas phase, ions are exchanged between the matrix and analyte allowing the formation of analyte ions (Leopold et al., 2018).

Soft ionisation techniques such as MALDI impart low internal/residual energy during the ion formation process resulting in less fragmentation of analyte molecules than hard ionisation and yields more or less direct measures of molecular weights of the molecules of interest via conversion to singly or multiply protonated [Molecular mass + H]+ or deprotonated ions [Molecular mass - H]- that are positively or negatively charged respectively (Medzihradszky et al., 2000; Yamaguchi et al., 2008). However, analyte molecules are typically singly protonated forming quasi-molecular ions with molecular weights corresponding to the original analyte molecular weight plus (or minus in the case of negatively charged ions) the mass of the ion required to produce a charge (Calvano et al., 2018; Caprioli et al., 1997; Leopold et al., 2018; Wang et al., 2013; Zimmerman et al., 2008).

Detection

The desorped ions are then detected by time-of-flight (TOF) analysers which are the most common type of mass analyser and are typically used for detecting neurotransmitters (Palanisamy et al., 2019). The mass of an ion is determined using its velocity, this involves accelerating the ions in an electrical field (typically ~20,000V; Hillenkamp et al., 1991; Leopold et al., 2018). After each laser pulse the resulting newly formed ions pass through a charged grid to accelerate them to a fixed amount of kinetic energy, these ions then travel freely over a field-free drift zone down a flight tube where separation based on their m/z ratio occurs (Hillenkamp et al., 1991; Leopold et al., 2018; Mann et al., 2001). As the acceleration energy is constant, the principle of ion mobility dictates that ions with a smaller mass will travel faster than ions with a larger mass and thus are detected before larger ions, this produces a TOF spectrum of peaks at different times corresponding to the density of a molecule at a particular mass (Chaurand et al., 2002; Mann et al., 2001; Zimmerman et al., 2008). Ion density images can then be produced for each detected molecule (Chaurand et al.

2002). The mass spectrometer determines the ions' molecular weights, thereby allowing their identification as well as their spatial localisation within the tissue.

The data array consists of a grid of laser spots, where each spot or pixel is a discrete area of tissue (typically 50x50microns), irradiated by the laser, which contains an entire mass spectrum of the ion masses detected at that discrete location and their densities (Caprioli et al., 1997; Chaurand et al., 2006, 2006a; Cornett et al., 2007). Thus, specific m/z values correspond to specific molecular weights. Resulting mass spectra typically yield 300-1000 signals of varying intensity each (Chaurand et al., 2002).

Currently laser beam width and matrix characteristics (e.g., size of droplets and crystals) limit spatial resolution to 10-20μm with most MALDI-TOF mass spectrometers using laser spot diameters of 50μm- this represents the pixel size of the ion image (Chaurand et al., 2006, 2006a; Cornett et al., 2007; Hanrieder et al., 2013; Schwamborn & Caprioli, 2010; Zimmerman et al., 2008).

Coupling MALDI with TOF-MS allows for a virtually unlimited mass range to be monitored (Wang et al., 2013), however, the mass range below 500Da is often obscured by matrix ions (Mann et el., 2001). MALDI-IMS also suffers from reduced sensitivity and compromised mass accuracy when resolving proteins at the higher molecular mass range with some researchers suggesting compromised accuracy at masses as low as 40kDa (Beavis & Chait, 1990; Schwartz et al., 2003). Proteins tend to fragment during MALDI-IMS which results in a broad range of peaks and a decrease in sensitivity, therefore, MALDI-IMS is mostly applied to the analysis of peptides (Mann et al., 2001).

Method

Animals

All animals were housed in the Victoria University of Wellington vivarium and in accordance with the Animal Welfare Act 1999, with all procedures being pre-approved by the VUW Animal Ethics Committee (AEC number 22375). A total of 9 adult (PND80, WT $n = 4$, HOM $n = 5$) and 10 adolescents (PND21, WT $n = 4$, HOM $n = 6$) male Wistars were used in this study, all bred from HET male – HET female pairings.

All animals were housed in polycarbonate cages with one dam and her offspring per cage in a temperature and humidity-controlled environment at 21°C ± 2°C and 55-60% respectively. A reverse 12-hour light cycle was used with lights on at 1900 - 0700 hours. Food and water were available to the animals ad libitum. All subjects were weaned at PND 21 from mothers. Tissue samples from an ear clipping were sent to be genotyped on PND 21 (Transnetyx, Cordova US).

Tissue preparation

After conscious decapitation (in order to prevent hypoxia-induced tissue degradation and prevent neurochemical alterations caused by general anaesthetics), brain tissue from adult male Wistar rats was rapidly extracted covered in foil and snap frozen within 45 seconds using liquid nitrogen to prevent degradation of neurotransmitters.

Samples were transferred to a cryostat (Lecia CM3050) at -20°C for adult brains and -21°C to -22°C for adolescent brains and allowed to acclimatise for a minimum of 60 minutes to improve sectioning quality. Following acclimatization, sections were halved down the longitudinal fissure with a cryomicrotome blade and subsequently the right hemisphere was mounted on a sample plate using OCT (optimal cutting temperature polymer) to attach the lateral side of the brain to the sample plate without any OCT surrounding the area where sections were to be taken from.

Sections were first trimmed to create an even cutting surface, following which, sagittal sections were obtained at 20μm thickness. Sectioned were carefully placed on cooled MALDI target slides which were also kept at -20° C in the cryomicrotome using a thin

(sparsely bristled, synthetic bristles are less likely to retain melted tissue) paintbrush and was then thaw-mounted by placing a gloved hand briefly under the slide, resulting in rapid tissue adherence to the sample slide. This also avoids residual ice crystal formation and the loss of water-soluble compounds associated with gradual adherence. Slides were stored in a -80°C freezer until they were to be imaged.

Materials

The chemicals 2-4-6-Triphenylpyrylium Tetrafluroborate (TPP), 2,4,6-trimethylpyrylium tetrafluoroborate (TMP), 4-hydroxy-3-methoxycinnamaldehyde (CA), 1,1'-thiocarbonyldiimidazole (TCDI), Triethylamine (TEA), α-cyano-4 hydroxycinnamic acid (CHCA) and N,N-Diisopropylethylamine (DIPEA) were purchased from Sigma Aldrich suppliers in Auckland, New Zealand.

Procedure

Before MALDI-IMS analysis, slides were dried at room temperature for 30 minutes using a vacuum desiccator to prevent condensation forming and delocalisation of compounds. After acquiring a scan of the slice, the slide was subjected to chemical derivatization followed by matrix deposition prior to MALDI-IMS analysis. The methods for each of derivatization reagents tested are described below.

To validate the accurate alignment of the spectra, reference points were added using a 1 mg/ml spot of dopamine (Dopamine hydrochloride; Sigma-Aldrich) dissolved in distilled water), and 1 mg/ml of serotonin (Serotonin hydrochloride; Sigma-Aldrich) dissolved in distilled water), spotted near the tissue.

The following derivatization reagents were selected optimized for their abilities to derivatize LW molecules including neurotransmitters: TPP, TMP, TCDI, CA (Chacon et al., 2011; Dueñas et al., 2019; Esteve et al., 2016; Manier et al., 2014; Palanisamy et al., 2019; Shariatgorji et al., 2014, 2015).

Derivatization method 1) TPP with TEA

To test the ability of TPP to derivatise low weight neurotransmitters, 1.8mg/ml of TPP was diluted in a 70% methanol and 0.5% Triethylamine solution. This solution was evenly sprayer over the slide with a flow rate of 0.07/ml per minute, 600mm/min, 8 passes, at 150 PSI and 50°C using an automatic sprayer (HTX TM-Sprayer). TPP was initially selected due to its ability to react with amines creating pyrylium salt structures. This derivatization reaction occurs at ambient temperature and pressure with no need for heat, stirring, agitation or extensive incubation, which helps to preserve the localization of endogenous molecular compounds in the sample (Shariatgorji et al., 2014, 2015).

The sample slide was incubated in an empty petri dish and placed in a dark cupboard for 2 hours. During this incubation phase, the derivatisation agent reacts with the amines through creating a basic environment, opening the chemical structure, and allowing for the formation of the pyrylium salt structure. Due to the acidic nature of the matrix, there was no need to re-acidify the sample as seen in prior studies (Kamath, Diedrich, & Hindsgaul, 1996), as this was likely to increase delocalisation.

Finally, α-cyano-4 hydroxycinnamic acid (CHCA, Sigma-Aldrich, 8mg/ml in a solution of 70% acetonitrile and 0.2% Trifloroacetic acid) was used as the matrix due its broad use in soft ionisation techniques. 2mL of the matrix solution was applied using the automatic sprayer (75°C, flow rate 0.07/ml per minute, velocity 600mm/min, 8 passes, 150 PSI) with the TPP acting as a proton donor allowing for the absorption of the laser resulting in the ionization and abolition of the sample tissue. Finally, the sample was incubated with 4ml of 50% methanol solution and 0.2 ml of acetic acid.

Following CHCA application and incubation the sample slide was loaded into the mass spectrometer (AB Sciex TOF/TOF 5800), which uses a Nd:YLF (neodymium-doped yttrium lithium fluoride) variable laser set at 349nm in a vacuum chamber to process the spectra using the time-of-flight principle. The laser ablates and ionises the sample with a resolution of 50μm^2 per pixel. Following the completion of the MALDI procedure, the output spectra were analyses using Bitmap software.

Derivatization method 2) TPP with DIPEA

Next, TPP was tested using the base DIPEA instead of TEA.

1.5mg/ml of TPP was diluted in a 100% methanol and DIPEA solution (0.658µL DIPEA per 1.5mg/mL TPP). This solution was evenly sprayed over the slide (flow rate 0.07/ml per minute, 600mm/min, 8 passes, 150 PSI, 50°C). After spraying, the slide was incubated for two hours in a covered petri dish inside a dark cupboard. After this, 2mL of CHCA (8mg/ml in a solution of 70% ACN and 0.2% TFA) was sprayed over the slide (flow rate 0.07/ml per minute, velocity 600mm/min, 8 passes, 150 PSI, 75°C). The slide was incubated again for two hours in a covered dish placed in a dark cupboard. Following incubation, the slide was loaded into the mass spectrometer to obtain the mass spectra.

Initial results

The ion images we obtained showed that our initial TPP derivatization method was not successfully derivatizing neurotransmitters. We observed little to no detection of neurotransmitters. Initially we thought this may be due to the TEA used being old as it had turned yellowish in colour. We next used the base DIPEA instead of TEA with TPP however we came across the same issue of little to no detection. While we did not have enough time to troubleshoot this problem further, the next steps are to try different derivatization reagents: TMP, TCDI, CA.

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Appendix

Table 1

Area means and SDs for each hippocampal subregion

Offspring genotype	Maternal genotype	DG		CA1		CA2 + CA3		
		M	*SD*	*M*	*SD*	*M*	*SD*	*n*
WT	WT	820.09	421.50	855.47	422.86	1241.70	530.74	4
	HET	846.50	446.12	1030.02	433.70	1238.73	561.23	8
HET	WT	848.24	337.49	754.14	311.57	1150.80	456.77	9
	HET	787.60	364.99	885.71	386.28	1169.13	489.91	9
	HOM	984.70	296	999	519.80	1427.40	378.73	4
HOM	HET	817.02	381.27	919.91	333.02	931.20	392.02	3
	HOM	838.63	381.04	993.80	461.15	1332.14	444.42	9

Table 2

Two-way ANOVA for differences between offspring and maternal genotype in hippocampal subregion areas

		Type IV sum of squares	df	Mean square	*F*	*p*-value
DG area	Genotype	46827.11	2	23413.56	0.164	0.849
	Maternal Genotype	215277.95	2	107638.98	0.755	0.473
	Genotype*Maternal genotype	109928.2	2	54964.100	0.385	0.681
	Error	15688595.9	110	142623.60		
	Total	99696422.47	117			
CA1 area	Genotype	104525.48	2	52262.74	0.303	0.739
	Maternal Genotype	477311.23	2	238655.61	1.385	0.255
	Genotype*Maternal genotype	9757.02	2	4878.51	0.028	0.972
	Error	16373180.5	95	172349.27		
	Total	102775083	102			
CA2+CA3 area	Genotype	481749.87	2	240874.93	1.057	0.351
	Maternal Genotype	1451273.10	2	725636.55	3.184	0.045
	Genotype*Maternal genotype	60863.76	2	30431.88	0.134	0.875
	Error	25069110.30	110	227901		
	Total	203980417	117			