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The Dynamics of Serotonin Receptor Expression in the Developing Forebrain

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Psychology

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ABSTRACT

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Serotonin (5-hydroxytryptamine, 5-HT) serves an important modulatory role in the entire central nervous system (CNS), and serotonergic abnormalities have been implicated in many mental disorders. All of the CNS supply of 5-HT originates in the brainstem raphe nuclei. The midbrain dorsal raphe nucleus (DRN) sends widespread projections to the forebrain but receives direct input from only a small number of forebrain regions, including the medial prefrontal cortex (mPFC). We hypothesized that prenatal perturbations of 5-HT levels may alter the forebrain expression of 5-HT receptors (5-HTRs) in a time-sensitive manner and that these changes may result in persistent changes of the forebrain control of the DRN. Specifically, this thesis investigated the susceptibility of 5-HTR expression in the embryonic mouse telencephalon to chronic and acute perturbations of 5-HT levels and examined the effects of altered 5-HT_4R expression on the synaptic structure of the mPFC-DRN projection. These studies demonstrated (i) that acute and chronic perturbations of 5-HT levels affect the expression of 5-HT_{1A}R, 5-HT_{2A}R, and 5-HT_4R in the embryonic

forebrain and (ii) that altered 5-HT_4R expression affects the development of the synaptic connectivity between mPFC terminals and DRN serotonergic neurons. These findings suggest that prenatal serotonergic perturbations can strongly influence the development of the prefrontal control of 5-HT signaling and have long-term consequences for behavioral responses later in life.

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Chapter 1

General Introduction

The Brain 5-HT System

The 5-HT (serotonin, 5-hydroxytryptamine) system is one of the most widely-distributed neurotransmitter systems in the central nervous system (CNS). 5-HT is synthesized by neurons in the brainstem raphe nuclei (RN), transported by long-distance axonal projections, and released diffusely or via specific synaptic contacts in virtually all CNS regions (Azmitia & Whitaker-Azmitia, 1991; Parnavelas & Papadopoulos, 1989). Given the widespread presence of serotonergic axons, it is not surprising that 5-HT has been found to play important roles in diverse brain functions, including the regulation of emotional states, sleep, and motivation (Fidalgo, Ivanov, & Wood, 2013; Olivier, 2004). The forebrain, including the prefrontal cortex (PFC), receives a robust serotonergic innervation (Fuxe, 1965; Jacobs & Azmitia, 1992; Lidov, Grzanna, & Molliver, 1980), which modulates various cognitive functions, such as learning, memory, categorization, inhibitory control, and cognitive flexibility.

The biosynthesis of 5-HT begins with an essential amino acid, L-tryptophan. Tryptophan is first hydroxylated into 5-hydroxytryptophan (5-HTP) by the rate-limiting enzyme tryptophan hydroxylase (TPH), which is found only in 5-HT-synthesizing cells. Two isoforms of TPH, TPH1 and TPH2, have been identified. TPH1 is expressed primarily by the gut enterochomaffin cells and in the placenta (Bonnin et al., 2011), while TPH2 is expressed almost exclusively by neurons in the central and enteric nervous systems (P. D. Patel, Pontrello, & Burke, 2004; Walther et al., 2003). Animals lacking a functional TPH1 maintain normal 5-HT levels in the brain, but exhibit greater than a 90% reduction of 5-HT in the blood and in the

gastrointestinal tract (Côté et al., 2003; Savelieva et al., 2008). In contrast, the blood and gut 5-HT levels are unaffected in mice lacking a functional TPH2, but more than 95% of 5-HT in the brain is depleted (Savelieva et al., 2008). Pharmacological agents that inhibit TPH (e.g., *p*CPA) can deplete 5-HT by blocking the hydroxylation step and are often used in experimental research. The TPH-mediated step is then followed by decarboxylation of 5-HTP into 5-HT by the enzyme aromatic L-amino acid decarboxylase (AADC). 5-HT is eventually metabolized by the enzyme monoamine oxidase A (MAOA) and aldehyde dehydrogenase (ALDH) into 5-hydroxyindoleacetic acid (5-HIAA). The level of 5-HIAA in the brain is often measured to assess the rate of 5-HT turnover, which may be altered in some brain abnormalities. In human studies, the level of 5-HIAA in the cerebrospinal fluid (CSF) is often used as an indirect measure of brain 5-HT levels (Wester et al., 1990).

Altered 5-HT synthesis or release have been implicated in many psychiatric disorders, including major depressive disorder (aan het Rot, Mathew, & Charney, 2009; Carr & Lucki, 2011; Duman, 2007; Jans, Riedel, Markus, & Blokland, 2007; Millan, 2004; Tamburella, Micale, Navarria, & Drago, 2009; Wong, Perry, & Bymaster, 2005), generalized anxiety disorder (Booij et al., 2010; Lowry et al., 2008; Lowry, Johnson, Hay-Schmidt, Mikkelsen, & Shekhar, 2005), schizophrenia (Fribourg et al., 2011; González-Maeso & Sealfon, 2009; González-Maeso et al., 2008; Williams, Rao, & Goldman-Rakic, 2002), and autism-spectrum disorders (ASDs) (Azmitia, Singh, & Whitaker-Azmitia, 2011; Chandana et al., 2005; Chugani, 2002, 2004; Whitaker-Azmitia, 2001). One post-mortem depression study has found

decreased brainstem 5-HT levels in suicide victims (SHAW, ECCLESTON, & CAMPS, 1967) and various others have reported robust correlations between low CSF 5-HIAA and suicidality, aggression, and impulsivity (Asberg, Thorén, Träskman, Bertilsson, & Ringberger, 1976; Å sberg, 1997; G. L. Brown et al., 1982). Importantly, many pharmaceutical agents targeting the 5-HT system, such as the selective serotonin reuptake inhibitors (SSRIs), have been shown to have antidepressant (Blier & De Montigny, 1999; Vaswani, Linda, & Ramesh, 2003), anxiolytic (Feighner & Boyer, 1989; Menard & Treit, 1999; Vaswani et al., 2003), and/or antipsychotic (Meltzer, 2012) properties, and have become a popular choice in pharmaceutical treatments of many neurobiological disorders.

The 5-HT Receptors

To date, fourteen subtypes of mammalian 5-HT receptors (5-HTRs), divided into 7 families, have been identified (Barnes & Neumaier, 2011). Some 5-HTRs, including 5-HT_4R, can be alternatively spliced into multiple functional isoforms (Bockaert, Claeysen, Bécamel, Dumuis, & Marin, 2006). These splice variants differ in their C-terminal domains that can recruit and interact with specific intracellular proteins, leading to finely regulated signal transduction (Bockaert et al., 2006). All 5-HTRs are G protein-coupled receptors (GPCRs) except for 5-HT_3R, which is a ligand-gated ion channel. The 5-HT GPCRs consist of 7 transmembrane domains with a ligand binding site on the N-terminus and a G-protein association site on the C terminus. 5-HT GPCRs are coupled to three types of G-proteins, Gs, Gi, and Gq. Upon ligand binding, the receptor changes its conformation and activates the

associated G-proteins, which detach from the C-terminus and activate various intracellular pathways. Downstream changes inside the neuron are induced via the activation of second messengers that can affect several cellular processes, including membrane ion exchange and gene transcription.

Since serotonergic axons are widely distributed throughout the CNS, 5-HTRs have been identified in most forebrain regions, including the PFC, basal ganglia, amygdala, and hippocampus (Beaudoin-Gobert & Sgambato-Faure, 2014). The 5-HTR family is larger than the GPCR receptor families of other neurotransmitters, including dopamine, norepinephrine, glutamate, and acetylcholine (Nichols & Nichols, 2008), suggesting a great functional versatility of 5-HT signaling. The regional distributions of 5-HTRs are subtype-specific. For example, 5-HT_{1A}Rs are highly expressed in the dorsal RN (DRN), where the expression of 5-HT_{2A}Rs and 5-HT_4Rs is low (Beaudoin-Gobert & Sgambato-Faure, 2014; Lucas et al., 2005). Because 5-HT_{1A}R activation leads to neuronal hyperpolarization and a reduced firing rate, DRN 5-HT_{1A}Rs play a regulatory role in the 5-HT synthesis and release. 5-HT_{1A}Rs are also expressed densely in the limbic system and in layers II and VI of the frontal cortex (Pazos & Palacios, 1985). 5-HT_{2A}Rs are highly expressed in layers III and V of several cortical areas in the human brain (Pazos, Probst, & Palacios, 1987). A high-resolution localization study of 5-HT_{2A}R in the primate cerebral cortex has shown that 5-HT_{2A}Rs are expressed on virtually all pyramidal cells, especially on their apical dendrites (Jakab & Goldman-Rakic, 1998). In the brain, 5-HT_4Rs are densely expressed in the basal ganglia, cortex, hippocampus, and substantia nigra

(Bonaventure et al., 2000; Eglen, Wong, Dumuis, & Bockaert, 1995). Because 5-HT_4R is Gs-protein-coupled and densely expressed in the hippocampus, many studies have examined its role in long-term potentiation (LTP) and synaptic plasticity (Kemp & Manahan-Vaughan, 2005; Kulla & Manahan-Vaughan, 2002; Wawra, Fidzinski, Heinemann, Mody, & Behr, 2014). Importantly, studies using 5-HT_4R knockout mice indicate that 5-HT_4Rs modulate 5-HT content in the rostral RNs; specifically, decreased 5-HT neuron activity and 5-HT content have been reported in 5-HT_4R knockout mice (Conductier et al., 2006). This is consistent with the finding that systemic injection of a 5-HT_4R agonist increases the firing rate of serotonergic DRN neurons (Lucas & Debonnel, 2002). Since 5-HT_4R is not expressed in the DRN, the modulating neurons are likely to be located in the mPFC that projects to the DRN (Conductier et al., 2006).

With at least nine splice variants in humans and four splice variants in mice, 5-HT_4R has the most complex genetic structure of all 5-HTRs (Bockaert, Claeysen, Compan, & Dumuis, 2004; Claeysen, Sebben, Becamel, Bockaert, & Dumuis, 1999). The four splice variants of 5-HT_4R in mice exhibit differences in their C-termini: The 5-$HT_{4(a)}$R and 5-$HT_{4(b)}$R have longer intracellular C-terminal ends and the 5-$HT_{4(e)}$R and 5-$HT_{4(f)}$R have shorter intracellular C-terminal ends (Claeysen et al., 1999). An important difference between the 5-HT_4R splice variants is their varying levels of constitutive activity. The constitutive activity of a receptor is the ability of a receptor to convert from an inactive conformation to an active one without the stimulation of a ligand. In the case of 5-HT_4R, the basal constitutive activity is measured by the

production of cAMP in the absence of a 5-HT_4R agonist. An *in vitro* study has shown that all 5-HT_4R splice variants are constitutively active, with the 5-$HT_{4(e)}$R and 5-$HT_{4(f)}$R splice variants having a 2-fold higher constitutive activity than the 5-$HT_{4(a)}$R and 5-$HT_{4(b)}$R splice variants (Claeysen et al., 1999). This splice-variant specific difference in activity is likely due to the lengths of the C-termini, since the shortest splice variants have a greater ability to isomerize to an active state without ligand-binding. This is supported by the finding that progressive deletions of the C-terminal part of 5-$HT_{4(a)}$R result in a corresponding increase in constitutive activity (Claeysen et al., 1999).

Aside from the difference in constitutive activity, the 5-HT_4R splice variants also differ in their internalization patterns. When stimulated with 5-HT, 5-$HT_{4(b)}$R internalizes more efficiently than 5-$HT_{4(a)}$R (Mnie-Filali et al., 2010). Upon endocytosis, 5-$HT_{4(a)}$Rs stay associated with β-arrestin, a protein that mediates internalization and post-endocytic trafficking of GPCRs, and accumulate around the perinuclear region (Mnie-Filali et al., 2010). In contrast, 5-$HT_{4(b)}$Rs dissociate from β-arrestin rapidly, recycle back to the cellular membrane, and become functional again (Mnie-Filali et al., 2010). The pattern of internalization and subsequent recycling has major implications at the transcriptional level because the splice variant that recycles to the membrane most efficiently is likely to require less receptor synthesis following ligand-induced internalization. Additionally, highly spontaneous, ligand-independent internalization of 5-HT_4R has been reported, and this constitutive internalization is more prominent in 5-$HT_{4(a)}$R than in 5-$HT_{4(b)}$R in cultured cortical

cells (Mnie-Filali et al., 2010). Lastly, RS67333, a 5-HT_4R partial agonist that is currently being investigated as a potential rapid-acting antidepressant (Lucas et al., 2007), produces less 5-HT_4R internalization than 5-HT but follows a similar pattern of internalization across the 5-HT_4R splice variants, as compared to 5-HT (Mnie-Filali et al., 2010).

We have previously demonstrated in our laboratory that 5-HT_4Rs show an mRNA splice variant-specific developmental expression in the mouse telencephalon (Hernandez & Janušonis, 2010). As shown in Figure 1.1, the mRNA levels of 5-$HT4_{(a)}$R are rapidly upregulated at two developmental times: between E14 and E15 and between E16 and E17. The mRNA levels of 5-$HT_{4(b)}$R are rapidly upregulated only once, between E14 and E15. The mRNA levels of 5-$HT_{4(e)}$R and 5-$HT_{4(f)}$R show an almost linear increase between E14 and E17. These upregulation patterns suggest that 5-HT_4Rs may control developmental processes in a time-sensitive manner.

Due to the many 5-HT_4R splice variant-specific differences and the involvement of 5-HT_4R in major brain disorders, such as depression and Alzheimer's disease, the characterization and developmental profiling of the 5-HT_4R splice variants are important for translational research. Because of these considerations, the 5-HT_4R splice variants were examined separately in this thesis.

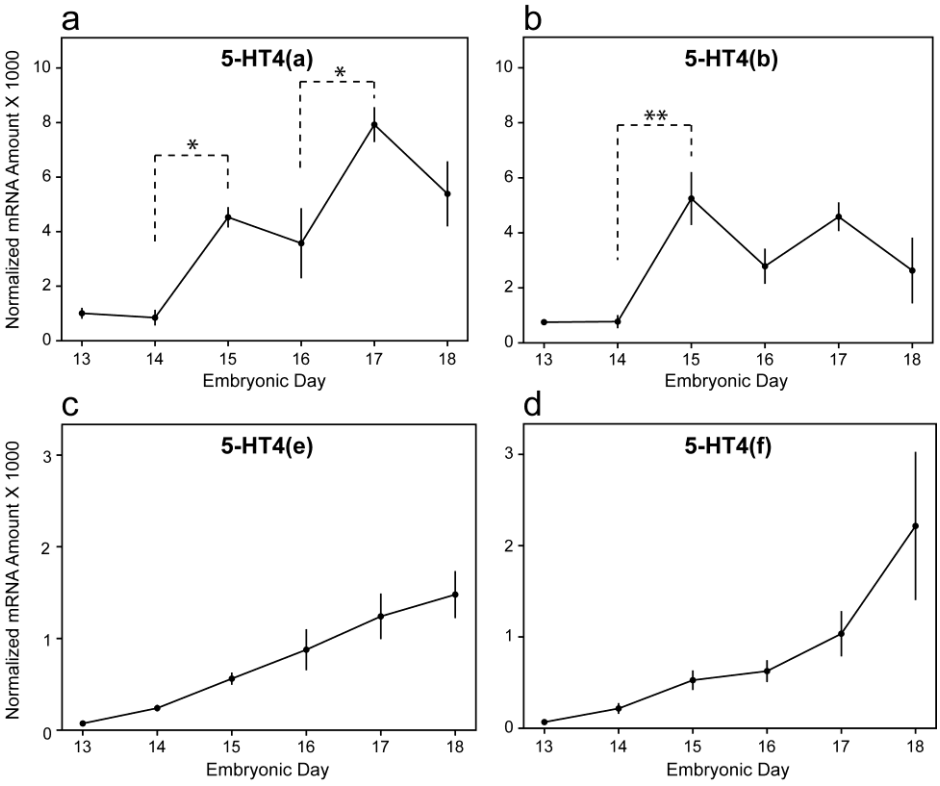

Figure 1.1. Quantitative RT-PCR (RT-qPCR) analysis has shown that the 5-HT_4R splice variants have distinct temporal patterns of expression in the mouse embryonic telencephalon. The dashed lines indicate all significant *post-hoc* (Tukey HSD) differences between consecutive time points. Error bars = SEM. *p<.05, **p<.01 (Hernandez & Janušonis, 2010).

5-HT Interactions with Other Neurotransmitters

Depending on the associated G-protein and the synaptic location, the metabotropic 5-HTRs can act as neurotransmitter release-inhibitors or -facilitators. For example, 5-HT_{1B}Rs can modulate the presynaptic or postsynaptic release of other neurotransmitters, such as acetylcholine, glutamate, dopamine, and norepinephrine (Pytliak, Vargová, Mechírová, & Felšöci, 2011). 5-HT_4Rs are found on cholinergic axon terminals in the hippocampus and can facilitate the release of acetylcholine in this region (Fink & Göthert, 2007). In addition, some 5-HT_{1A}Rs and 5-HT_{2A}/$_{2C}$Rs are expressed on local GABAergic interneurons in brain regions such as the PFC, which can inhibit neurotransmitter release from projection neurons (J. De Almeida & Mengod, 2007; Julián De Almeida & Mengod, 2008). Because one neurotransmitter system can easily regulate another, this type of cross-talk between multiple neurotransmitter systems introduces further complexity in the study of the 5-HT system.

Another, less explored mechanism of cross-talk between various neurotransmitter systems is the formation of receptor heterodimers. It has been demonstrated that many GPCRs, including metabotropic 5-HTRs, can form homo- and hetero-dimers. Dimerized receptors become a single signaling unit that can exhibit a complex pharmacological dynamic. For example, it has been shown that 5-HT_{2A}R and the dopamine receptor 2 (D_2R) can form a heterodimer, and that specific 5-HT_{2A}R or D_2R ligands can change the recognition and signaling at the other receptor (Borroto-Escuela et al., 2010; Lukasiewicz et al., 2010). Specifically,

Borroto-Escuela et al. (2010) has shown that 5-HT stimulation of the heterodimer leads to inhibition of D_2R function, suggesting a 5-$HT_{2A}R$-mediated D_2R trans-inhibition phenomenon. Also, it has been demonstrated with immunofluorescence double-labeling techniques that the two receptors colocalize in the medial PFC (mPFC) (Lukasiewicz et al., 2010), one of the few brain regions that directly project to the DRN.

The human 5-HT_4R has been reported to homodimerize, as well as heterodimerize, with the β2 adrenergic receptor (β2-AR) (Berthouze et al., 2005). Therefore, 5-HT_4Rs and β2-ARs are well-positioned to integrate serotonergic and noradrenergic signals at the single-neuron level. Additionally, the β2-AR gene is nested within an untranslated intron of the 5-HT_4R gene (*Htr4*) (Bockaert et al., 2004). It should be noted that, in the mammalian genome, it is rare for two proteins with similar functions to have a nested gene relationship (Jaworski, Beem-Miller, Lluri, & Barrantes-Reynolds, 2007). As a single heterocomplex signaling unit, the two receptors may affect each other's internalization and recycling. For these reasons, transcriptional interactions between 5-HT_4R and β2-AR were investigated in this thesis.

The Role of 5-HT in Brain Development

Many lines of research show that long before 5-HT assumes its role as a neurotransmitter in the mature brain, it regulates brain development. The 5-HT system is one of the first neurotransmitter systems to develop during embryonic development. Neurons immunopositive for 5-HT can be detected as early as

embryonic day (E) 10-12 in the mouse brain (Pattyn et al., 2004), not long after the RN neurons begin to differentiate (Sundström et al., 1993). The axons of the serotonergic neurons in the RN then extend rapidly, reaching toward both the hindbrain and forebrain. It has been reported that 5-HT expression in development, occurs prior to synaptic neurotransmission or synaptogenesis (Bonnin, Peng, Hewlett, & Levitt, 2006), indicating that 5-HT can act as an important signal for immature brain cells. Studies have implicated 5-HT in various developmental processes, including cell proliferation and differentiation. Lauder et al. (1978) has shown that maternal exposure to a TPH inhibitor, which blocks 5-HT synthesis, delays and prolongs the onset of cell differentiation in some brain regions known to receive dense 5-HT innervation. The advancing serotonergic axons also appear to contact proliferating cells in the ventricular zone (Wallace & Lauder, 1983) and Cajal-Retzius cells in the cortical marginal zone (Janušonis, Gluncic, & Rakic, 2004).

Many studies have demonstrated the role of 5-HT in developmental cell differentiation and growth. In snail cell cultures, some neurons with actively growing neurites display inhibition of growth cone activity, suppression of neurite outgrowth, and reduced synapse formation following addition of 5-HT to the culture medium (Haydon, McCobb, & Kater, 1984; McCobb, Haydon, & Kater, 1988). Another study has shown that when snail embryos are exposed to a 5-HT-synthesis inhibitor, cells that are normally innervated by 5-HT axons exhibit aberrations in the dendritic morphology and in the strength of electrical synaptic connections (Goldberg & Kater,

1989). Taken together, 5-HT has a growth-inhibitory effect in the snail nervous system.

Conversely, addition of 5-HT to cultures of rat neurons stimulates neurite outgrowth and synaptogenesis. Another study has shown that pharmacological depletion of 5-HT with systemic *p*CPA injections from E12 to E17 or exposure to a low-tryptophan diet leads to a decrease in the dendritic arborization of rat cortical neurons (González-Burgos, Del Angel-Meza, Barajas-López, & Feria-Velasco, 1996; Vitalis, Cases, Passemard, Callebert, & Parnavelas, 2007). The difference between the 5-HT effects in these nervous systems may be due to the expressed 5-HTRs, or to other differences between invertebrate and vertebrate neurons. Irrespective of the specific effects, these studies show the importance of 5-HT in the development and differentiation of neural cells.

Recently, more research has been done to identify which 5-HTRs and corresponding mechanisms are involved in these developmental processes. Juvenile and adult mice lacking functional 5-HT_{1A}Rs exhibit increased arborization of hippocampal pyramidal neurons (Cumbo, Mittaud, Ackermann, Hen, & Hornung, 2008). Similar changes have been observed in mice exposed to a 5-HT_{1A}R antagonist from the third to fifth postnatal weeks (Ferreira, Iacono, & Gross, 2010). A study has shown that the reduction in dendritic length and spine density in hippocampal neurons following exposure to 5-HT depletion can be prevented by administration of a 5-HT_{1A}R antagonist (Yan, Wilson, & Haring, 1997). Hayashi et al. (2010) have found that a 5-HT_3R agonist decreases dendritic growth and branching of non-GABAergic

cells in the cerebral cortex, and that this effect can be reversed by a 5-HT_3R antagonist. Activation of 5-HT_4R has been shown to cause neurite retraction and cell rounding (Ponimaskin, Profirovic, Vaiskunaite, Richter, & Voyno-Yasenetskaya, 2002), as well as a decrease in the length and number of neurites (Kvachnina et al., 2005). These findings suggest that the effects of 5-HT in developmental processes are highly receptor type-specific.

Finally, several groups (including ours) have shown that 5-HTRs can be expressed by developing brain projections, such as thalamocortical afferents (TCAs) (Bonnin, Torii, Wang, Rakic, & Levitt, 2007; Slaten, Hernandez, Albay, Lavian, & Janusonis, 2010; Wai, Lorke, Kwong, Zhang, & Yew, 2011). Because 5-HTRs have been shown to guide axonal growth during development (Bonnin et al., 2007), alterations in 5-HTR expression can lead to axonal mis-wiring. The gain or loss of function of certain 5-HTRs at critical developmental periods causes changes in the formation of neural connections that last into adulthood. A study by Bonnin et al. (2006) has shown that upregulating and downregulating 5-$HT_{1B/1D}$R expression creates two opposing effects. Upregulating 5-$HT_{1B/1D}$Rs causes the TCAs to be ventrally shifted/expanded in the internal capsule, while downregulating 5-$HT_{1B/1D}$Rs causes the TCAs to follow a more restricted dorsal-medial path. Another neural projection that has been studied in this regard is the projection from the rostral RN to the PFC. Witteveen et al. (2013) have demonstrated that the serotonergic innervation of the mPFC is dramatically increased in rats lacking the 5-HT transporter (5-HTT).

These studies support the idea that 5-HT can have a profound influence on the formation of specific forebrain circuits.

5-HT Perturbations in Embryonic Development

With 5-HT implicated in major developmental processes, it is not surprising that prenatal perturbations in the 5-HT system can lead to neurodevelopmental and behavioral consequences in the offspring. A series of studies by Vataeva et al. (Vataeva, Khozhaĭ, Makukhina, & Otellin, 2007; Vataeva et al., 2008; Vataeva, Kudrin, et al., 2007) have shown that 5-HT depletion with *p*CPA (*para*-chlorophenylalanine, a TPH inhibitor) during embryonic development results in abnormal adult behaviors such as locomotor hyperactivity in the open field test, increased immobility in the forced swim test, impaired learning in the water maze task, hypersensitivity to novel or aversive stimuli, and an increase in saccharin preference. Depletion of 5-HT also has led to a significant decrease in the level of a DA metabolite (Vataeva et al., 2008), which demonstrates that 5-HT can have a strong effect on other neurotransmitter systems. Another study has found that mice lacking TPH2, the enzyme responsible for the synthesis of 5-HT in the CNS, exhibit increased depression-like behavior, decreased anxiety-like behavior, and aggressiveness (Mosienko et al., 2012).

Prenatal Perturbations: Exposure to Stress and Antidepressants

There is evidence that the disruption of proper embryonic 5-HT signaling has negative consequences for humans. Pregnant women are commonly exposed to

factors that can result in altered 5-HT signaling in the fetal brain, including maternal stress and antidepressants, such as SSRIs. These two factors can affect the embryonic 5-HT system, which in turn may lead to behavioral consequences in adolescent and adult offspring.

Many lines of investigation have shown that exposing pregnant women to physical and emotional stress can have neurostructural and behavioral effects on the offspring, including a predisposition to disorders such as depression, anxiety, schizophrenia, and autism spectrum disorder (ASD) (Gillott & Standen, 2007; Koenig, Kirkpatrick, & Lee, 2002; Kofman, 2002; Walker, Mittal, & Tessner, 2008). Other experiments in mice have shown that prenatal stress (PS) increases 5-HT synthesis in the fetal brain (D. A. V. Peters, 1990) and induces region-specific changes in brain 5-HT and 5-HIAA levels during infancy (Huang et al., 2012; D. A. V. Peters, 1982), which can contribute to lasting PS effects. Huang et al. (2012) have shown that exposure to chronic unpredictable stress from E0 to E19 results in a decreased ratio of 5-HIAA to 5-HT, decreased 5-HT_{1A}R mRNA expression, and decreased 5-HTT protein expression in the rat embryonic brain at E20. Miyagawa et al. (2011) have used the elevated-plus maze and the open field test to show increased anxiety-like behavior in mice that have been exposed to PS. In this study, PS-exposed mice spent significantly less time in open arms and exhibited less exploratory behaviors in the elevated plus maze, indicating an emotional abnormality. This study also has found that the adult offspring of PS-exposed mice have morphological changes in serotonergic DRN neurons. Specifically, 5-HT- and TPH- immunoreactivities in the

DRN appear to be significantly higher in PS-exposed mice compared to control mice. van den Hove et al. (2011) have reported that heterozygous 5-HTT deficient mice, which display a 50% reduction in 5-HTT binding in the brain (Bengel et al., 1998), show an increase in depressive-like behavior in female offspring, a reduced basal corticosterone level, and impaired object memory performance in all offspring when prenatally exposed to restraint stress from E13 to E17. Holloway et al. (2013) reported that mice exposed to variable stress conditions from E9 to birth display schizophrenia-like behaviors, such as increased head-twitch and locomotor activity, and show a decrease in 5-HT_{2A}R expression in the adult frontal cortex. These findings indicate that PS-induced alterations of the 5-HT system may contribute to the etiology of psychiatric disorders in the offspring.

Up to 16% of women experience the onset of affective disorders during the period of pregnancy (Kitamura, Shima, Sugawara, & Toda, 2009), and various effects of prenatal depression on the fetus and newborn have been reported, including elevated fetal activity, delayed prenatal growth, prematurity, and low birth weight (T. Field, Diego, & Hernandez-Reif, 2006). Currently, the percentage of women using antidepressants during pregnancy is around 7-13% and on the rise (S. E. Andrade et al., 2008), which leads to a greater focus on the effects of these pharmacological agents on prenatal brain development. A number of commonly used SSRIs can readily cross the placental barrier and alter 5-HT signaling in the embryo (Rampono et al., 2009); SSRIs can also be passed onto neonatal infants through breastfeeding (Kristensen et al., 2001). Since the nervous system continues to develop well after

birth, the neonatal stage is a critical period when 5-HT perturbations can have major consequences that may last well into the adult life. The findings reported by prenatal SSRI studies are currently somewhat inconsistent (for review, see Kepser & Homberg, 2014). Some studies have found that prenatal SSRI exposure increases abnormal behaviors in adolescent and adult offspring, while others have reported no such abnormalities. This may be due to differences in the type and dose of SSRI, the dosing interval, the route of administration, the specific developmental period in which SSRI exposure occurs, and sample sizes (Kepser & Homberg, 2014). Despite this lack of consensus, SSRI exposure during critical developmental times is likely to disrupt the development of the 5-HT system. This in turn may lead to an abnormal development of critical brain structures and pathways and predispose the offspring to maladaptive behavior later in life.

Stress induced by prolonged maternal separation has reduced 5-HT_{1A}R and 5-HT_{2A}R mRNA in the mPFC of rat pups (Ohta et al., 2013). Also, chronic prenatal exposure of rats to dexamethasone, which mimics PS, has decreased 5-HT_{1A}R mRNA in the mPFC at postnatal week 4 (Nagano, Liu, Inagaki, Kawada, & Suzuki, 2012). While these results can be viewed as simple homeostatic responses to possibly elevated 5-HT (D. A. V. Peters, 1990), the abnormally low 5-HT_{1A}R mRNA levels after the dexamethasone treatment have been restored with chronic neonatal treatment with fluoxetine (an SSRI), which increases extracellular 5-HT (Nagano et al., 2012). In adult rats, chronic treatment with fluoxetine has produced a decrease in 5-HT_{1A}R mRNA levels in the cortex (Shishkina, Kalinina, & Dygalo, 2012), and similar

treatments with fluoxetine and paroxetine (another SSRI) have reduced 5-HT_4R binding in the forebrain (Licht et al., 2009; Vidal, Valdizán, Mostany, Pazos, & Castro, 2009). It is important to note that the expression levels of 5-HT_{1A}R, 5-HT_{2A}R, and 5-HT_4R may be interdependent (Fox, Stein, French, & Murphy, 2010; Janušonis, 2014). In summary, 5-HT signaling may be an important component of the fetal programing of mental diseases, which makes it imperative that we understand the type and timing of developmental perturbations that can alter 5-HTR expression in the developing brain.

Forebrain Control of the 5-HT System

In contrast to the wide-spread efferent 5-HT projections, the rostral RN receives direct afferent projections from a limited set of brain regions. One major and functionally important afferent projection to the DRN is the glutamatergic projection that originates in the mPFC, including its infralimbic and prelimbic parts (Aghajanian & Wang, 1977; Gabbott, Warner, Jays, Salway, & Busby, 2005; Gonçalves, Nogueira, Shammah-Lagnado, & Metzger, 2009; Hajós, Richards, Székely, & Sharp, 1998; Sesack, Deutch, Roth, & Bunney, 1989; Vertes, 2004). The mPFC afferents in the DRN can make direct synapses with serotonergic neurons, or synapse onto GABAergic interneurons (Jankowski & Sesack, 2004) that, in turn, inhibit serotonergic neurons (Celada, Puig, Casanovas, Guillazo, & Artigas, 2001; Varga, Székely, Csillag, Sharp, & Hajós, 2001). Since this mPFC input can have either direct excitatory effects or indirect inhibitory effects on DRN serotonergic neurons, it is well-positioned to control their baseline activity. Additionally, mPFC pyramidal cells

project to many major brain areas important for cognitive and emotional processes (Bossert et al., 2012; J. Peters, Kalivas, & Quirk, 2009; Sesack et al., 1989), thus forming a complex regulatory network of neurotransmitter release.

Serotonergic brain abnormalities have been implicated in a number of brain disorders, including ASD (Anderson, 2002; Chugani, 2002, 2004; Whitaker-Azmitia, 2001), schizophrenia (González-Maeso & Sealfon, 2009; González-Maeso et al., 2008; Williams et al., 2002), and depression (Carr & Lucki, 2011; Duman, 2007; Tamburella et al., 2009). Likewise, altered mPFC function has been implicated in all of these disorders (F Artigas, 2010; Courchesne et al., 2007; Drevets, Savitz, & Trimble, 2008). A study of autistic brains has shown that they may have a reduced number of long-distance axons in the mPFC (Zikopoulos & Barbas, 2010). Intriguingly, the mPFC is currently considered to be a part of the brain "default-mode network," a major neural hub that appears to be affected in schizophrenia, depression and other brain disorders (Broyd et al., 2009; H. Lu et al., 2012; Zhang & Raichle, 2010). In this regard, the neurobiology of these disorders may have more in common than has been previously thought. The potential importance of the mPFC-DRN projection to these disorders has been directly studied or noted by a number of researchers (Amat et al., 2005; Amat, Paul, Watkins, & Maier, 2008; F Artigas, 2010; Celada, Puig, Martín-Ruiz, Casanovas, & Artigas, 2002; Duman, 2007; Lucas et al., 2005; Vollenweider & Kometer, 2010).

Gross et al. (2002) have demonstrated that, in mouse prenatal and early postnatal development, normal expression of forebrain 5-HT_{1A}Rs is required for the formation

of typical (non-anxiety-like) behavior in offspring. This study has used constitutive and conditional 5-HT_{1A}R-knockout models and shown that rescuing 5-HT_{1A}Rs in the forebrain at postnatal day (P) 21) does not result in a significant difference in anxiety-like behaviors compared to the constitutive knockout mouse. Another study has found that increased 5-HT signaling resulting from exposure to SSRIs during P2 to P11 alters mPFC function in adult mice by reducing the excitability of pyramidal neurons in the infralimbic cortex and results in increased anxiety behaviors in the offspring (Rebello et al., 2014). Because some infralimbic pyramidal neurons project to the DRN, the altered excitability can lead to dysregulated 5-HT synthesis and release.

Exposure to repeated stress has been shown to have robust effects on the branching patterns of apical dendrites in mPFC, including dendritic retraction in the prelimbic and infralimbic cortices in the rat brain (Izquierdo, Wellman, & Holmes, 2006; Radley et al., 2006). In humans, reduced glia and neuron density in the mPFC of suicide victims has been reported (Rajkowska, Halaris, & Selemon, 2001). 5-HTRs are expressed in the fetal telencephalon before serotonergic axons reach the forebrain, including the future mPFC. Since the mPFC eventually projects back to the DRN to regulate 5-HT synthesis and release, disruption of the optimal 5-HT supply during critical developmental times can affect the proper wiring of the mPFC-DRN projection. This in turn may result in an altered dynamic of 5-HT signaling in the adult offspring and their abnormal behavioral responses (Figure 1.2).

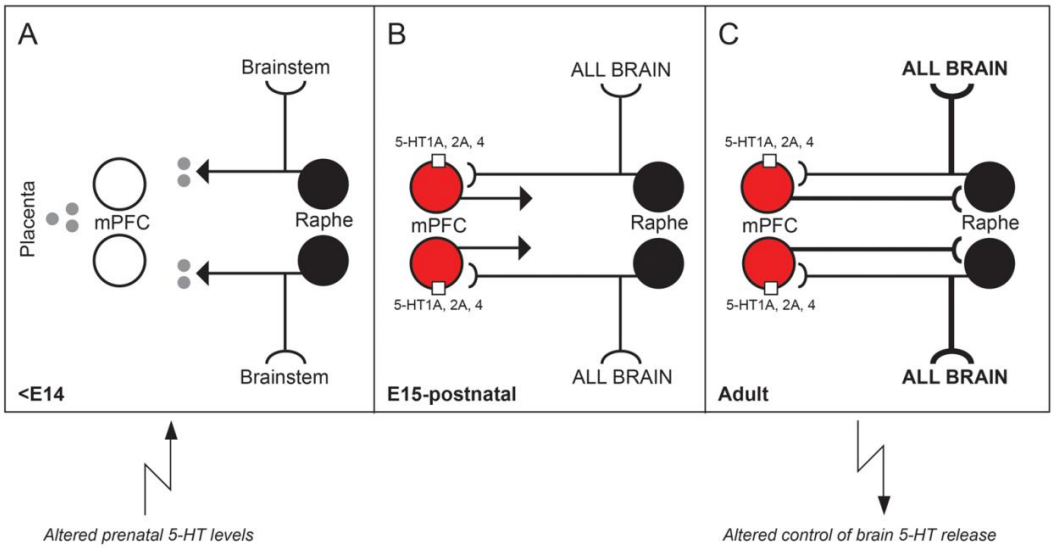

Figure 1.2. The developing forebrain control of the 5-HT system. (A) Exogenous and endogenous 5-HT triggers the upregulation of 5-HTR synthesis in the mPFC. (B) These receptors control the development of the mPFC-DRN projection. (C) The mature mPFC-DRN projection controls 5-HT release in the entire brain. Environmental factors such as PS or maternal exposure to SSRIs may affect the expression of 5-HTRs in the mPFC and alter the development of the mPFC-DRN projection.

Specific Aims

Specific Aim 1: To characterize the effects of repeated perturbations of prenatal 5-HT levels on 5-HTR and β_2AR mRNA expression in the embryonic telencephalon. The 5-HT_4R splice variants in the embryonic telencephalon exhibit distinct developmental trajectories (Hernandez & Janušonis, 2010) and thus might respond differently to prenatal perturbations. Based on these considerations, the susceptibility of these splice variants to PS and 5-HT depletion was investigated. Also, a region within the 5-HT_4R gene contains the gene coding for the adrenergic β_2 receptor (β_2-AR) Therefore, it was further investigated whether prenatal 5-HT perturbations can affect β_2-AR transcription, and, conversely, whether agents targeting β_2-AR can affect 5-HT_4R transcription. Pregnant mice received a combination of restraint stress, a TPH inhibitor, a β_2-AR agonist, a β_2-AR antagonist, or a vehicle from E14 to E16, and quantitative reverse-transcription polymerase chain reaction (RT-qPCR) was used to assess the mRNA levels of all 5-HT_4R splice variants (*a*, *b*, *e*, *f*) and β_2AR in the embryonic telencephalon at E17.

Specific Aim 2: To characterize the effect of acute prenatal 5-HT perturbation on 5-HTR mRNA expression in the embryonic telencephalon. Based on the findings in Specific Aim 1, it was investigated whether 5-HTRs show a receptor-specific susceptibility to acute 5-HT perturbations at specific developmental times. Embryonic 5-HT levels were perturbed at two developmental times (E14-15 and E16-17) by depleting 5-HT with a TPH inhibitor and elevating 5-HT levels with an SSRI, and

RT-qPCR was be used to assess the mRNA levels of 5-HT_{1A}R, 5-HT_{2A}R, and all 5-HT_4R splice variants (*a, b, e,* and *f*) in the embryonic telencephalon.

Specific Aim 3: To identify changes in the DRN synaptic structure of mice with constitutively altered 5-HT_4R expression. The results of Specific Aims 1 and 2 showed that prenatal 5-HT perturbations can affect the expression of 5-HTRs in the developing telencephalon. Since these alterations can disrupt the normal development of major neural projections, it was next investigated how the lack of a specific 5-HTR, 5-HT_4R, alters the structure and synaptic connectivity of the mPFC-DRN projection in adult mice. First, the ultrastructural morphology of the DRN of adult wild-type (*Htr4*$^{+/+}$), 5-HT_4R-heterozygous (*Htr4*$^{+/-}$), and 5-HT_4R-knockout (*Htr4*$^{-/-}$) mice was examined using electron microscopy. Second, the synaptic microstructure of the mPFC projection to the DRN was studied in adult *Htr4*$^{+/+}$, *Htr4*$^{+/-}$, and *Htr4*$^{-/-}$ mice using multiple-label immunofluorescence, confocal microscopy, and automatic colocalization detection.

Chapter 2

Effects of Prenatal Stress and Monoaminergic Perturbations on the Expression of Serotonin 5-HT_4 and Adrenergic β_2 Receptors in the Embryonic Mouse Telencephalon

Introduction

The 5-HT4R is coded by a complex gene that generates at least nine mRNA splice variants in humans and pigs and four splice variants in mice (5-$HT_{4(a)}$R, 5-$HT_{4(b)}$R, 5-$HT_{4(e)}$R, 5-$HT_{4(f)}$R) (Bockaert et al., 2006, 2004; Claeysen et al., 1999; Ray et al., 2009). This receptor shows a highly dynamic expression in the development of the mouse and human brain (Lambe, Fillman, Webster, & Shannon Weickert, 2011; Manzke, Preusse, Hülsmann, & Richter, 2008; Slaten et al., 2010; Waeber, Sebben, Bockaert, & Dumuis, 1996; Waeber, Sebben, Nieoullon, Bockaert, & Dumuis, 1994) and, at least in the mouse brain, the 5-HT_4R splice variants have different developmental trajectories (Hernandez & Janušonis, 2010). This variant-specificity may carry important temporal and spatial information because 5-HT_4R splice variants differ in their constitutive activity (Claeysen et al., 2001, 1999), internalization properties (Mnie-Filali et al., 2010), and association with intracellular proteins (Joubert et al., 2004).

Altered 5-HT_4R function has been associated with ASD (Vincent et al., 2009), major depressive disorder (Lucas et al., 2007; Rosel et al., 2004), bipolar disorder (Hayden & Nurnberger, 2006), and attention deficit/hyperactivity disorder (ADD/ADHD) (J. Li et al., 2006). A recent study has suggested that 5-HT_4R is a molecular network hub that controls multiple neural processes (Hu, Addington, & Hyman, 2011), including those important for the neurobiology of several brain disorders. Different levels of 5-HT_4R expression have been reported in the amygdala

of males and females, which may contribute to sex differences in the prevalence of affective disorders (Madsen et al., 2011).

Evidence suggests that 5-HT_4R may be a key component in the forebrain control of the brain serotonergic system. Some of this control is mediated by a projection that originates in the mPFC and terminates in the DRN (Gabbott et al., 2005; Gonçalves et al., 2009; Hajós et al., 1998; Vertes, 2004). In the adult brain, cortical 5-HT_4Rs modulate the activity of this projection (Bockaert, Claeysen, Compan, & Dumuis, 2011; Lucas et al., 2005) and can alter brain 5-HT levels by affecting DRN serotonergic neurons directly or through GABAergic interneurons (Celada et al., 2001; Jankowski & Sesack, 2004). It has implications for a number of brain disorders, including the biological action of antidepressants (Lucas et al., 2010; Vidal et al., 2009). In the embryonic mouse brain, young mPFC neurons express 5-HT_4Rs before they establish synapses with raphe neurons (Slaten et al., 2010); therefore, a transient change in 5-HT_4R expression in the fetal mPFC can potentially result in permanent dysregulation of 5-HT levels in many brain regions.

This experiment investigated the effects of maternal PS and 5-HT depletion on the mRNA levels of all 5-HT_4R splice variants in the embryonic mouse telencephalon. Receptor mRNA levels were measured instead of receptor protein levels due to the lack of highly specific 5-HT_4R antibodies appropriate for quantitative studies. Previous studies have shown that maternal stress and serotonergic perturbations can cause various alterations in the developing brain (Altamura et al., 2007; Miyagawa et al., 2011; D. A. V. Peters, 1990; Vitalis et al., 2007). This experiment also

investigated whether maternal stress and other environmental signals can affect 5-HT_4R expression in the embryonic telencephalon by way of β_2-ARs. Evidence suggests that 5-HT_4Rs may be functionally associated with β_2-ARs, since the β_2-AR gene is nested within the 5-HT_4R gene (Bockaert et al., 2004) and 5-HT_4Rs can form heterodimers with β_2-ARs (Berthouze et al., 2005, 2007). Also, concerns have been raised regarding a possible association between terbutaline (a selective β_2-AR agonist used to treat preterm labor) and ASD (Witter, Zimmerman, Reichmann, & Connors, 2009), a disorder in which serotonergic abnormalities have long been noted (Anderson, 2002).

Experimental Procedures

Animals

Timed-pregnant C57BL/6 mice were purchased from Charles River Laboratories and housed individually on a 12:12 light–dark cycle (lights on at 07:00, off at 19:00) with free access to water and food. All procedures have been approved by the UCSB Institutional Animal Care and Use Committee.

Treatment Groups

Dams were randomly assigned to one of five treatment groups. In the first (control) group, dams were given an intraperitoneal (i.p.) injection of saline once every day beginning at E14. In the second group, embryonic 5-HT was depleted by treating dams with *para*-chlorophenylalanine (*p*CPA; Sigma-Aldrich #C3635), a TPH inhibitor, dissolved in saline. Based on published reports (Vataeva et al., 2008; Vitalis

et al., 2007), dams were given an injection of *p*CPA (200 mg/kg, i.p.) once every day beginning at E14. In the third group, dams were given an intraperitoneal injection of saline and immediately exposed to restraint stress by placing them in a 50 mL polystyrene centrifuge tube with air-holes for 1 h every day beginning at E14. Restraint stress has been used as a controlled stressor in many published studies (Darnaudéry, Dutriez, Viltart, Morley-Fletcher, & Maccari, 2004; Holson, Gough, Sullivan, Badger, & Sheehan, 1995; Jones et al., 2010; Ribes, Fuentes, Torrente, Colomina, & Domingo, 2010; Vallée et al., 1997). In the fourth group, dams were exposed to restraint stress as dams in the third group, but instead of saline they were given an injection of ICI 118,551, a selective adrenergic β_2 antagonist (Sigma-Aldrich #I127), dissolved in saline. The dose of ICI 118,551 (2 mg/kg, i.p.) was based on published studies (Yalcin et al., 2010; Yu et al., 2010). In the fifth group, dams were treated with terbutaline hemisulfate, a selective adrenergic β_2 agonist (Sigma-Aldrich #T2528), dissolved in saline. Based on published reports (Thaker et al., 2006), dams were given an injection of terbutaline (5 mg/kg, i.p.) every day beginning at E14. Both terbutaline and ICI 118,551 could reach the embryonic brain because the mouse blood-brain barrier matures after birth (Daneman et al., 2010) and it is permeable to terbutaline and ICI 118,551 in adulthood (Hsu, Robinson, & Basmadjian, 1994; Moresco et al., 2000). In all groups, the injected volume was kept constant (0.5 cm^3).

Tissue Collection and RNA Isolation

At E17, dams were terminally anesthetized with a mixture of ketamine (200 mg/kg) and xylazine (20 mg/kg), and their uterus was dissected and kept in 0.1 M

phosphate-buffered saline (PBS, pH 7.4) on ice. Embryos were removed from the uterus, decapitated, and their brains were dissected with a fine forceps under a stereoscope. The telencephalon was isolated by carefully transecting the telencephalon-diencephalon junction and the total RNA was immediately extracted from the telencephalon with the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA quality (the A260/A280 ratio) and concentration were measured with a NanoDrop spectrophotometer and the samples were stored at −75 ℃ until further processing. From each sample, an estimated 100 ng of total RNA was reverse-transcribed to cDNA in an Eppendorf Mastercycler pro S using the iScript cDNA Kit (Bio-Rad) according to the manufacturer's instructions. Three embryos were used from each dam.

Quantitative RT-PCR Analysis

The RT-qPCR analysis was based on our published protocol (Hernandez & Janušonis, 2010). Five mRNAs of interest (the four 5-HT_4R splice variants and β_2-AR) and three reference (housekeeping) mRNAs (β_2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase, and TATA box binding protein) were analyzed. The amplification was performed in a MyiQ single color real-time PCR detection system (Bio-Rad). Each PCR reaction (20 μL) was performed in triplicate and contained the cDNA equivalent of 20 ng RNA, forward and reverse splice variant-specific primers (0.5 μM each; Integrated DNA Technologies, Inc.), 0.2 mM dNTPs, 0.25 U PlatinumTaq DNA polymerase (Invitrogen), 0.7X SYBR Green I, and 10 nM fluorescein in a PCR buffer containing 10mM Tris–HCl (pH 9.0 at 25 ℃), 50mM

KCl, 2.5mMMgCl2, and 0.1% Triton X-100. The primers were designed in Beacon Designer (Premier Biosoft International) and are given in Table 2.1. The amplification conditions were as follows: 94 ℃ (10min); 45 cycles of 94 ℃ (10 s), 56 ℃ (10 s), 72 ℃ (90 s), 78 ℃ (20 s), 82 ℃ (20 s); 95 ℃ (1 min); and 60 ℃ (1 min). In order to minimize non-specific signal, fluorescence values used in the analysis were obtained at the highest available temperature at which no melting of the product double-stranded DNA was detected. Controls with no reverse transcription indicated no contamination with genomic DNA.

The efficiency of each amplification was calculated automatically by using a Mathematica (Wolfram Research, Inc.) program (Hernandez & Janušonis, 2010) based on a published algorithm (Tichopad, Dilger, Schwarz, & Pfaffl, 2003) with modifications. Briefly, linear regression was used to model the initial baseline of the non-transformed fluorescence data, and the beginning of the exponential phase was defined as the amplification cycle in which the first of three consecutive regression outliers was detected (the outliers were defined as points whose externally studentized residual was significant at the 0.005 level). The exponential phase was modeled by linear regression of the log-transformed fluorescence data and the end of the exponential phase was defined as the amplification cycle in which the first regression outlier was detected (the outlier was defined as the first point whose externally studentized residual was significant at the 0.10 level). The mRNA amount in the initial tissue sample was calculated as the triplicate mean of (RFU−*baseline*) × E^{-x}, where *baseline* is the mean baseline fluorescence before the exponential phase, *x*

mRNA accession	Target mRNA	Forward primer (5'-3')	Reverse primer (5'-3')	bp	E
Y09587	5-$HT_{4(a)}$R	ATCCTCTGCTGTGATGATGAG	ACTGTGCAAAACTGTATACCTTAG	120	1.696
Y09585	5-$HT_{4(b)}$R	CCTGGACAATGACCTAGAAGAC	TTGCCTCTGCTCTTGGAAAG	121	1.734
Y09588	5-$HT_{4(e)}$R	ATCCTCTGCTGTGATGATGAG	GGAACAGGTCTATTGCGGAAG	134	1.713
AJ011369	5-$HT_{4(f)}$R	ACCTGTTCCCGTCTAACTGAG	TAGTAACCTGTTCATGCAGACAC	190	1.766
NM_007420	β2-AR	TCTGTCTGTCTGTCTGGATGATG	CCCATTGTCACAGCAGAAAGG	167	1.731
NM_009735 (reference)	B2M	GGAGAATGGGAAGCCGAACATAC	AGAAAGACCAGTCCTTGCTGAAG	143	1.812
NM_008084 (reference)	GAPDH	AATGTGTCCGTCGTGGATCTGA	AGTGTAGCCCAAGATGCCCTTC	117	1.794
NM_013684 (reference)	TBP	GATCTTTGCAGGCAAGCAGCT	TTCTCTATGGTGTCACTGGGCTC	197	1.801

Table 2.1. The quantitative RT-PCR primers and the amplification efficiencies. Since amplification efficiencies depend only on primers and amplification conditions, all experimental conditions were used to obtain the estimates. Abbreviations: bp, amplicon length in base pairs; E, mean amplification efficiency; B2M, β2-microglobulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA box binding protein.

and RFU are the cycle number and the fluorescence at the mid-point of the exponential phase, and *E* is the amplification efficiency of the mRNA species. The normalized mRNA amount was calculated by dividing the mRNA amount by the geometric mean of the mRNA amounts of the reference genes in the same sample (Vandesompele et al., 2002). For convenience, the obtained value was multiplied by 1000.

Statistical Analysis

Statistical analyses were performed in R 2.14 (The R Foundation for Statistical Computing) and in IBM SPSS 19 (IBM, Inc.). Mixed-effects models were analyzed with the R *nlme* package (Pinheiro, Bates, DebRoy, Sarkar, & Team, 2013) and non-homogeneity of variances was accounted for by choosing the *varIdent* variance structure (Zuur, Ieno, Walker, Saveliev, & Smith, 2009). These approaches are superior to traditional statistical tests that are based on mathematical convenience rather than biological relevance and are strongly recommended for experimental research in neuroscience (Lazic, 2010; Nakagawa & Hauber, 2011). By following a well-established procedure (Zuur et al., 2009), statistical models were built sequentially by using restricted maximum likelihood estimation (REML) and maximum likelihood estimation (ML): (i) first, a "beyond optimal" model was constructed that included all relevant fixed effects; (ii) the structure of variances and random effects was optimized by comparing nested REML-fitted models with ANOVA and testing the significance of the likelihood ratios (*L*) (in mixed-effects analyses, the significance of *L* was calculated "on the boundary"); (iii) the structure of

fixed effects was optimized by comparing nested ML-fitted models with ANOVA and testing the significance of L (the nonsignificant fixed effects were removed); (iv) the final model was refitted with REML and validated (tested for the normality and homogeneity of residuals). In all tests, the significance level was set at 0.05. For post-hoc analyses, the Bonferroni correction was used.

Results

Effects of PS, β_2-AR Agents, and 5-HT Depletion on β_2-AR mRNA Levels

Since the transcription of the 5-HT_4R gene can potentially be affected by the transcription of the β_2-AR gene due to the nested arrangement of the genes (Gibson, Thomson, Abrams, & Kirkham, 2005; Lucas et al., 2005), we first investigated whether the β2-AR mRNA amount in the embryonic telencephalon was altered by any of the treatments used in the study (maternal PS, selective β_2-AR agents (terbutaline and ICI 118,551), and 5-HT depletion with *p*CPA) (see Figure 2.1). The initial statistical model included these conditions as the fixed effect (Treatment). Adding a random litter effect to the intercept significantly improved the model (L=16.8, p<0.0001). Allowing each condition to have a different variance did not further improve the model (L=4.60, df=4, p>0.3). The final validated model revealed no significant Treatment effect (F(4,17)=0.65, p=0.63). In summary, the analysis indicated the presence of inter-litter variability in the baseline β_2-AR mRNA levels (with an estimated standard deviation of 17% of the baseline), but these levels were not significantly altered by any of the used experimental perturbations.

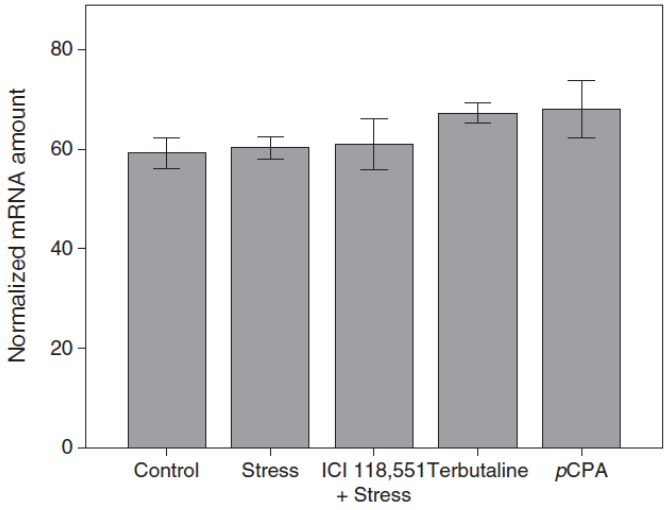

Figure 2.1. Effects of PS, β_2-AR agents, and 5-HT depletion on β_2-AR mRNA levels. The normalized β_2-AR mRNA amounts in the telencephalon of embryos (E17) from control dams injected with saline (Control) and from dams (i) injected with saline and exposed to maternal prenatal stress (Stress), (ii) injected with a β_2-AR antagonist and exposed to maternal prenatal stress (ICI 118,551+Stress), (iii) injected with a β_2-AR agonist (Terbutaline), and (iv) injected with a TPH inhibitor (*p*CPA). Error bars = SEM.

Effects of PS and β_2-AR Agents on 5-HT_4R Splice Variants

We next examined whether maternal restraint stress and a selective β_2-AR agonist (terbutaline) affect the mRNA levels of the 5-HT_4R splice variants in the embryonic telencephalon. We also investigated whether a selective β_2-AR antagonist (ICI 118,551), administered prior to restraint stress, attenuates stress effects on 5-HT_4R mRNA levels (see Figure 2.2). The selective β_2-AR agents were included because stress-related signals can act on β_2-ARs (Qu, Guo, & Li, 2008), β_2-ARs have been shown to form heterodimers with 5-HT_4Rs (Berthouze et al., 2005, 2007), and the activity of one receptor in a heterodimer can affect the internalization and trafficking of the other receptor, with potential changes in its transcription (Renner et al., 2012; Rozenfeld & Devi, 2011). The initial statistical model (with Variant, Treatment, and Variant×Treatment as the fixed effects) was significantly improved by allowing each splice variant to have a different variance (L=215.8, df=3, p<0.0001) and was further improved by adding correlations (compound symmetry) among all splice variants (L=87.7, df=1, p<0.0001). No further improvement was obtained by adding a random litter effect to the intercept (L=10^{-7}, p>0.4), suggesting no variability among the litters. The final validated model showed no significant Variant×Treatment interaction (F(9,212)=1.60, p=0.12) and no Treatment effect in the absence of the interaction term (F(3,221)=0.34, p=0.79).

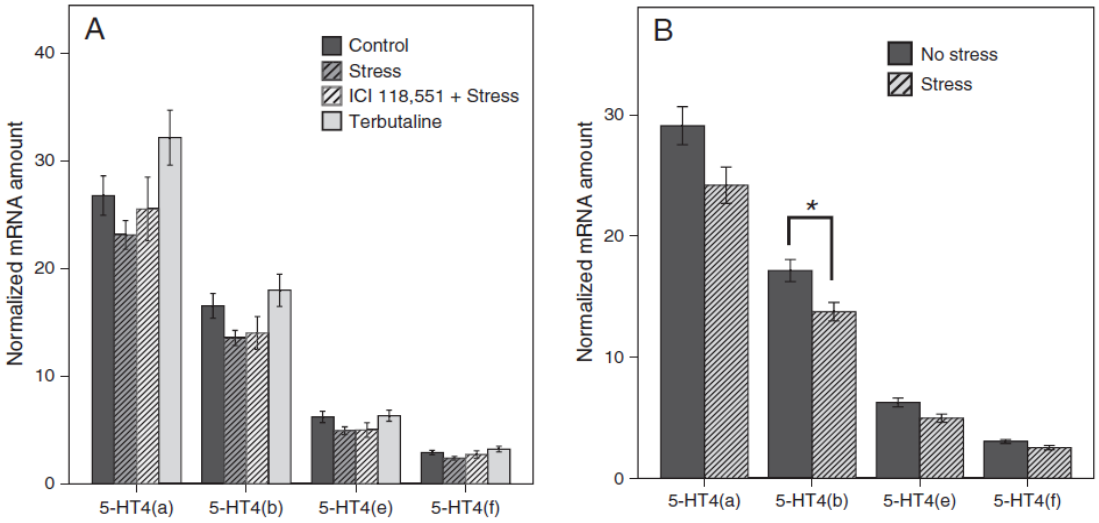

Figure 2.2. The effects of PS and β_2-AR agents on the 5-HT_4R splice variants. (A) The normalized mRNA amounts of the four 5-HT4R splice variants in the telencephalon of embryos (E17) from control dams injected with saline (Control) and from dams (i) injected with saline and exposed to maternal prenatal stress (Stress), (ii) injected with a β_2-AR antagonist and exposed to maternal prenatal stress (ICI 118,551+Stress), and (iii) injected with a β_2-AR agonist (Terbutaline). (B) The data obtained by pooling the two treatments with no stress (Control and Terbutaline) and the two treatments with stress (Stress and ICI 118,551+Stress). Error bars = SEM; *p<0.012 (after the Bonferroni correction for the four comparisons (0.05/4)).

Since similar differences were observed between stressed and unstressed embryos irrespective of whether or not they had been treated with the selective β_2-AR agents (see Figure 2.2A), we next combined these data into two groups disregarding the β_2-AR treatment (see Figure 2.2B). This approach was further supported by the finding that neither the β_2-AR agonist nor the β_2-AR antagonist had a significant effect on the β_2-AR mRNA levels (see Figure 2.1). Also, the correlations between the β_2-AR mRNA levels and the mRNA levels of each 5-HT_4R splice variant were not significantly different from zero, in contrast to the strong correlations among the 5-HT_4R splice variants (see Table 2.2). The two pooled conditions (no stress and stress) produced the same structure of the statistical model (the initial model: Variant, Treatment, Variant×Treatment; different variances for each splice variant: L=229.5, df=3, p<0.0001; correlations between the variants: L=90.9, df=1, p<0.0001; a random litter effect added to the intercept: $L=10^{-7}$, p>0.4). In the final validated model, the Variant×Treatment interaction was significant ($F(3,220)=2.80$, p=0.04). There was no Treatment effect in the absence of the interaction ($F(1,223)=0.57$, p=0.45). Consistent with this finding, only the decrease in the 5-$HT_{4(b)}$R levels was significant after the Bonferroni correction for the four comparisons (5-$HT_{4(a)}$R: $t(55)=2.25$, p=0.028; 5-$HT_{4(b)}$R: $t(55)=2.83$, p=0.007; 5-$HT_{4(e)}$R: $t(55)=2.55$, p=0.014; 5-$HT_{4(f)}$R: $t(55)=2.09$, p=0.041). The decrease in 5-$HT_{4(b)}$R mRNA was 20%.

	5-$HT_{4(b)}$R	5-$HT_{4(e)}$R	5-$HT_{4(f)}$R	β_2-AR
5-$HT_{4(a)}$R	0.579* ($p<10^{-6}$)	0.707* ($p<10^{-10}$)	0.756* ($p<10^{-12}$)	0.059 (p=0.64)
5-$HT_{4(b)}$R		0.478* ($p<10^{-4}$)	0.472* ($p<10^{-4}$)	0.061 (p=0.63)
5-$HT_{4(e)}$R			0.576* ($p<10^{-6}$)	0.066 (p=0.60)
5-$HT_{4(f)}$R				0.067 (p=0.59)

Table 2.2. The correlations between the mRNA amounts of the 5-HT_4R splice variants and β_2-AR at E17. Since correlation measures only association strength, all experimental conditions were used. In each cell, the top number is the Pearson correlation (65 cases) and the bottom number is the *p*-value. By taking into account the Bonferroni correction for the ten cross-correlations, the significance level was set at 0.005 (*).

Effects of 5-HT Depletion on 5-HT$_4$R Splice Variants

We next assessed the susceptibility of all 5-HT$_4$R splice variants to low 5-HT levels in the developing brain. Depletion of 5-HT with *p*CPA strongly reduced the mRNA amount of the 5-HT$_{4(b)}$R splice variant in the embryonic telencephalon, with virtually no effect on the other three 5-HT$_4$R splice variants (see Figure 2.3).

The initial statistical model (with Variant, Treatment, and Variant×Treatment as the fixed effects) was significantly improved by allowing each splice variant to have a different variance (L=86.7, df=3, p<0.0001). Adding correlations between all splice variants further improved the model (L=27.1, df=1, p<0.0001). Adding a random litter effect to the intercept did not further improve the model (L=0.023, p>0.4), suggesting no significant variability among the litters. The Variant×Treatment interaction in the final validated model was highly significant (F(3,92)=4.65, p=0.0045). The decrease in 5-HT$_{4(b)}$R mRNA was 37% and significant after the Bonferroni correction for the four comparisons (t(23)=3.14, p=0.005). The Variant×Treatment interaction remained significant if the data were analyzed with a mixed-design ANOVA with the Greenhouse–Geisser correction (A. Field, 2009), to account for the violation of the sphericity assumption (F(1.8,41.4)=3.72, p=0.037).

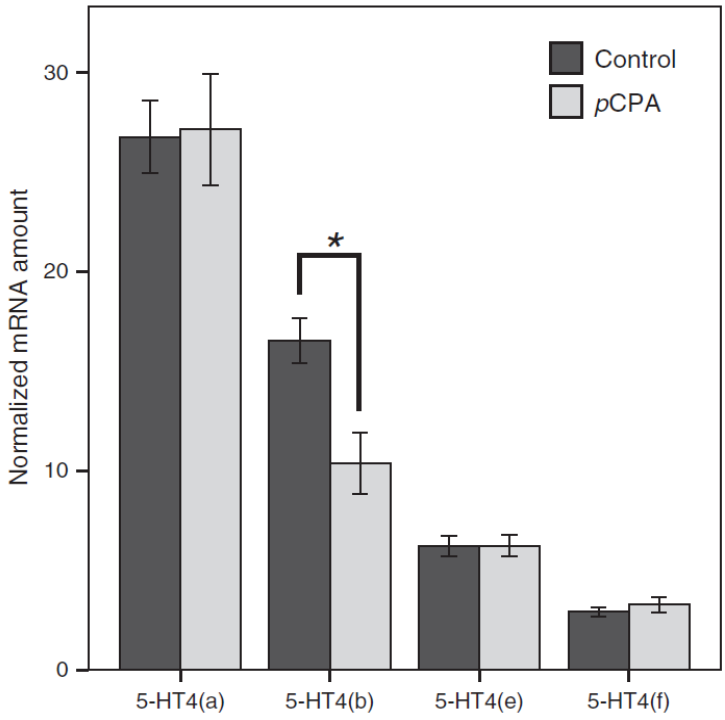

Figure 2.3. The effects of 5-HT depletion on the 5-HT_4R splice variants. The normalized mRNA amounts of the 5-HT_4R splice variants in the telencephalon of embryos (E17) from control dams and from dams treated with *p*CPA. Error bars = SEM; *p<0.012 (after the Bonferroni correction for the four comparisons (0.05/4)).

Discussion

The obtained results demonstrate that PS and reduced 5-HT levels can alter 5-HT_4R expression in the developing telencephalon and that some 5-HT_4R splice variants may be more susceptible than others. They also suggest that these effects are not mediated or modulated by β_2-AR receptors.

Binding studies in the adult brain have found that altered 5-HT levels typically cause compensatory changes in the availability of 5-HT_4Rs to ligands. On the one hand, an increase in 5-HT_4R binding has been observed in some brain regions of adult rats and young pigs following 5-HT depletion with *p*CPA (Ettrup, Kornum, Weikop, & Knudsen, 2011; Licht et al., 2009) and in mice overexpressing the 5-HTT (Jennings et al., 2012). On the other hand, a decrease in 5-HT_4R binding has been observed in the adult rat brain following chronic treatment with the selective 5-HT reuptake inhibitors fluoxetine and paroxetine (Licht et al., 2009; Vidal et al., 2009) and in knockout mice lacking 5-HTT (Jennings et al., 2012). PS can increase 5-HT levels in the embryonic rat brain (D. A. V. Peters, 1990) and it may also increase the number of 5-HT immunoreactive neurons in the adult brains of prenatally exposed mice (Miyagawa et al., 2011). Taken together, these results appear to indicate that PS is likely to decrease 5-HT_4R expression in the embryonic telencephalon and that 5-HT depletion is likely to increase it.

Our results are not consistent with these predictions and suggest a more complex developmental mechanism. Both maternal stress and 5-HT depletion caused a decrease in 5-HT_4R mRNA levels, even though the strength of the effect varied

among the conditions and splice variants. These shifts are remarkably similar to the results obtained in another study that has investigated 5-HT_{1A}Rs in the developing rat brain (Lauder, Liu, & Grayson, 2000). In this study, prenatal exposure to 5-methoxytryptamine (5-MT), a non-specific 5-HTR agonist, has caused a moderate (but significant) decrease in 5-HT_{1A}R mRNA levels and prenatal depletion of 5-HT with *p*CPA has reduced 5-HT_{1A}R mRNA levels by nearly 50%. These changes are comparable to our findings not only in their direction, but also in their relative magnitudes (Figures 2.2B and 2.3). Taken together, these findings suggest that, in development, the expression of at least some 5-HTRs requires an optimal 5-HT level and that a deviation from this optimum may result in a lower number of mRNA transcripts. Abnormally low or high 5-HT levels may induce this change through the same mechanism or two different mechanisms. For example, low 5-HT levels might result in a failure to developmentally upregulate mRNA levels, perhaps in a splice-specific manner (Hernandez & Janušonis, 2010), whereas high 5-HT levels might lead to compensatory downregulation of mRNA levels. Generally, quantitative relationships between mRNA transcripts and receptor availability can be complex or counterintuitive due to the dynamics of the system (Janušonis, 2012a). In this regard, several technical considerations are important for the interpretation of our and other related studies.

Most published studies have examined 5-HT_4R binding, whereas receptor mRNA levels were analyzed in our study and in Lauder et al. (2000). The measures obtained by these two technical approaches are not directly comparable. First, 5-

HT_4R binding can change due to receptor internalization and recycling (Mnie-Filali et al., 2010), with no change in mRNA levels. Second, mRNA analysis cannot detect 5-HT_4Rs on the terminals of long-range projections that originate outside the telencephalon. There is strong evidence that 5-HT_4Rs can be expressed not only postsynaptically, but also presynaptically in distal axonal segments (Slaten et al., 2010; Vilaró, Cortés, & Mengod, 2005). Third, binding studies cannot distinguish among 5-HT_4R splice variants; therefore, changes affecting only some of the variants can be masked by other, abundantly expressed variants.

A recent study has shown that early forebrain development depends on maternal 5-HT signals that reach the embryo through the placenta (Bonnin et al., 2011). While no information is currently available about how placental 5-HT synthesis is affected by maternal stress, *p*CPA is known to inhibit placental 5-HT synthesis (Bonnin et al., 2011). It cannot be ruled out that placental 5-HT might have contributed to our findings, but we note that all experimental treatments began at E14, around the time when the mouse forebrain begins to switch to its endogenous 5-HT source, brainstem serotonergic afferents (Bonnin & Levitt, 2011).

The relatively small effect of maternal stress on 5-HT_4R levels may be due to the restraint length (one hour per day), which was low in comparison to that used in other reports. Daily restraint stress for a total of 1.5–3.0 h is considered standard or "weak" (Darnaudéry et al., 2004; Holson et al., 1995; Miyagawa et al., 2011; Ribes et al., 2010; Vallée et al., 1997), and some researchers have used daily six-hour exposures to produce "strong" PS (Miyagawa et al., 2011). However, such long exposures

increase the contribution of various confounding factors, such as muscle fatigue, thermoregulation, blood circulation, and food intake, and may consequently reduce the relevance of the findings to human research. Achieving an optimal balance between experimental effects in rodents and relevance for human research will require a better understanding of the dose-dependent effects of maternal PS (Mychasiuk, Ilnytskyy, Kovalchuk, Kolb, & Gibb, 2011).

The susceptibility of the 5-$HT_{4(b)}$R splice variant to altered 5-HT levels is interesting because in the adult brain this variant is readily internalized and recycled (Mnie-Filali et al., 2010). Consequently, it is well positioned to rapidly respond to fluctuations of 5-HT levels at the protein level and may depend on a relatively stable mRNA pool. If the reduced availability of 5-$HT_{4(b)}$R mRNA persists into adulthood, it may have implications for brain disorders associated with dysfunction of 5-HT homeostasis.

Chapter 3

Transcriptional Responses of Serotonin Receptors in the Embryonic Mouse Forebrain to Acute Changes of Prenatal Serotonin Levels

Introduction

Forebrain 5-HTRs support various neural functions, including global brain states (R. E. Brown, Basheer, McKenna, Strecker, & McCarley, 2012; Jacobs & Azmitia, 1992), and have been implicated in a number of mental disorders, such as depression, obsessive-compulsive disorder, schizophrenia, ASDs, and others (Azmitia et al., 2011; Fribourg et al., 2011; Vollenweider & Kometer, 2010). This experiment focused on three 5-HTRs, 5-HT_{1A}R, 5-HT_{2A}R, and 5-HT_4R, all of which are expressed in the forebrain (R. Andrade, 2011; Suwa, Bock, Preusse, Rothenberger, & Manzke, 2014). The mouse 5-HT_4R gene produces four splice variant (5-$HT_{4(a)}$R, 5-$HT_{4(b)}$R, 5-$HT_{4(e)}$R, 5-$HT_{4(f)}$R) that differ in their functional properties (Claeysen et al., 1999; Mnie-Filali et al., 2010). In the mPFC, these receptors control the activity of the direct prefrontal projection to the DRN (Hajós et al., 1998; Soiza-Reilly & Commons, 2011a) and modulate the dynamics of 5-HT signaling in the brain (Celada et al., 2002; Hajós, Hajós-Korcsok, & Sharp, 1999; Holmes, 2008; Lucas et al., 2005).

In the developing human brain, the expression of 5-HT receptors is likely to be affected by environmental exposures. We therefore investigated whether the expression of 5-HT_{1A}R, 5-HT_{2A}R, and 5-HT_4R in the embryonic telencephalon is affected by acute prenatal exposures to an SSRI and a 5-HT synthesis inhibitor. Since the mouse 5-HT_4R gene produces four mRNA splice variants (5-$HT_{4(a)}$R, 5-$HT_{4(b)}$R, 5-$HT_{4(e)}$R, 5-$HT_{4(f)}$R) that differ in their functional properties (Claeysen et al., 1999; Mnie-Filali et al., 2010), we analyzed these splice variants separately. Acute exposures were used because chronic models reflect near-equilibrium states

and can be fundamentally misleading, especially in the inherently dynamic developmental processes (Janušonis, 2012b; Kumar, Vlachos, Aertsen, & Boucsein, 2013).

Based on the experimental and conceptual considerations presented in Chapter 1, this experiment sought to understand the immediate mRNA responses of 5-HT_{1A}R, 5-HT_{2A}R, and 5-HT_4R in the mouse telencephalon to acute alterations of prenatal 5-HT levels.

Experimental Procedures

Animals

Timed-pregnant C57BL/6 mice were purchased from Charles River Laboratories and housed individually on a 12:12 light-dark cycle with free access to water and food. All procedures have been approved by the UCSB Institutional Animal Care and Use Committee.

Treatment Groups

Dams were randomly assigned to one of the six groups, each of which represented one of the two embryonic developmental times and one of the three treatments. In the morning of either E14 or E16, dams were given a single intraperitoneal (i.p.) injection of one of the following: (i) saline, (ii) *p*CPA (Sigma-Aldrich C3635; dissolved in saline), or (iii) fluoxetine hydrochloride (Sigma-Aldrich F132; dissolved in saline). The doses of *p*CPA (200 mg/kg) and fluoxetine (0.5 mg/kg) were based on published reports that have used these agents in studies of rodent

embryonic development (Kornum, Licht, Weikop, Knudsen, & Aznar, 2006; Noorlander et al., 2008; Smit-Rigter et al., 2012; Vataeva et al., 2008; Vitalis et al., 2007). Both *p*CPA and fluoxetine have a high placental transfer (Lauder & Krebs, 1976; Noorlander et al., 2008; Rampono et al., 2009; Vitalis et al., 2007). Consistent with previous reports (Noorlander et al., 2008; Vitalis et al., 2007), embryos showed no gross developmental abnormalities.

Tissue Collection and RNA Isolation

Twenty-four hours after the injection, dams were terminally anesthetized with a mixture of ketamine (200 mg/kg) and xylazine (20 mg/kg), and their uterus was dissected and kept in 0.1M PBS on ice. Embryos were removed from the uterus, decapitated, and their brains were dissected with fine forceps under a stereoscope. The telencephalon was isolated by transecting the telencephalon-diencephalon junction, and the total RNA was immediately extracted from the telencephalon with the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA quality (the A260/A280 ratio) and concentration were measured with a NanoDrop spectrophotometer and the samples were stored at −75℃ until further processing. From each sample, an estimated 100 ng of total RNA was reverse-transcribed to cDNA in an Eppendorf Mastercycler pro S using the iScript cDNA Kit (Bio-Rad) according to the manufacturer's instructions. Three embryos were used from each dam, which were assigned unique numbers for further analyses of litter effects.

Quantitative RT-PCR Analysis

The quantitative (real-time) reverse-transcription polymerase chain reaction (RT-qPCR) analysis was based on our published protocol (Chen, Kelley, & Janušonis, 2012). Six mRNAs of interest (5-HT_{1A}R, 5-HT_{2A}R, and the four 5-HT_4R splice variants) and two reference mRNAs (glyceraldehyde-3-phosphate dehydrogenase and ubiquitin C) were analyzed. The amplification was performed in a MyiQ single color real-time PCR detection system (Bio-Rad) or in a CFX96 Touch real-time PCR detection system (Bio-Rad). Each PCR reaction (20 µL) was performed in triplicate and contained the cDNA equivalent of 20 ng RNA, forward and reverse splice variant-specific primers (0.5 µM each; Integrated DNA Technologies, Inc.), 0.2 mM dNTPs, 0.25 U Platinum Taq DNA polymerase (Invitrogen), 0.7X SYBR Green I, and 10 nM fluorescein in a PCR buffer containing 10 mM Tris-HCl (pH 9.0 at 25℃), 50 mM KCl, 2.5 mM MgCl2, and 0.1% Triton X-100. The primers were designed in Beacon Designer (Premier Biosoft International) and Primer-BLAST (National Center for Biotechnology Information) and are given in Table 3.1. The amplification conditions were as follows: 94℃ (10 min); 45 cycles of 94℃ (10 sec), 56℃ (10 sec), 72℃ (90 sec), 78℃ (20 sec), 82℃ (20 sec); 95℃ (1 min); 60℃ (1 min). In order to minimize non-specific signal, fluorescence values used in the analysis were obtained at the highest available temperature at which no melting of the product double-stranded DNA was detected (for all mRNAs, this temperature was 82℃).

mRNA accession	Target mRNA	Forward primer (5'-3')	Reverse primer (5'-3')	bp	E
NM_008308	5-HT_{1A}R	CAGCGCGAGACA GATATTAC	CATCTGAGAGGA GCACTCAC	152	1.802
NM_172812	5-HT_{2A}R	TGACTGATTCCTC TCTGTGC	CCCCTCTCTTTGA GCTTCTA	196	1.798
Y09587	5-$HT_{4(a)}$R	ATCCTCTGCTGTG ATGATGAG	ACTGTGCAAAAC TGTATACCTTAG	120	1.800
Y09585	5-$HT_{4(b)}$R	CCTGGACAATGA CCTAGAAGAC	TTGCCTCTGCTCT TGGAAAG	121	1.799
Y09588	5-$HT_{4(e)}$R	ATCCTCTGCTGTG ATGATGAG	GGAACAGGTCTA TTGCGGAAG	134	1.810
AJ011369	5-$HT_{4(f)}$R	ACCTGTTCCCGTC TAACTGAG	TAGTAACCTGTTC ATGCAGACAC	190	1.804
NM_008084 (reference)	GAPDH	AATGTGTCCGTCG TGGATCTGA	AGTGTAGCCCAA GATGCCCTTC	117	1.770
NM_019639 (reference)	UBC	GATCTTTGCAGGC AAGCAGCT	TTCTCTATGGTGT CACTGGGCTC	174	1.740

Table 3.1. The primer sequences for the RT-qPCR. Abbreviations: bp, amplicon length in base pairs; E, mean amplification efficiency; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; UBC, Ubiquitin C.

The efficiency of each amplification was calculated automatically by using a Mathematica (Wolfram Research, Inc.) program (Hernandez & Janušonis, 2010) based on a published algorithm (Tichopad et al., 2003) with modifications. Briefly, linear regression was used to model the initial baseline of the non-transformed fluorescence data, and the beginning of the exponential phase was defined as the amplification cycle in which the first of three consecutive regression outliers was detected (the outliers were defined as points whose externally studentized residual was significant at the 0.005 level). The exponential phase was modeled by linear regression of the log-transformed fluorescence data and the end of the exponential phase was defined as the amplification cycle in which the first regression outlier was detected (the outlier was defined as the first point whose externally studentized residual was significant at the 0.10 level). The mRNA amount in the initial tissue sample was calculated as the triplicate mean of $(RFU - \text{baseline}) \times E^{-x}$, where *baseline* is the mean baseline fluorescence before the exponential phase, x and RFU are the cycle number and the fluorescence at the mid-point of the exponential phase, and E is the mean amplification efficiency of the mRNA species. The normalized mRNA amount was calculated by dividing the mRNA amount by the geometric mean of the mRNA amounts of the reference genes in the same sample (Vandesompele et al., 2002). For convenience, the obtained value was multiplied by 1000.

Statistical Analysis

For statistical analyses, normalized mRNA amounts were log-transformed. This was done to avoid an interaction effect if two different expression baselines (E15 and

E17) were altered by comparable factors (without this transformation, a change from 1 to 2 and a change from 10 to 20 would be treated as different by additive statistical models). Statistical analyses were performed in R 3.0.2 (The R Foundation for Statistical Computing) and in IBM SPSS 19 (IBM, Inc.). Mixed-effects models were analyzed with the R *nlme* package (Pinheiro et al., 2013) and non-homogeneity of variances was included in statistical models by choosing the varIdent variance structure (Chen et al., 2012; Flood et al., 2012; Zuur et al., 2009). In contrast to traditional tests motivated by mathematical simplicity, these statistical approaches are biologically-oriented and are strongly recommended for experimental research in neuroscience (Lazic & Essioux, 2013; Lazic, 2010; Nakagawa & Hauber, 2011). By following a recommended procedure (Zuur et al., 2009), statistical models were built sequentially by using restricted maximum likelihood estimation (REML) and maximum likelihood estimation (ML): (i) first, a "beyond optimal" model was constructed that included all relevant fixed (deterministic) effects; (ii) the structure of variances and random effects was optimized by comparing nested REML-fitted models with ANOVA and testing the significance of the likelihood ratios (L) (in mixed-effects analyses, the significance of L was calculated "on the boundary"); (iii) the structure of fixed effects was optimized by comparing nested ML-fitted models with ANOVA and testing the significance of L (the non-significant fixed effects were removed); (iv) the final model was refitted with REML and validated (tested for the normality of residuals with the Shapiro-Wilk test and for the homogeneity of residuals with the Fligner-Killeen and Bartlett tests). The effects of individual

treatments were assessed by estimating the significance of the coefficients at the appropriate dummy variables in the final model. In all tests, the significance level was set at 0.05.

Results

Experimental Groups

This experiment investigated whether acute changes in prenatal 5-HT levels alter 5-HT_4R expression in the embryonic telencephalon. Since in the adult brain DRN-projecting mPFC neurons are also strongly modulated by 5-HT_{1A}R and 5-HT_{2A}R, these receptors were added to the set.

Timed-pregnant mice were pharmacologically treated at E14 or E16 with a *p*CPA or an SSRI (fluoxetine) to achieve a decrease or increase in extracellular 5-HT levels, respectively. After 24 hours, the mRNA levels of 5-HT_{1A}R, 5-HT_{2A}R, and the four 5-HT_4R splice variants were quantified in the embryonic telencephalon Figures 3.1 and 3.2.).

5-HT_{1A}R Expression

In the analysis of 5-HT_{1A}R expression, allowing the variance to depend on Age or Treatment did not significantly improve the statistical model ($L(1) = 0.22$, $p = 0.64$ and $L(2) = 4.95$, $p = 0.08$, respectively). The model was significantly improved when the intercept (the baseline mRNA expression) was allowed to randomly vary across litters ($p = 0.0004$; the estimated standard deviation of this variation was 0.087). In the presence of the litter effects, the Age×Treatment interaction was not significant

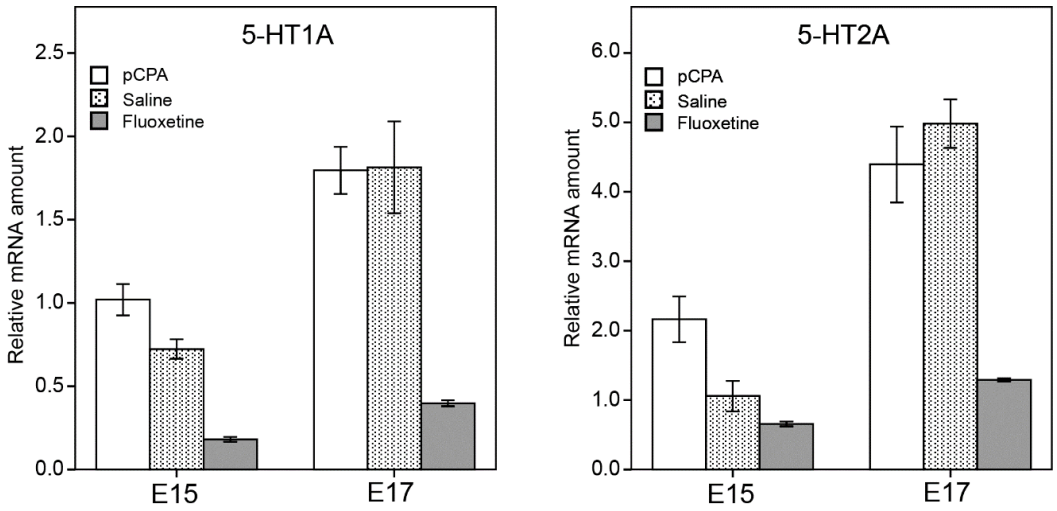

Figure 3.1. The mRNA amounts of the 5-HT_{1A}R and 5-HT_{2A}R in the telencephalon of embryos prenatally exposed to *p*CPA and fluoxetine for 24 hours (E14-15 and E16-17). Error bars = SEM; the plotted means assume independence among data points. In statistical analyses, litter effects were included.

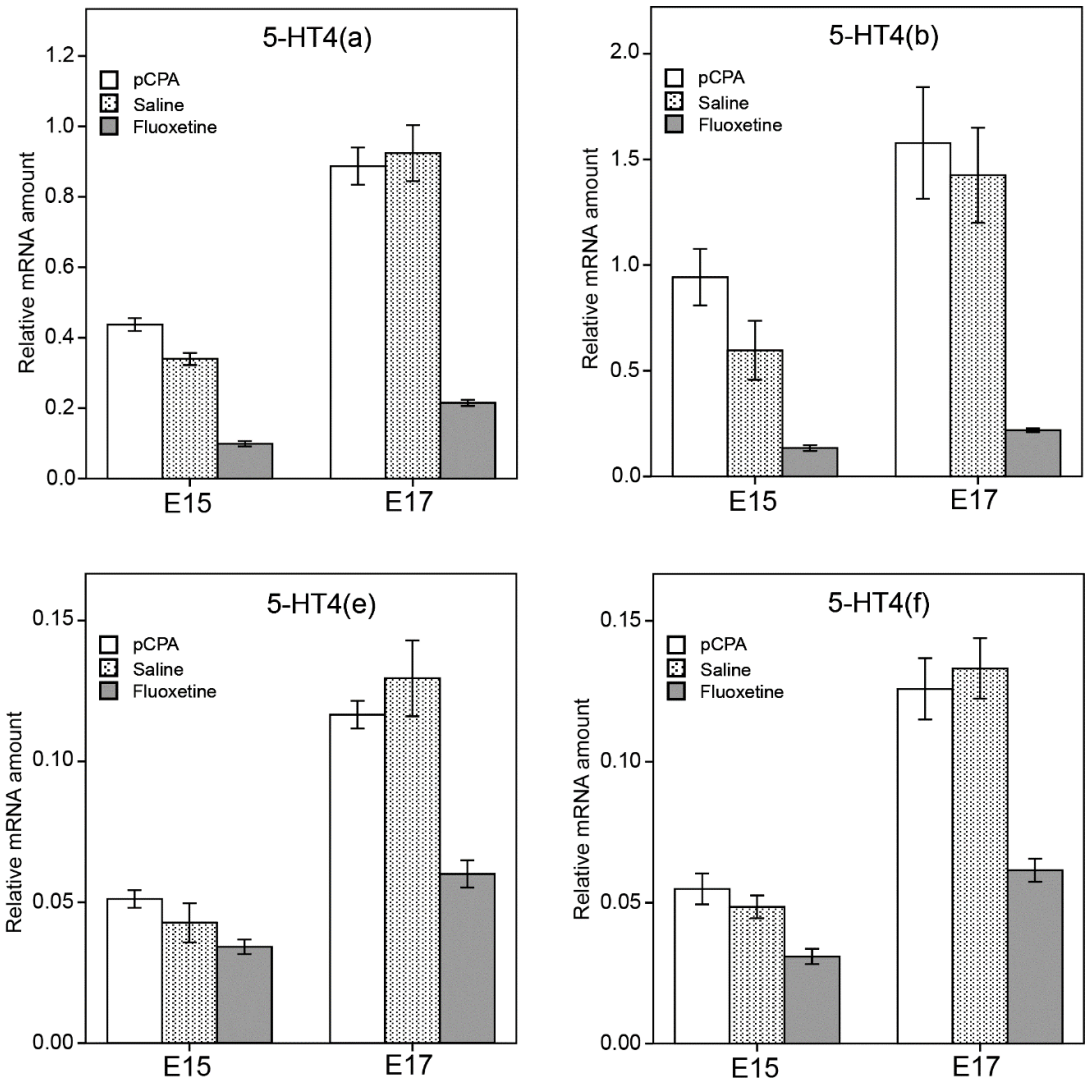

Figure 3.2. The mRNA amounts of the 5-HT_4R splice variants in the telencephalon of embryos prenatally exposed to *p*CPA and fluoxetine for 24 hours (E14-15 and E16-17). Error bars = SEM; the plotted means assume independence among data points. In statistical analyses, litter effects were included.

($L(2) = 1.60$, $p = 0.45$). Without the interaction term, the overall Treatment effect was significant ($L(2) = 54.4$, $p < 0.0001$). Taken separately, only the fluoxetine treatment significantly affected 5-HT_{1A}R mRNA levels ($t(17) = -11.1$, $p < 0.0001$), but the *p*CPA treatment had no significant effect ($t(17) = 1.50$, $p = 0.15$).

5-HT_{2A}R Expression

In the analysis of 5-HT_{2A}R expression, allowing the variance to depend on Age and Treatment significantly improved the model ($L(5) = 63.7$, $p < 0.0001$). The model was further improved when the intercept was allowed to randomly vary across litters ($p = 0.002$; the estimated standard deviation of this variation was 0.217). In the presence of the litter effects, the Age×Treatment interaction was not significant ($L(2) = 5.76$, $p = 0.06$). Without the interaction term, the overall Treatment effect was significant ($L(2) = 14.8$, $p = 0.0006$). Taken separately, only the fluoxetine treatment significantly affected 5-HT_{2A}R mRNA levels ($t(17) = -2.87$, $p = 0.01$), but the *p*CPA treatment had no significant effect ($t(17) = 0.95$, $p = 0.35$).

5-$HT_{4(a)}$R Expression

In the analysis of 5-$HT_{4(a)}$R expression, allowing the variance to depend on Age or Treatment did not significantly improve the model ($L(1) = 0.31$, $p = 0.58$ and $L(2) = 2.04$, $p = 0.36$, respectively). The model was significantly improved when the intercept was allowed to randomly vary across litters ($p = 0.015$; the estimated standard deviation of this variation was 0.050). In the presence of the litter effects, the Age×Treatment interaction was not significant ($L(2) = 4.01$, $p = 0.13$). Without the

interaction term, the overall Treatment effect was significant ($L(2) = 69.6$, $p < 0.0001$). Taken separately, only the fluoxetine treatment significantly affected 5-$HT_{4(a)}$R mRNA levels ($t(17) = -16.8$, $p < 0.0001$), but the *p*CPA treatment had no significant effect ($t(17) = 1.51$, $p = 0.15$).

5-$HT_{4(b)}$R Expression

In the analysis of 5-$HT_{4(b)}$R expression, allowing the variance to depend on Age did not significantly improve the model ($L(1) = 0.043$, $p = 0.84$), but the model was significantly improved when the variance was allowed to depend on Treatment ($L(2) = 13.2$, $p = 0.001$). The model was further improved when the intercept was allowed to randomly vary across litters ($p = 0.005$; the estimated standard deviation of this variation was 0.116). In the presence of the litter effects, the Age×Treatment interaction was not significant ($L(2) = 1.75$, $p = 0.42$). Without the interaction term, the overall Treatment effect was significant ($L(2) = 45.3$, $p < 0.0001$). Taken separately, only the fluoxetine treatment significantly affected 5-$HT_{4(b)}$R mRNA levels ($t(17) = -8.38$, $p < 0.0001$), but the *p*CPA treatment had no significant effect ($t(17) = 1.54$, $p = 0.14$).

5-$HT_{4(e)}$R Expression

In the analysis of 5-$HT_{4(e)}$R expression, allowing the variance to depend on Age did not significantly improve the model ($L(1) = 2.57$, $p = 0.11$), but the model was significantly improved if the variance was allowed to depend on Treatment ($L(2) = 9.74$, $p = 0.008$). The model was further improved when the intercept was allowed to

randomly vary across litters ($p = 0.004$; the estimated standard deviation of this variation was 0.084). In the presence of the litter effects, the Age×Treatment interaction was only marginally significant ($L(2) = 6.60$, $p = 0.04$) and, considering the lack of this effect in the other five genes, was not included in the final model. Without the interaction term, the overall Treatment effect was significant ($L(2) = 17.9$, $p = 0.0001$). Taken separately, only the fluoxetine treatment significantly affected 5-$HT_{4(e)}$R mRNA levels ($t(17) = -3.28$, $p = 0.004$), but the *p*CPA treatment had no significant effect ($t(17) = 0.65$, $p = 0.52$).

5-$HT_{4(f)}$R Expression

In the analysis of 5-$HT_{4(f)}$R expression, allowing the variance to depend on Age or Treatment did not significantly improve the model ($L(1) = 3.65$, $p = 0.06$ and $L(2) = 2.04$, $p = 0.36$, respectively). The model was significantly improved when the intercept was allowed to randomly vary across litters ($p = 0.04$; the estimated standard deviation of this variation was 0.066). In the presence of the litter effects, the Age×Treatment interaction was not significant ($L(2) = 2.27$, $p = 0.32$). Without the interaction term, the overall Treatment effect was significant ($L(2) = 25.8$, $p < 0.0001$). Taken separately, only the fluoxetine treatment significantly affected 5-$HT_{4(f)}$R mRNA levels ($t(17) = -5.31$, $p = 0.0001$), but the *p*CPA treatment had no significant effect ($t(17) = 0.102$, $p = 0.92$).

Effect of the pCPA Treatment on 5-HT_{2A}R Expression at E15

The previous analyses showed that the *p*CPA treatment had no significant effect on the three 5-HTRs. However, Figures 3.1 and 3.2 suggest that, at E15, this treatment may have increased the mRNA levels of at least some receptors, but this effect was undetectable due to insufficient statistical power. To further investigate it, we chose the receptor with the strongest apparent change (5-HT_{2A}R) and reanalyzed its mRNA levels at E15, considering only the *p*CPA and control conditions (Figure 3.1). The mRNA amounts were not log-transformed. If all data points were considered independent, the *p*CPA effect was indeed significant ($t(19) = -2.59$, $p = 0.018$). However, a mixed-effects model detected a strong litter effect ($p < 0.0001$). When this effect was included, the difference between the *p*CPA and control conditions became not significant ($L(1) = 2.24$, $p = 0.13$). It was therefore concluded that the present data do not provide evidence that inhibition of 5-HT synthesis rapidly alters the mRNA levels of the studied receptors.

Discussion

We found that inhibition of 5-HT synthesis for 24 hours did not significantly affect the mRNA abundance of all studied 5-HTRs in the embryonic telencephalon, but that their mRNA levels were significantly reduced by elevation of extracellular 5-HT for the same length of time. We detected no significant interaction between the treatment condition and the developmental time (E15 and E17). The observed mRNA responses may represent changes not only in gene transcription, but also in mRNA trafficking and degradation (Di Liegro, Schiera, & Di Liegro, 2014; Joseph, Spicer, &

Tholanikunnel, 2013; Tholanikunnel et al., 2010). Functionally, a change in mRNA abundance does not necessarily indicate a similar change in protein levels (Schwanhäusser et al., 2011) or the receptor availability to ligands (Tholanikunnel et al., 2010). However, mRNA levels can be measured with the level of specificity and quantitative accuracy that is difficult to achieve in protein analyses.

The lack of significant changes following the inhibition of 5-HT synthesis is consistent with the unchanged 5-HT_{1A}R binding in genetic mouse models that lack brain serotonergic neurons (Massey et al., 2013) and the unaltered 5-HT_{1A}R, 5-HT_{2A}R, and 5-HT_4R binding in many studied brain regions in young pigs chronically treated with *p*CPA (Ettrup et al., 2011). Likewise, chronic prenatal treatment with *p*CPA has produced no significant changes in the mRNA levels of three out of the four 5-HT_4R splice variants (5-$HT_{4(a)}$R, 5-$HT_{4(e)}$R, 5-$HT_{4(f)}$R) in the mouse telencephalon (Chen et al., 2012). However, chronic prenatal treatment with *p*CPA has strongly decreased 5-HT_{1A}R mRNA in the rat brain (Lauder et al., 2000) and 5-$HT_{4(b)}$R mRNA in the mouse telencephalon (Chen & Janušonis, 2014). This suggests that unaltered mRNA levels may not indicate insensitivity to 5-HT levels, but may rather represent the initial or final points of dynamic processes that could produce different results if different developmental and exposure times were used (Janušonis, 2012b). For example, one hour after a single dose of fluoxetine, 30-40% of membrane 5-HT_{1A} autoreceptors become internalized in the rat DRN, but three weeks of treatment with fluoxetine results in a normal membrane density of 5-HT_{1A}R in these neurons (Descarries & Riad, 2012). Likewise, the apparent contradiction between the

similar responses of 5-$HT_{4(a)}$R and 5-$HT_{4(b)}$R in the acute *p*CPA treatment and the different responses of 5-$HT_{4(a)}$R and 5-$HT_{4(b)}$R in the repeated *p*CPA treatment (Chapter 2) might be explained by the different internalization and recycling of these splice variants (Mnie-Filali et al., 2010). These differences may become apparent only with longer treatment times. Our results suggest that 5-HTR mRNA levels might increase if 5-HT synthesis were inhibited for more than 24 hours (Figures 3.1-3.2) and perhaps eventually return to their normal levels, if receptor binding signals can be used as proxies (Ettrup et al., 2011; Massey et al., 2013).

The strong decrease in mRNA levels of all three receptors in response to acute fluoxetine treatment mirror the results of studies that have used chronic fluoxetine exposures (Licht et al., 2009; Shishkina et al., 2012; Vidal et al., 2009). However, this apparent simplicity may conceal more complex processes. Chronic fluoxetine treatment has decreased 5-HT_{1A}R protein levels in the rat hippocampus, but the hippocampal 5-HT_{1A}R protein levels have been significantly increased in rats that had been exposed to a mimicked prenatal infection (which reduces 5-HT_{1A}R expression) (Lin, Lin, & Wang, 2012). Also, no change in 5-HT_{2A}R mRNA has been found in adult mice lacking 5-HTT (Q. Li et al., 2003).

On a methodological note, this study demonstrates the importance of including litter effects in developmental analyses. The baseline mRNA amounts of all 5-HTRs significantly varied from litter to litter despite the genetic uniformity of the inbred C57BL/6 strain. Disregard for litter effects continues to undermine the validity of inferences in many studies, despite the simplicity of mixed-effects analyses and calls

to incorporate basic biology into statistical reasoning (Lazic & Essioux, 2013; Lazic, 2010; Nakagawa & Hauber, 2011).

The time-dependent biological factors that can affect the transcription and translation of 5-HTRs remain poorly understood. A more systematic sampling of immediate responses to altered 5-HT levels at different developmental times may reveal how the dynamics of 5-HTRs is driven by environmental stimuli.

Chapter 4

Genetically Altered 5-HT_4R Expression Affects the Synaptogenesis of the mPFC-DRN Projection

Introduction

In rodents, the mPFC projects directly to the DRN (Hajós et al., 1998; Vertes, 2004) and dynamically controls behavioral decisions on a fine temporal scale (Challis, Beck, & Berton, 2014; Warden et al., 2012). This projection has been implicated in the neurobiology of depression, social avoidance, and other related brain states (Challis et al., 2014; Lammel, Tye, & Warden, 2014). Strong anatomical and functional evidence suggests that the rodent mPFC is homologous to the human agranular mPFC (Passingham, Passingham, & Wise, 2012; Preuss, 1995).

DRN-projecting neurons in the mPFC express several 5-HTRs, the activity of which has different effects on DRN targets. Activation of 5-HT_{1A}R in the mPFC decreases the firing rate of DRN neurons (Celada et al., 2002; Hajós et al., 1999), whereas activation of 5-HT_{2A}R and 5-HT_4R has the opposite effect (Bockaert et al., 2011; Celada et al., 2002; Lucas et al., 2005). The three receptors (5-HT_{1A}R, 5-HT_{2A}R, 5-HT_4R) are coupled to different G-proteins (Gi, Gq, and Gs, respectively) and can collectively regulate neuronal excitability (R. Andrade, 2011; Holmes, 2008; Janušonis, 2014). Importantly, their expression is upregulated prenatally (Hernandez & Janušonis, 2010; Lauder et al., 2000; Waeber et al., 1996), before mPFC projection neurons can establish synapses with their brainstem targets (Polleux, Dehay, & Kennedy, 1997). Therefore, these receptors are likely to affect the formation and stabilization of synapses between mPFC terminals and DRN neurons, and their altered expression in perinatal development may result in an abnormal behavioral dynamic later in life. To gain a better understanding of these processes, this

experiment studied the structure of synaptic contacts between mPFC terminals and serotonergic DRN neurons in mice lacking functional 5-HT_4Rs. The mPFC terminals in the DRN were visualized by taking advantage of the finding that they express a specific vesicular glutamate transporter, vGluT1 (Soiza-Reilly & Commons, 2011a).

Experimental Procedures

Animals

5-HT_4R heterozygous mutant (*Htr4*$^{+/-}$) mice were obtained by breeding 5-HT_4R receptor homozygous mutant (*Htr4*$^{-/-}$) mice (strain B6.129P2-Htr4tm1Dgen/J on the C57BL/6J background; The Jackson Laboratory) with wild-type (*Htr4*$^{+/+}$) mice (strain C57BL/6J). These transgenic mice have been used by other researchers (Kobayashi et al., 2010). Adult *Htr4*$^{+/-}$ mice were bred and monitored during pregnancy until the birth of offspring. Pups were weaned at postnatal day (PD) 21 and housed with 2-5 same-sex littermates. All animals were housed on a 12:12 light-dark cycle (lights on at 07:00, off at 19:00) with free access to water and food. All experiments were approved by the UCSB Institutional Animal Care and Use Committee.

Immunoelectron Microscopy

In order to identify how the lack of functional 5-HT_4Rs alters the synaptic structure of the mPFC-DRN projection in adulthood, we first attempted to visualize vGluT1-positive synapses in the DRN by using immunoelectron microscopy. Electron microscopy was used because it allows direct observation of synapses. Primary antibodies targeting vGluT1 and 5-HT were used to identify mPFC

glutamatergic terminals and serotonergic neurons, respectively. Several tissue embedding methods were attempted.

Electron Microscopy with No Immunolabeling. At PD56, offspring were terminally anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and tails were collected and stored at -20℃ for genotyping. Anesthetized animals were transcardially perfused with 0.9% saline, followed by 100 mL of cold 4% paraformaldehyde and 1% glutaraldehyde in 0.1M phosphate buffer (PB). The brains were dissected from the skull, post-fixed in the same fixative mixture overnight, and sectioned on a vibratome at 50 μm thickness. The sections were post-fixed for 30 minutes in 2% glutaraldehyde in PBS; rinsed 3 times (10 min each) in PBS; incubated for 30 minutes in 1% osmium tetroxide in PBS; rinsed 3 times (10 min each) in PBS; dehydrated in a graded series of ethanol; and transferred into propylene oxide. After infiltrating with Durcupan ACM (Fluka, Saint Louis, MO), the sections were flat-embedded on glass slides, coverslipped with Liquid Release Agent-coated coverslips, and polymerized for 48 hours at 55-60℃.

Thin-sectioning, Contrasting, and Image Acquisition. Glass knives were cut from 38 mm wide glass strips with the LKB 7801B Knifemaker (Vienna, Austria) immediately before thin-sectioning. By using the Reichert Ultracut E Microtome (Buffalo, NY), embedded sections were semi-thin sectioned at 1 μm thickness, stained with a cresyl violet-based stain, and inspected under a light microscope. This step was repeated until the desired sectioning depth was reached. The cured sections were then ultra-thin sectioned at 90 nm, flattened with chloroform, and collected onto

copper grids. After drying, the ultra-thin sections were contrasted with 5% uranyl acetate for 20 minutes and Reynold's lead citrate for 10 minutes, and imaged with the JEOL 1230 Transmission Electron Microscope (Tokyo, Japan). Montages were assembled manually in high digital magnification, and the editing of images was limited to global brightness and contrast. This method was used several times, and this section is referred to throughout this chapter.

Pre-embedding Immunoelectron Microscopy with 3,3'-Diaminobenzidine as a Chromogen. In order to develop an immunolabeling protocol for electron microscopy, we first used an anti-5-HT antibody. At PD56, offspring were terminally anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and tails were collected and stored at -20℃ for genotyping. Anesthetized animals were transcardially perfused with 0.9% saline, followed by 100 mL of cold 4% paraformaldehyde and 1% glutaraldehyde in PB. The brains were dissected from the skull, post-fixed in the same fixative mixture overnight and sectioned on a vibratome at 50 μm thickness. The sections were blocked for 30 minutes in 3% NDS in PBS at room temperature; incubated for 48 hours in 1:1000 goat anti-5-HT IgG (ImmunoStar, #200800) with 2% NDS in PBS at 4^0C; rinsed three times (10 min each) in PBS; incubated for 90 minutes in 1:2000 biotinylated donkey anti-goat IgG with 2% NDS in PBS at room temperature; rinsed 3 times (10 min each) in PBS; incubated in the VECTASTAIN Elite ABC solution (1:100, Vector Laboratories) for 1 hr; rinsed 3 times (10 min each) in PBS; reacted with 0.05% 3,3'-diaminobenzidine tetrahydrochloride hydrate (ISOPAC, Sigma-Aldrich) and 0.01% H_2O_2 in PBS for 5

min; rinsed 3 times (10 min each) in PBS; incubated in 1% osmium tetroxide for 30 minutes; rinsed 3 times (10 min each) in PBS; dehydrated in a graded series of ethanol; and transferred into propylene oxide. Sections were flat-embedded with Durcupan ACM and polymerized; the rest of the procedure is described in the section "Thin-sectioning, Contrasting, and Image Acquisition."

Pre-embedding Immunoelectron Microscopy with Immunogold. Since the previous procedure failed to produce a readily detectable electron-dense signal, we next tried an immunogold labeling method. At PD56, offspring were terminally anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and tails were collected and stored at -20℃ for genotyping. Anesthetized animals were transcardially perfused with 0.9% saline, followed by 100 mL of cold 4% paraformaldehyde and 1% glutaraldehyde in PB. The brains were dissected from the skull, post-fixed in the same fixative mixture overnight and sectioned on a vibratome at 50 μm thickness. The sections were incubated for 15 minutes in 50 mM glycine in PBS for aldehyde inactivation; rinsed three times (10 min each) in PBS; blocked for 30 minutes in 1% BSA and 2% NDS in PBS at room temperature; incubated for 48 hours in 1:1000 goat anti-5-HT IgG (ImmunoStar, #20080) with 2% NDS in PBS at 4^0C; rinsed three times (10 min each) in PBS; incubated overnight in 1:100 nanogold-anti-goat Fab' (NanoProbes, #2006) with 2% NDS in PBS at 4^0C; rinsed 3 times (10 min each) in 2% BSA in PBS; rinsed 2 times (10 min each) in PBS; incubated for 1 hour in 2% glutaraldehyde; incubated for 15 minutes in 50 mM glycine in PBS for aldehyde inactivation; rinsed 2 times (10 min each) in PBS; rinsed 2 times (10 min

each) in H_2O; enhanced with GoldEnhance EM (NanoProbes, #2113) in the dark; rinsed 2 times (10 min each) in H_2O; incubated in 1% osmium tetroxide for 30 minutes; rinsed 3 times (10 min each) in PBS; dehydrated in a graded series of ethanol; and transferred into propylene oxide. Sections were flat-embedded with Durcupan ACM and polymerized; the rest of the procedure is described in the section "Thin-sectioning, Contrasting, and Image Acquisition."

Post-embedding Immunoelectron Microscopy with Immunogold. The results were still unsatisfactory with the previous procedure, so we next attempted a post-embedding method. The difference between the two methods is in whether immunolabeling is performed prior or post embedding. In order to allow immunolabeling in tissue after it has been cured in an embedding medium, a water-soluble resin such as L.R. White (Ted Pella, Redding, CA) is required. At PD56, offspring were terminally anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and tails were collected and stored at -20℃ for genotyping. Anesthetized animals were transcardially perfused with 0.9% saline, followed by 100 mL of cold 1% paraformaldehyde and 1% glutaraldehyde in PBS. The brains were removed from the skull and a 1 mm^3 tissue block was dissected out. The tissue block was post-fixed for 1 hour in the same fixative mixture; washed 3 times (10 min each) in the same fixative mixture; dehydrated in a graded series of methanol (15%, 30%, 50%); immersed for 1 hour in uranyl acetate fixative; dehydrated in another graded series of methanol (85%, 95%, 100%); infiltrated overnight with 1:1 ratio of 100% methanol:L.R. White on a rotator at 4℃; infiltrated for 2 hours with 100% L.R.

White on a rotator at 4℃. The last infiltration step was repeated two more times before curing the tissue block for 2 days at 52℃. The cured tissue block was trimmed with a razor; the rest of the procedure is described in the section "Thin-sectioning, Contrasting, and Image Acquisition."

Immunofluorescent Confocal Microscopy

Since none of the immunoelectron procedures has produced a reliable electron-dense signal *and* preserved ultrastructure in the same preparation, we next used multiple-label fluorescent immunohistochemistry with a high-precision confocal analysis. Confocal microscopy, unlike epifluorescent microscopy, utilizes improved optical methods that can block out the unfocused fluorescent signals above and below the optical section and also allows image acquisition in three dimensions with sub-micrometer accuracy. Once the image has been acquired, advanced image analysis software, such as IMARIS (Bitplane), can be used to separate biological signals from background noise and distinguish true co-localization from spuriously overlapping pixels. This allows unbiased image segmentation and accurate quantitative analyses.

DNA extraction and genotyping. Genomic DNA (gDNA) was extracted from tails using the Genomic DNA Isolation Kit (Lamda Biotech, St. Louis, MO). Briefly, 1 cm of the tail was digested in a lysis buffer at 56℃ overnight; the DNA was precipitated with ethanol, dissolved in 150 μL of Tris-EDTA buffer (TE, pH 8.0), and stored at -20℃. The altered *Htr4* region was amplified by PCR using the primers 5'-GGGCCAGCTCATTCCTCCCACTCAT-3', 5'-AGGGAAAGAGGGCTTTGGGTCATGG-3', and 5'-

ATCCAGAGAGGTCCGGACCAGGCAG-3', as specified by the protocol provided by The Jackson Laboratory. These primers yielded two amplification products, a mutant variant of 313 bp and a wild-type variant of 400 bp. Each PCR reaction (20 μL) contained 20 ng gDNA, 0.5 μM of each primer, 0.2 mM dNTP, 0.25U Platinum Taq DNA polymerase (Invitrogen), in a PCR buffer containing 10mM Tris-HCl (pH 9.0 at 25℃), 50mM KCl, 2.5mM $MgCl_2$, and 0.1% Triton X-100. The amplification was performed in a Mastercycler pro thermal cycler (Eppendorf, Hauppauge, NY) with the following conditions: initial activation at 95℃ for 15 min, 35 cycles of amplification (94℃ for 30 sec; 60℃ for 1 min; 72℃ for 1 min), and final extension at 72℃ for 2 min. The PCR products were run at 120 V on a 1.5% agarose gel in Tris-acetate-EDTA buffer (TAE) containing 5% ethidium bromide, and digitally imaged with a DigiDoc-It UV transillumination system (UVP, Upland, CA).

Sample Preparation and Immunofluorescence. At PD56, homozygous and heterozygous mice were terminally anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and their tails were collected and stored at -20℃ for genotyping. Anesthetized animals were transcardially perfused with 0.9% saline, followed by 100 mL of cold 4% paraformaldehyde in PB. The brains were dissected from the skull, post-fixed in the same fixative overnight at 4℃, cryoprotected in 30% sucrose for two days at 4℃, and used immediately or stored in a cryoprotectant containing 30% sucrose, 1% polyvinylpyrrolidone (PVP-40), and 30% ethylene glycol in PBS.

Brains were sectioned coronally at 40 µm thickness on a freezing microtome and sections were kept in PBS for immediate processing or stored at -20℃ in the cryoprotectant. All rinses and incubations were performed at room temperature. Sections were rinsed in PBS (0.1 M, pH 7.2), blocked for 30 minutes in 0.5% bovine serum albumin and 0.25% Triton X-100 in PBS, and incubated in a mixture of primary antibodies (see Table 4.1) in the same blocking solution overnight. After several washes, sections were incubated in a mixture of Alexa Fluor-conjugated secondary antibodies (see Table 4.1) in the same blocking solution for 90 minutes. After several washes, sections were mounted onto gelatin/chromium subbed glass slides, allowed to air dry, and coverslipped with ProLong Gold Antifade mounting media with DAPI (Invitrogen). The specificity of the secondary antibody was tested by omitting the primary antibody. The used procedure was based on published reports (Calizo et al., 2011; Crawford, Craige, & Beck, 2011; Gibson et al., 2005; Soiza-Reilly, Anderson, Vaughan, & Commons, 2013).

Image Acquisition, Segmentation, and Analysis. Sections were preliminarily examined and imaged on a Zeiss Axio Imager Z1 system equipped with standard epifluorescence filter sets and a color Zeiss AxioCam HRc camera. In selected slides, the confocal imaging was performed on a Fluoview 1000 Spectral confocal microscope (Olympus) with 10× (0.4 NA, dry) or 60× (1.4 NA, oil) objectives. The signals for DAPI, Alexa Fluor 488, Alexa Fluor 568, and Alexa Fluor 633 were acquired sequentially, stack by stack, using the laser lines of 405, 488, 546, and 633 nm, respectively. The laser intensity was adjusted to ensure that the signals were not

Antibody	Type	Host	Manufacturer	Dilution
anti-5-HT	Primary	Rabbit	ImmunoStar (20080)	1:1000
anti -GAD67	Primary	Mouse	Millipore (MAB5406)	1:2000
anti-VGluT1	Primary	Guinea pig	Millipore (AB5905)	1:1000
AF488-conj. anti-rabbit	Secondary	Donkey	Invitrogen (A21206)	1:200
AF568-conj. anti-mouse	Secondary	Donkey	Invitrogen (A10037)	1:200
AF633-conj. anti-guinea pig	Secondary	Donkey	Invitrogen (A21105)	1:200

Table 4.1. The antibodies used in immunohistochemistry. Abbreviations: vGluT1, vesicular glutamate transporter 1; GAD67, glutamic acid decarboxylase; AF, Alexa Fluor.

over-exposed, to reduce non-linear effects in the fluorescent signal. The offset was adjusted to slightly underexpose the background, and image resolution was set to 1024x1024 pixels with a 2x magnification. Starting with the section side closest to the coverslip, a *z*-stack of 12-13 optical sections (0.45 µm in thickness) were obtained for each physical section.

In the first analysis step, we assessed the consistency of the vGluT1 signal in each section through the collected *z*-stack. Each *z*-stack was cropped to a volume of 52.8µm x 52.8µm x 1µm. The built-in IMARIS spot function (Bitplane) was used to automatically detect vGluT1-positive puncta in each cropped image. The intensity threshold, used in the detection, was automatically calculated with the spot function, based on a published algorithm (Costes et al., 2004). To assess the reliability of the signal, the numbers of vGluT1-puncta in consecutive pairs of optical sections were plotted for each physical section (Figure 4.3A). The consistency plot for each physical section was inspected blindly to the genotype. Sections with clearly inconsistent numbers of vGluT1-puncta were eliminated from the sample set. Sections with outlier numbers only at an extreme end of the *z*-stack were cropped appropriately and included in the sample set. An outlier at the beginning or end of a stack could be caused by the closeness of the optical section to the surface of the physical section (which may contain sectioning microdefects) or by the limited penetration of antibodies (if the optical section is deep with respect to the surface).

The final selected set consisted of 9 *Htr4*-/- mice, 10 *Htr4*+/- mice, and 12 *Htr4* +/+ mice. First, the total vGluT1-puncta in each section were automatically detected

and counted. This number was normalized by the number of optical sections in the *z*-stack. Second, the IMARIS ImarisColoc[1] (Bitplane) tool was used to build a vGluT1/5-HT co-localization channel. Once the colocalization channel has been built, the spot function was used again to obtain the number of co-localized puncta, which was normalized by the number of optical sections in the *z*-stack. All images shown in the figures were processed with minor modifications in global brightness, contrast, and gamma adjustments.

Statistical analysis. Statistical analyses were performed in R 3.0.2 (The R Foundation for Statistical Computing) and in IBM SPSS 19 (IBM, Inc.). The data were analyzed with one- and two-way ANOVAs.

[1] The ImarisColoc is a fully automatic tool that utilizes a sophisticated colocalization threshold selection algorithm proposed by Costes et al. (2004). This tool allows more accurate threshold selections and minimizes the false-positive error, producing a result that is both robust and reproducible (Dunn, Kamocka, & McDonald, 2011). This method uses Pearson's correlation coefficient (PCC) to objectively identify the background threshold, then Mander's correlation coefficients are calculated as the fraction of overlapping pixels (colocalization) above the background threshold set by PCC.

Results

Immunoelectron Microscopy

When the Durcupan resin was used to embed the tissue and no immunolabelling was used, well-preserved ultrastructure was observed in the DRN. As shown in Figure 4.1A, synapses, myelinated axons, and synaptic vesicles were clearly visible. The immunolabeling with DAB greatly reduced the quality of the ultrastructure, and unspecific electron-dense areas were observed (see Figure 4.1B). No specific signal was obtained with the Nanogold immunolabeling, which resulted in uniformly distributed electron-dense particles of the correct size (see Figure 4.1C). Lastly, when the water-soluble embedding resin (L.R.White) was used, , the ultrastructure was not preserved (see Figure 4.1D). Because of these technical challenges, we took a different approach, which is described next.

Immunofluorescent Confocal Microscopy

We investigated the density of contacts between mPFC afferents (positive for vGluT1) and serotonergic neurons in the DRN of *Htr4*$^{-/-}$, *Htr4*$^{+/-}$, and *Htr4*$^{+/+}$ mice (Figure 4.2A). Consistent with previous reports (Crawford et al., 2011; Soiza-Reilly & Commons, 2011a), vGluT1-positive puncta were especially dense in the ventromedial DRN (vmDRN) (Figure 4.2B). Therefore, we focused on this DRN subdivision, in which we automatically detected and counted puncta that were immunoreactive for vGluT1 only, or for both vGluT1 and 5-HT (Figure 4.2C).

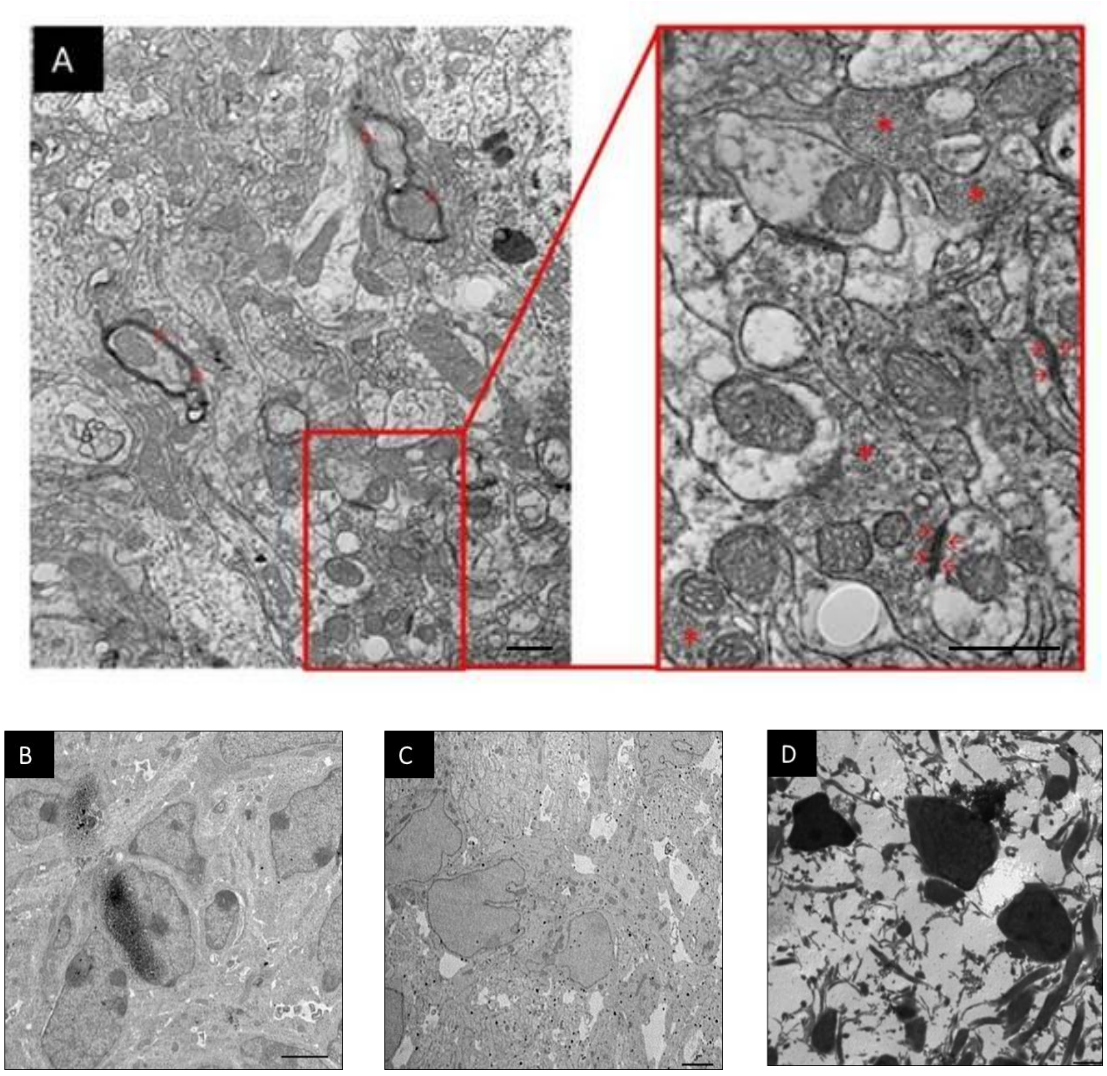

Figure 4.1. (A) An electron micrograph of the DRN. Shown are synapses (arrows), myelinated axons (circles) and vesicles (asterisks). The ultrastructure is well preserved. (B) An electron micrograph of the DRN, labeled using a pre-embedding staining protocol with an anti- 5-HT antibody and DAB as a chromogen. The ultrastructure is reasonably well preserved, but non-specific electron-dense areas obscure the immunosignal. (C) An electron micrograph of the DRN, labeled using a pre-embedding staining protocol with an anti-5-HT antibody and Nanogold. The ultrastructure is less well preserved and Nanogold particles are uniformly distributed. (D) An electron micrograph of the DRN, embedded in L.R. White resin. The ultrastructure is not preserved. Scale bars = 2 μm.

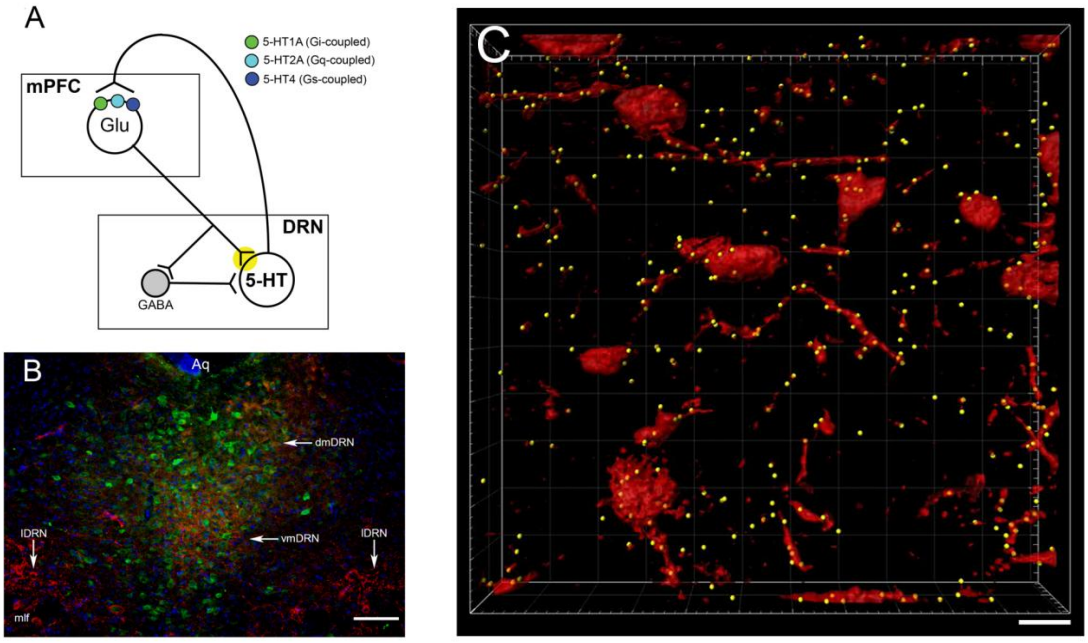

Figure 4.2. (A) A schematic representation of the direct projection from the mPFC to the DRN. The study investigated contacts (yellow) between glutamatergic mPFC afferents and serotonergic neurons using vGluT1 and 5-HT as immunomarkers. (B) A typical section through the DRN showing the distribution of 5-HT-positive neurons (green), vGluT1-positive puncta (red), and cell nuclei (blue). Abbreviations: Aq, cerebral aqueduct; dm, dorsomedial DRN; mlf, medial longitudinal fasciculus; vm, ventromedial DRN; lDRN, lateral DRN. Scale bar = 100 μm. (C) Automatic detection of 3D-colocalization between vGluT1 and 5-HT (pseudo-colored red) immunoreactive profiles. Scale bar = 10 μm.

Immunosignals often vary in intensity at different distances from the section surface due to sectioning microartifacts, limited antibody penetration, depth-dependent optical properties, and other factors. In order to avoid this artifact in our high-resolution analyses, we first examined the genotype-blind counts of vGluT1-positive puncta through the entire *z*-stacks of all individual mice and eliminated the mice in which these counts were clearly inconsistent (Figure 4.3A). The final set included 9 *Htr4*$^{-/-}$ mice, 10 *Htr4*$^{+/-}$ mice, and 12 *Htr4*$^{+/+}$ mice. In the wild-type (*Htr4*$^{+/+}$) mice, the mean counts of vGluT1-positive puncta and vGluT1/5-HT-positive puncta per optical section were 54.7 ± 3.5 and 19.1 ± 3.3, respectively. The proportion of the vGluT1-positive puncta colocalized with 5-HT with respect to all vGluT1-positive puncta was therefore around 35%, which is consistent with an array tomography analysis of wild-type C57BL/6 mice (Soiza-Reilly & Commons, 2011b).

A two-way ANOVA showed no significant effects of sex or genotype on the counts of vGluT1-positive puncta ($F(1, 25) = 0.010$, $p = 0.92$ and $F(2, 25) = 0.443$, $p = 0.65$, respectively) (Figure 4.3B). A two-way ANOVA revealed a significant effect of genotype on the counts of vGluT1-positive puncta colocalized with 5-HT ($F(2, 25) = 5.727$, $p = 0.009$) (Figure 4.3C), but the effects of sex and the genotype-sex interaction were not significant ($F(1, 25) = 0.843$, $p = 0.37$ and $F(2, 25) = 1.292$, $p = 0.29$, respectively). A post-hoc analysis confirmed that the heterozygous mice had significantly more vGluT1/5-HT-positive puncta than the two homozygous groups ($p < 0.03$).

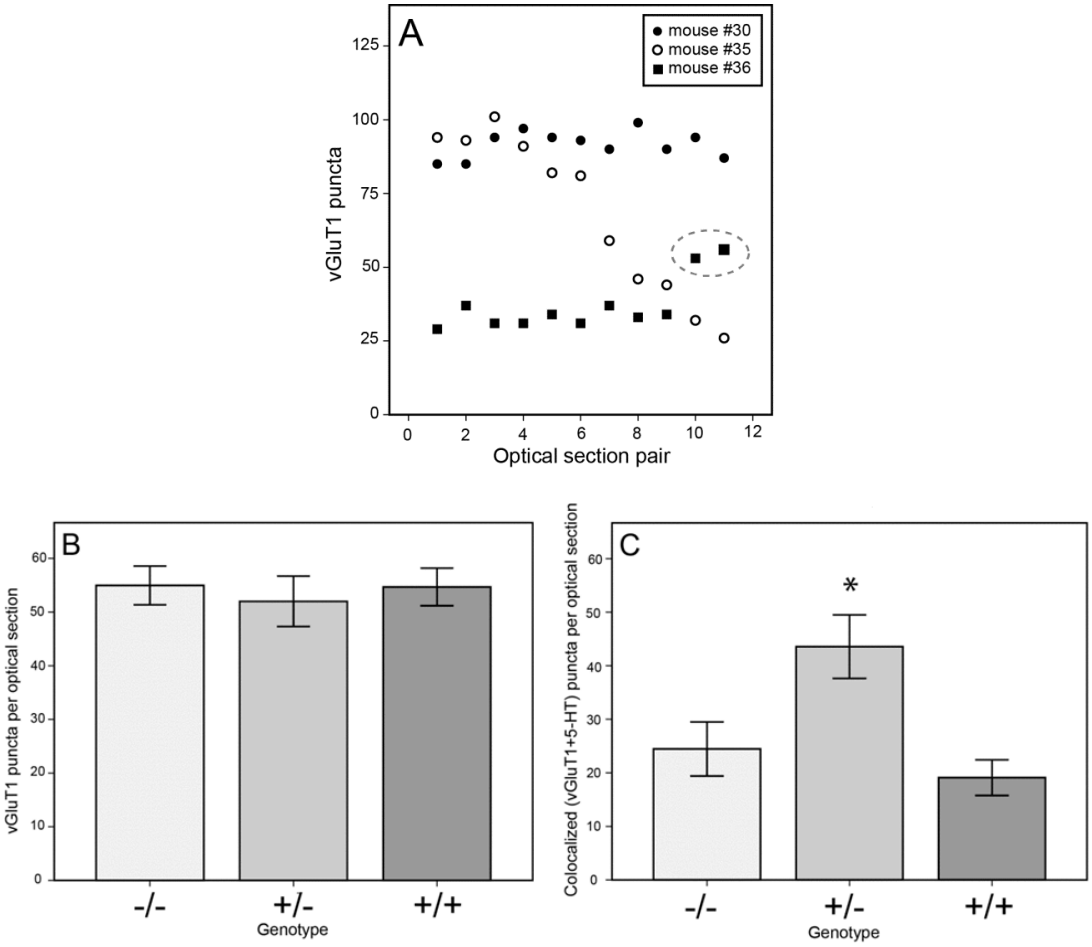

Figure 4.3. (A) The reliability analysis of automatically-depicted vGluT1-positive puncta in serial optical sections through the same physical section. Cases were included in the analysis only if the counts of puncta were relatively stable across the pairs of adjacent optical sections (mouse #30) or if the counts became relatively stable after the elimination of a small number of optical sections at an extreme end of the *z*-stack (mouse #36; the eliminated points are circled). Cases with unstable counts (mouse #35) or with outlier counts in the middle of the *z*-stack were not included in the analysis. (B) vGluT1-positive puncta in the vmDRN of *Htr4*$^{-/-}$, *Htr4*$^{+/-}$, and *Htr4*$^{+/+}$ mice. The genotypes were not significantly different (one-way ANOVA: $F(2,28) = 0.165$, $p = 0.85$). (C) Puncta with colocalized vGluT1 and 5-HT immunoreactivity in the vmDRN of *Htr4*$^{-/-}$, *Htr4*$^{+/-}$, and *Htr4*$^{+/+}$ mice. The heterozygous mice had significantly more contacts between vGluT1-positive terminals and 5-HT-positive cells bodies and dendrites (one-way ANOVA: $F(2, 28) = 7.542$, $p = 0.002$; Tukey's HSD post-hoc tests for +/- vs. -/- and +/- vs. +/+: *$p < 0.03$). Error bars = SEM (B and C).

Discussion

We found that the absence of functional 5-HT_4Rs had no significant effect on the overall density of mPFC terminals in the DRN, but that the density of mPFC terminals colocalized with serotonergic neurons was significantly increased in heterozygous mice. This increase was unlikely to be due to a larger proportion of serotonergic neurons in the DRN because the densities of 5-HT-positive neurons are not significantly different in the DRN of 5-HT_4R-knockout and wild-type mice (Conductier et al., 2006). Also, it is unlikely that the observed change was caused by altered 5-HT_4R signaling in the DRN itself because its expression of 5-HT_4R is very low (Lucas et al., 2005; Suwa et al., 2014).

Since many mPFC afferents terminate on GABAergic neurons in the DRN (Calizo et al., 2011; Fu et al., 2010; Jankowski & Sesack, 2004), the unchanged overall density of mPFC terminals may reflect a lower density of mPFC-GABAergic synapses in 5-HT_4R heterozygous mice. These synapses appear to play an important role in real-time behavioral control (Challis et al., 2013, 2014). In addition to the presented data, we attempted to directly quantify the colocalization between vGluT1-positive puncta and GAD67-positive profiles in the DRN with an anti-GAD67 antibody that has been used in other studies (Fu et al., 2010; Shikanai et al., 2012), but could not obtain a sufficiently reliable signal for automatic colocalization detection (Costes et al., 2004). However, a large proportion of GABAergic axons in the DRN form synaptic “triads” with vGluT1-positive terminals and the same postsynaptic target (Soiza-Reilly et al., 2013), which suggests that many mPFC-

GABAergic synapses eventually modulate the activity of the same serotonergic neurons that receive direct mPFC projections.

The finding that the density of contacts between mPFC terminals and serotonergic neurons was affected in heterozygous but not homozygous mice was unexpected, but it allows interesting interpretations. The brain is a complex system and it is likely to show a non-linear behavior when pushed far away from its equilibrium (such as when a functional gene is missing). A stronger perturbation can result in a smaller effect due to simple dynamical properties of the system (Janušonis, 2012a), or it may recruit major compensatory mechanisms that remain dormant if the gene is functional (even if its activity is lower than normal). This has been observed in other studies; for example, a null-mutation of the parkin gene alters the synaptic activity in the hippocampus of heterozygous but not homozygous knockout mice (Hanson, Orr, & Madison, 2010). These observations may be particularly relevant to human studies, where the expression of serotonin receptors is more likely to be moderately affected by environmental factors (including pharmacological agents) than completely abolished by genetic mutations.

While the electron microscopy analysis did not produce quantifiable results, excellent brain ultrastructure was obtained in unlabeled samples. Given that functional changes in structure have been observed in the DRN of 5-HT_4R deficient mice (Conductier et al., 2006), this technique may be useful for the quantification of morphological changes that do not require immuno-labeling, such as the ratio

between excitatory and inhibitory synapses, or the patterns of myelination. This analysis also showed that further refinement is needed in the selection of regions of interest, even if they are located in the same DRN subdivision (e.g., the ventromedial DRN), because the DRN ultrastructure is remarkably heterogeneous (Marinelli et al., 2004).

Chapter 5

General Discussion

The obtained results demonstrate that chronic and acute developmental 5-HT perturbations alter 5-HTR mRNA expression in the embryonic telencephalon, and that altered 5-HTR expression in development affects the formation of synapses between mPFC terminals and serotonergic neurons in the DRN. A combination of pharmacology, mRNA quantification, genetic models, immunofluorescence, and confocal microscopy showed that an optimal 5-HT environment is required for the normal up- and down-regulation of 5-HTRs in forebrain development and for the normal development of the prefrontal control of the 5-HT system. Our findings suggest that common maternal factors that can affect the 5-HT system, such as prenatal stress or SSRIs, can lead to abnormalities in the wiring of the fetal brain. This implies that such factors may compromise the mPFC control of neurotransmitter systems in adolescence and adulthood. Because the mPFC is implicated in most, if not all, cognitive deficits in psychiatric disorders, it is of great clinical importance to understand which developmental perturbations can induce long-lasting 5-HTR changes and may result in functional changes in prefrontal efferent projections.

The Role of 5-HT: Temporal Sensitivity in Development

It has long been known that 5-HT is involved in various developmental processes. In particular, 5-HT plays an important role in fetal brain development, an especially complex and dynamic process. The homeostatic regulatory system, which tightly controls the mature CNS, is not yet completely functional in the embryonic or fetal brain. Because of the highly dynamic nature of development, coupled with the lack of fully-developed regulatory mechanisms, the brain is susceptible to various

environmental or pharmacological perturbations. Since such perturbations are not uncommon in modern society, their pros and cons have to be well understood, especially because pregnant women can make conscious decisions about some of them.

Specific events must occur at specific times and in a specific order for a biological system to develop and function properly. This is also true regarding the maturation and integration of the brain 5-HT system during development. All CNS 5-HT is synthesized in the RN, and, in the mouse, serotonergic axons do not reach the developing telencephalon until approximately E15 (Aitken & Törk, 1988; Lidov et al., 1980). However, 5-HTR expression can be detected in the forebrain many days before serotonergic axons reach this part of the brain. A series of elegant studies (Bonnin & Levitt, 2011, 2012; Bonnin et al., 2011) have shown that, in mice, the maternal placenta supplies 5-HT to the embryonic forebrain prior to E15, and that only around E17 does the endogenous RN supply become the primary 5-HT source. Combined with the findings of Hernandez and Janušonis (Hernandez & Janušonis, 2010), which showed rapid upregulation of some 5-HT_4R splice variants at E15 and E17, it appears that 5-HTR expression should be highly susceptible to environmental factors at specific developmental times. The experiments in this thesis showed that this susceptibility is not only temporally specific, but that it is also 5-HTR specific. A significant decrease in mRNA levels of the 5-$HT_{4(b)}$R splice variant at E17 was observed following PS and 5-HT depletion from E14 to E16, while the other 5-HTR splice variants showed no significant changes in mRNA levels. These findings

contribute to the growing body of literature on embryonic 5-HTand provide further evidence that common prenatal perturbations can affect 5-HTRs in the developing forebrain in a time-sensitive manner. The clinical relevance of these effects requires more translational research.

Susceptibility to Prenatal Perturbation

It has been shown that various types of maternal adversity, including PS, can impact the 5-HT system of the mother and can consequently affect the development of neural circuits in the fetal brain. Approximately 50% of women report an increase in stress-related symptoms during pregnancy (Evans, Heron, Francomb, Oke, & Golding, 2001; Faisal-Cury & Rossi Menezes, 2007; Heron, O'Connor, Evans, Golding, & Glover, 2004; Rubertsson, Wickberg, Gustavsson, & Rådestad, 2005). Increased stress levels during pregnancy may have short-term and long-term consequences in the development of the fetal brain. In general, common negative factors during pregnancy include pregnancy anxiety, catastrophic events, major life events, chronic stress, and depressive symptoms (Dunkel Schetter & Tanner, 2012). It has been shown in human studies that PS is associated with major depressive disorder, generalized anxiety disorder, and schizophrenia in the offspring later in life (Markham & Koenig, 2011). Using animal models, similar findings have been reported. Animals that have experienced PS often show anxiety- and depression-like behaviors, such as decreased time spent in the open-field test and in the open arms of the plus-maze test, as well as increased immobility in the forced-swim test (Bogoch, Biala, Linial, & Weinstock, 2007; Morley-Fletcher et al., 2003; Neumann, Krömer, &

Bosch, 2005; Ordyan & Pivina, 2004; Rayen, van den Hove, Prickaerts, Steinbusch, & Pawluski, 2011; Zagron & Weinstock, 2006). Also, many effects of PS exposure on the adolescent and adult 5-HT systems have been demonstrated. PS has been associated with altered 5-HT levels and synthesis, 5-HTR expression and binding, and changes in behavioral outcomes following 5-HTR activation (Hayashi et al., 2010; D. A. V. Peters, 1986a, 1986b, 1990).

An increasing number of women are prescribed SSRI medications (S. E. Andrade et al., 2008) and SSRIs are often used to treat depression during pregnancy and the postpartum period. Many SSRIs readily cross the placental barrier and affect the fetus prenatally; also, SSRIs have been reported to affect infants postnatally due to their presence in breast milk (Gentile, 2005). Even though the effects of SSRI exposure to developing embryos are not yet fully understood, increasing clinical data associate SSRI exposure with abnormalities such as premature birth and low birth weight (Oberlander, Warburton, Misri, Aghajanian, & Hertzman, 2006; Wisner et al., 2009). Many studies have shown that prenatal and early postnatal exposure to SSRIs are linked to depression- and anxiety-like behaviors in adolescent and adult offspring (Ansorge, Morelli, & Gingrich, 2008; Hansen, Sánchez, & Meier, 1997; Karpova, Lindholm, Pruunsild, Timmusk, & Castrén, 2009; Rayen et al., 2011; Smit-Rigter et al., 2012), as also observed with PS exposure. Paradoxically, it has been shown in animal studies that early *postnatal* SSRI exposure can reverse the neurobiological and behavioral effects of PS (Nagano et al., 2012; Rayen et al., 2011). Early studies have established the role of 5-HT in stress-related disorders, while recent studies have

identified some forebrain regions, including the mPFC, to be involved in 5-HT-mediated stress responses. It has been shown that acute stress increases glutamate in several regions of the forebrain, including the PFC (Bagley & Moghaddam, 1997; Lowy, Wittenberg, & Yamamoto, 1995; Reznikov et al., 2007), while chronic treatment with SSRIs abolishes the stress-induced upregulation of glutamate release (Bonanno et al., 2005; Michael-Titus, Bains, Jeetle, & Whelpton, 2000; Tokarski, Bobula, Wabno, & Hess, 2008).

Even though a large body of research has examined the effects of PS and SSRI on offspring, the effects of these prenatal exposures on the developing 5-HT system remain poorly understood. The present thesis reports some interesting and unexpected results, the clinical relevance of which warrants further investigation.

A paradoxical result was obtained when PS, which increases 5-HT levels in the embryonic brain (D. A. V. Peters, 1990), and *p*CPA, which decreases 5-HT levels in the embryonic brain, led to a decrease in mRNA levels of the 5-$HT_{4(b)}$R splice variant. A similar study has found that both an increase and a decrease in prenatal 5-HT levels results in lower 5-HT_{1A}R mRNA levels in the embryonic rat brain (Lauder et al., 2000). Surprisingly, the acute 5-HT depletion with a TPH inhibitor had no effect on the mRNA levels of 5-HT_{1A}R, 5-HT_{2A}R, and all 5-HT_4R splice variants, while the mRNA levels of all examined 5-HTRs decreased in response to the acute SSRI treatment. This suggests that a decrease in 5-HT levels may produce smaller deviations from the normal developmental 5-HTR expression, compared to an increase in 5-HT levels, for which the response appears to be more drastic. Perhaps

this is because a low-5-HT state more closely approximates early developmental conditions when the 5-HT concentration may still be low. Many perplexing and conflicting findings have been reported by other researchers, possibly due to differences in the experimental parameters, such as treatment length and dosage. Because the developing system changes dynamically, acute, pulse-like exposures at different developmental times are essential in characterizing and understanding receptor susceptibility to prenatal perturbations.

5-HT and the mPFC-DRN Projection

Upon confirming that prenatal perturbations can affect the mRNA expression of some 5-HTRs, we next studied whether the normal 5-HTR expression is necessary for the development of neural circuits. Specifically, we investigated whether 5-HT_4Rs are required for the normal formation of synapses between mPFC terminals and serotonergic neurons in the DRN. In addition to being susceptible to prenatal perturbations, 5-HT_4R is expressed in DRN-projecting mPFC pyramidal cells. The mPFC is one of the few brain areas that send reciprocal projections to the DRN and function as 5-HT synthesis/release regulators. Because the PFC is the executive headquarters of the CNS and receives information directly and indirectly from all cognitive areas, it can integrate this information to decide whether more or less 5-HT has to be released from the DRN (Sharp, 2009). The mPFC then sends a signal to the DRN via its glutamatergic projections to modulate the activity of serotonergic neurons.

Local application of a 5-HT_{1A}R agonist in the mPFC has been shown to reduce the firing rate of serotonergic neurons in the DRN (Hajós et al., 1999). A potential mechanism for this reduction is the inhibition of mPFC GABA interneurons and the consequent disinhibition of mPFC glutamatergic projections to DRN GABA interneurons, which in turn inhibit DRN serotonergic neurons (Aznar, Qian, Shah, Rahbek, & Knudsen, 2003; Santana, Bortolozzi, Serrats, Mengod, & Artigas, 2004). A systemic injection of a 5-HT_{2A}R agonist, DOI, has been shown to induce an increase in *c-fos* expression in DRN GABA neurons (Boothman & Sharp, 2005), which in turn inhibit the firing of serotonergic neurons in the DRN. Lastly, overexpression of 5-HT_4R in the mPFC with a viral transfection method has been shown to increase the firing rate of serotonergic neurons in the DRN (Lucas et al., 2005), likely by stimulating the mPFC glutamatergic afferents that terminate directly on serotonergic neurons. The vesicular glutamate transporter (vGluT) is present in all glutamatergic terminals. Fremeau et al.(2001) have reported that vGluT1 mRNA is mainly expressed in telencephalic areas, including the mPFC, while vGluT2 mRNA is expressed mainly in subcortical areas. This information was used to quantify the synaptic structure of the mPFC-DRN projection.

We found that the number of synapses between mPFC terminals and serotonergic DRN neurons was markedly increased in *Htr4*$^{+/-}$ mice, showing an abnormal connectivity between the mPFC and the DRN. This confirms that developmental perturbations in 5-HTR expression can permanently alter the prefrontal control of brain 5-HT. Interestingly, no changes were observed in the

number of mPFC-DRN synapses in *Htr4*$^{-/-}$ mice, indicating that the complete absence of a 5-HTR may trigger major compensatory processes. This is supported by the results of a study that has found that heterozygous, but not homozygous, parkin-deficient mice exhibit functional synaptic impairments in the hippocampus (Hanson et al., 2010).

Potential Implications for 5-HT Research

In conclusion, our results indicate that acute and chronic prenatal 5-HT perturbations can affect 5-HTR mRNA expression in the embryonic telencephalon, and that constitutively low 5-HT_4R expression (due to a genetic deletion on one chromosome) may lead to changes in the mPFC-DRN projection. To achieve a better clinical interpretation of these results, it is important to understand whether changes in 5-HTR expression that are not related to genetic mutations can also persist into adolescence and adulthood. 5-HT_{1A}R has long been a target for research investigating the neurobiological basis of depression. Studies have suggested that the SSRI therapeutic lag corresponds to the time required for the DRN 5-HT_{1A} autoreceptor to desensitize (Piñeyro & Blier, 1999). 5-HT_{1A}Rs regulate 5-HT release by inhibiting serotonergic neurons in the DRN; therefore, an increase in synaptic SSRI would lead to increased inhibition by 5-HT_{1A}Rs. It has therefore been proposed that inhibiting DRN 5-HT_{1A} autoreceptors with a 5-HT_{1A}R antagonist could induce a more rapid onset of SSRI effects (Francesc Artigas, Romero, De Montigny, & Blier, 1996). The research of 5-HT_{2A}R in the PFC has focused on its role in modulating other neurotransmitters, such as dopamine and glutamate release; specifically,

antagonism at 5-HT_{2A}Rs has been shown to inhibit dopamine release in the PFC (Pehek, Nocjar, Roth, Byrd, & Mabrouk, 2006). Additionally, selective 5-HT_{2A}R antagonists have been shown to have antidepressant properties in rodents (Albinsson, Björk, Svartengren, Klint, & Andersson, 1994; J. G. Patel, Bartoszyk, Edwards, & Ashby, 2004), and thus have also been proposed as an adjunct treatment to SSRIs (Marek, Carpenter, McDougle, & Price, 2003; Marek, Martin-Ruiz, Abo, & Artigas, 2005). A 5-HT_4R partial agonist, RS67333, has been demonstrated to have rapid-onset antidepressant-like effects in rodents. A 3-day exposure to 5-HT_4R agonists has been shown to effectively desensitize 5-HT_{1A} autoreceptors and decrease depression-like behaviors, such as reduced immobility in the forced swimming test (Lucas et al., 2007).

To achieve better clinical relevance, it is important to know which prenatal alterations of brain development persist into adolescence and adulthood. Understanding the regulatory processes and relationships among the various components of the 5-HT system, especially with regard to brain development, is crucial in and of itself. It will provide us with a stronger grasp on one of the most abundant neurotransmitters, including its role in shaping neural communication networks.

General Limitations and Future Directions

Measurement of Receptor Expression with qRT-PCR.

For many 5-HTRs, including 5-HT_4R, the specificity of antibodies remains questionable or poorly characterized, which greatly limits the utility of

immunohistochemistry, especially in quantitative applications. Because of these limitations, in this thesis the mRNA levels of 5-HTRs were used to assess receptor expression levels. The mRNA levels were measured with qRT-PCR, currently the most accurate and specific quantitative technique. However, measures of receptor protein levels would provide more direct information needed for functional interpretations. It has been reported that mRNA transcription levels predict approximately 40% of protein production, and that translation efficiency more accurately reflects protein expression (Schwanhäusser et al., 2011). A separate study has found that changes in protein levels are often not accompanied by changes in the corresponding mRNA levels (R. Lu et al., 2009). These studies show that translational and post-translational processes are highly important, potentially more so than transcription itself. Since finding changes in mRNA expression cannot be immediately generalized to changes in receptor protein expression, findings reported in this thesis should be interpreted with caution.

Spatial Precision of Sampling is Lacking in the qRT-PCR studies.

Even though the qPCR method provided accurate quantitative information about mRNA levels, the spatial anatomical information was limited. Because the entire telencephalon (both hemispheres for E13-E15, one hemisphere for E16-E18) was used to extract mRNA, potentially important changes in telencephalic regions may not have been detected or may have been obscured by high receptor expression in adjacent regions. In the future, punching smaller telencephalic areas may be used for more targeted expression analyses. This way, more detailed information can be

obtained from the quantification data, making findings more amenable to functional interpretations. Also, techniques with a better spatial resolution, such as *in situ* hybridization, could be used in conjunction with qRT-PCR to measure mRNA levels in small anatomical regions. Recent advancements in commercially available fluorescent *in situ* hybridization (FISH) allow for the precise identification of multiple gene expression patterns within a single sample, in addition to better spatial localization.

Maternal Restraint Stress as Prenatal Stress (PS).

In the repeated perturbation study in Chapter 2, PS was used instead of pharmaceuticals to increase embryonic 5-HT levels, because PS commonly occurs in pregnant women. However, PS can lead to a wide range of neurological responses, including the activation of the hypothalamic-pituitary-adrenal axis. Also, the very different forms (restraint stress, social stress, food/drink deprivation, swim stress, reversal of the light-dark cycle, tail pinch, bright light stress, motion stress, dexamethasone injection, or some combination of the above) and lengths of laboratory stress treatments have possibly contributed to conflicting results in PS research. The maternal restraint stress regime (1 hour/day) used here was relatively mild in comparison to other PS studies. Exposure to short-lasting, mild, and long-lasting (severe) PS has led to opposite outcomes in studies on neonatal neurogenesis and differentiation of hippocampal neurons (Fujioka, Fujioka, Ishida, Maekawa, & Nakamura, 2006). Also, it is possible that animals can become desensitized to the same stress regime after some time. Restraint stress also does not translate well to

dynamic real-world stressors, such as physical and emotional stressors, that pregnant women encounter on a daily basis. Preferably, a combination of stressors should be used in PS experiments in order to minimize stress desensitization and mimic real-world stressful conditions.

Choice of Pharmacological Agents for 5-HT Perturbations.

An important consideration for future research is determining which pharmaceutical agents are best suited for 5-HT manipulation. One potential issue with acute SSRI and *p*CPA exposures is the time-course of the effects. SSRIs increase 5-HT neurotransmission by blocking reuptake transporters, thus rapidly increasing synaptic 5-HT levels. In contrast, *p*CPA decreases 5-HT neurotransmission by inhibiting 5-HT synthesis, and thus decreases 5-HT levels gradually, as the 5-HT supply becomes depleted. Due to this difference, an alternative 5-HT-depleting agent or a 5-HTR antagonist should be used for a better comparison. Using high performance liquid chromatography (HPLC) to measure regional 5-HT and 5-HIAA contents may provide important information about the effectiveness of these treatments.

Lack of Functional Data in the Study of the mPFC-DRN Projection in 5-HT_4R -deficient Mice.

To further elucidate the effects of altered 5-HT_4R expression due to the mutation in the 5-HT_4Rgene, methods that directly measure regional neurotransmitter levels, such as microdialysis with HPLC, could be used. Measuring the glutamate and 5-HT levels in the mPFC and DRN of 5-HT_4R-transgenic mice would provide important

data about the role of 5-HT_4R in the prefrontal control of 5-HT signaling. It would also be informative to utilize regionally specific gene-knockdown methods over a short period of time. In this approach, the activation of compensatory mechanisms would be less significant, allowing for more precise analyses of the effects of decreased 5-HT_4R expression during critical developmental times.

Another important question regarding the findings in Chapter 4 is the developmental time at which 5-HT_4Rs are first expressed in the mPFC. 5-HT_4Rs are expressed on pyramidal mPFC cells in the adult mouse brain, but it remains unclear whether 5-HT_4Rs are expressed on cortical pyramidal cells prenatally. In order to achieve this, high-precision methods, such as single-cell PCR, could be utilized to extract information about 5-HT_4R expression in specific cortical cells. Lastly, functional data can be obtained by using electrophysiological or optogenetic methods to determine how the altered synaptic connectivity in the DRN of 5-HT_4R heterozygous mice has affected the mPFC regulation of DRN neuronal activity.

Future Directions for the Study of the 5-HT_4R System.

Future research will benefit from increasingly sophisticated transgenic models that target specific components of the 5-HT system. Recently, a 5-HT_4R BAC-transgenic mouse model has been produced (Tg(*Htr4*-EGFP)AU103Gsat/Mmmh, www.mmrrc.org). In this model, the enhanced green fluorescent protein (EGFP) reporter gene is inserted immediately upstream of the coding sequence of the 5-HT_4R gene, which allows the visualization of 5-HT_4R directly with conventional epifluorescent and confocal microscopy. This model can be used to profile the

developmental trajectory of 5-HT_4R, as well as its susceptibility to prenatal perturbations, and therefore may complement the findings reported in this thesis. Neurons expressing 5-HT_4Rs in 5-HT_4R-EGFP mice can be cultured to dynamically track receptor interactions with other proteins by using live imaging.

Since 5-HT_4R splice variants differ in their functional properties, (Claeysen et al., 2001; Mnie-Filali et al., 2010), including their different responses to PS and 5-HT depletion (reported in this thesis), the development of 5-HT_4R splice variant-specific ligands is critical in further research. A 5-HT_4R partial agonist has been proposed as a rapid-onset antidepressant (Lucas et al., 2007), but the expression of these receptors in the gastrointestinal system cause side effects that may outweigh the antidepressant effect. Since the mouse 5-$HT_{4(e)}$R and 5-$HT_{4(f)}$R splice variants appear to be brain-specific, development of 5-HT_4R splice-variant specific ligands may lead to breakthroughs in the development of new treatment options for depression and other mental disorders.

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