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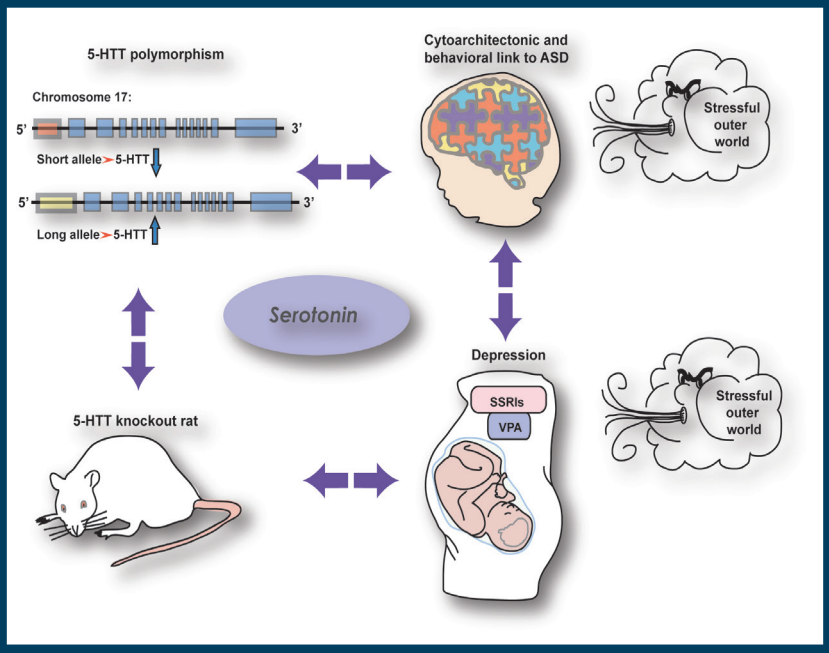

DECIPHERING SEROTONIN´S ROLE IN NEURODEVELOPMENT

Topic Editors
Judith R. Homberg, Sharon M. Kolk and Dirk Schubert

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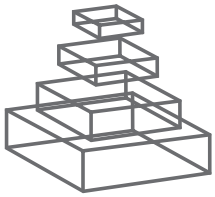

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ISSN 1664-8714
ISBN 978-2-88919-276-2
DOI 10.3389/978-2-88919-276-2

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DECIPHERING SEROTONIN´S ROLE IN NEURODEVELOPMENT

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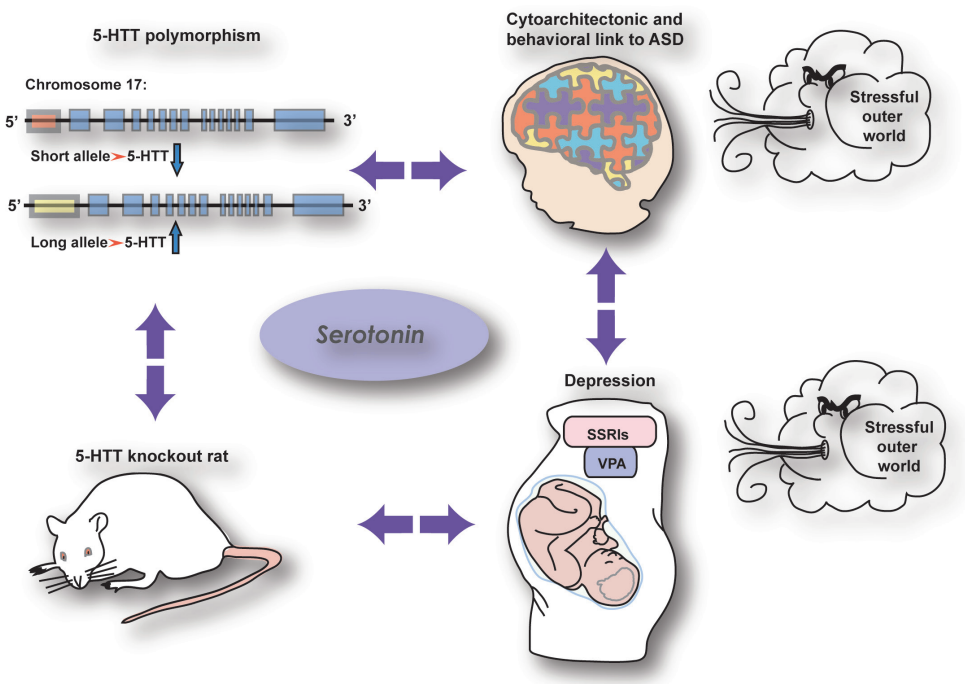

The relationship between genetic and pharmacological manipulations affecting the serotonin system showing similar effects on brain wiring and behaviour.

One of the most challenging questions in neurobiology to tackle is how the serotonergic system steers neurodevelopment. With the increase in serotonergic anxiolytic and antidepressant drugs, serotonin was thought to signal adversity or to serve as an emotional signal. However, a vast amount of literature is accumulating showing that serotonin rather mediates neuroplasticity and plays a key role in early developmental processes. For instance, selective serotonin reuptake inhibitors (SSRIs), serving as antidepressants, increase neurogenesis and trigger autism-related brain and behavioural changes during embryonic and perinatal exposure. Moreover, serotonin transporter gene variation is associated with alterations in corticolimbic neuroplasticity, autism-related neuroanatomical changes, as well alterations in social behaviour. Hence, the view is emerging that early life changes in serotonin levels influence the developmental course of socio-emotional brain circuits that are relevant for autism and other neurodevelopmental disorders. It is particularly exciting that the effects of embryonic and perinatal SSRI exposure and serotonin transporter gene variation on neurodevelopment seem to overlap to a large extent, at the cellular as well as the behavioural level. Yet, the precise mechanisms by which serotonin mediates neurodevelopment in the normal and ´autistic´ brain is unclear. Whereas serotonin has a placental origin during early gestation, serotonergic neurons develop during midgestation under the control of a cascade of transcription factors determining the fate of mid-hindbrain neurons that together form the Raphe nuclei. These neurons are among the earliest neurons to be generated, and because serotonin is released before any conventional synapses are formed, serotonin is suspected to influence crucial neurodevelopmental processes such as proliferation,migration and network formation. During late gestation they target their final destinations in, for instance, the cortex, where they affect the secretion of reelin. Reelin is a secreted extracellular matrix glycoprotein that helps to regulate processes of neuronal migration and positioning in the developing cortex by controlling cell–cell interactions. During the late prenatal and early postnatal phase (in rodents) serotonin further shapes the outgrowth of projecting neurons, synaptic connectivity, and the morphology of white fiber tracts. This is under the influence of transient serotonin transporter expression in (thalamo)cortical projections, sensory and prefrontal cortices and the hippocampus, as well as the local expression patterns of 5-HT1A, 5-HT1B and 5-HT3A receptors that each exert their specific roles in neuronal migration, remodeling of axons, and controlling dendritic complexity. There is also evidence that serotonin influences neural activity in locus ceroeleus neurons. Hence, serotonin appears to influence the development of both short- and long-distance connections in the brain.

This ResearchTopic is devoted to studies pinpointing the neurodevelopmental effects of serotonin in relation to prenatal SSRI exposure, serotonin transporter gene variation, and autism/neurodevelopmental disorders, using a wide-variety of cellular and molecular neurobiological techniques like, (epi)genetics, knockout, knockdown, neuroanatomy, physiology, MRI and behaviour in rodents and humans. We especially encouraged attempts to cross-link the neurodevelopmental processes across the fields of prenatal SSRI exposure, serotonin transporter gene variation, and autism/neurodevelopmental disorders, as well as new views on the positive or beneficial effects on serotonin-mediated neurodevelopmental changes.

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frontiers in
CELLULAR NEUROSCIENCE

EDITORIAL
published: 18 November 2013
doi: 10.3389/fncel.2013.00212

Editorial perspective of the Research Topic "Deciphering serotonin's role in neurodevelopment"

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Keywords: serotonin, neurodevelopment, placental serotonin, sensory system, prefrontal cortex, raphe nuclei, cortical integrity, autism

Serotonin is implicated in many, if not all, psychiatric disorders and is therefore the most studied neurotransmitter in our brain. Nevertheless, the developing serotonergic system and especially its role during brain maturation are still poorly understood. The role of serotonin in psychiatric conditions like anxiety, depression, and autism is either investigated in advanced stages of the disorder or in the context of selective serotonin reuptake inhibitor (SSRI) treatment. However, there is ample evidence for serotonin playing a crucial role in the early development of the nervous system, and that this role is different from the function of serotonin in the mature brain.

In this Research Topic entitled "Deciphering serotonin's role in neurodevelopment" we, together with leaders in the field, have brought together the most recent insights in serotonin's diverse roles especially during development, by means of both reviews and new empirical data. (Smidt and van Hooft, 2013) provide an overview of the development of the serotonergic system in rodents. Serotonergic neurons are born at embryonic day 10.5 caudal to the mid-hindbrain border (the isthmus). A plethora of transcription factors are temporally and spatially expressed here which determine the fate of the newborn neurons and their serotonergic phenotype. Yet, local synthesis within the raphe nuclei is not the only source for serotonin during embryonic brain development. As reviewed by Velasquez et al. (2013) the placenta is additionally involved in the synthesis of serotonin using maternally derived tryptophan. This placental source of serotonin may be a critical link between early genetic and environmental perturbations and their impact on brain maturation, including the development of the serotonergic system itself. As such, in the paper by Witteveen et al. (2013) the outgrowth of serotonergic neurons from the rostral raphe cluster to the medial prefrontal cortex (mPFC) is investigated as a function of genetic variance in the gene encoding the serotonin transporter (5-HTT) using dorsal/median raphe and prefrontal explants. It was found that whereas the dorsal raphe serotonergic outgrowing neurites remained unaffected by the loss of 5-HTT, the median raphe serotonergic neurites switched from a strong repulsive toward an attractive interaction when cocultured with the mPFC. As a result, the mPFC of 5-HTT deficient rats may receive more serotonergic innervation from the median raphe nucleus compared to wild-type rats. Furthermore, it was shown that the number of Satb2-positive callosal projection neurons was reduced in absence of the 5-HTT. Besides the development of the raphe- prefrontal network formation also the anatomical and physiological properties of the somatosensory system is affected by 5-HTT ablation. Miceli et al. (2013) report that thalamocortical afferents (TCA's) innervating their main target structures in layer 4 of the somatosensory cortex, the "barrels" representing the whiskers, are more diffuse and less topologically organized in absence of the 5-HTT. Accordingly, the barrel cortex pattern, although clearly present in 5-HTT deficient rats, was more diffuse with smaller barrels and increased inter-barrel widths. It is well possible that these extensive structural alterations in the topological organization affect somatosensory (whisker-mediated) perceptions. Intriguingly, these perceptions are indeed reduced in 5-HTT knockout mice [reviewed by Kinast et al. (2013)]. The somatosensory system is not the only sensory system showing a dependency on serotonin during brain development, as was demonstrated by Zhang et al. (2013). The serotonergic raphe nuclear complex projects directly to the olfactory bulb and olfactory performance is known to depend strongly on serotonin. The authors found that neonatal SSRI application caused a gender specific reduction in the 5-HTT expressing fibers that innervate the olfactory bulb in rats.

Serotonin can act through one of the 15 identified 5-HT receptor subtypes. Besides the 5-HT_{1B} receptor, the 5-HT_3, and 5-HT_6 receptors may play specific roles in the serotonin-mediated neurodevelopmental processes as nicely reviewed by Vitalis et al. (2013). Different aspects of cortical construction such as neuronal migration or dendritic differentiation are steered through the 5-HT_{3A} and the 5-HT_6, receptor, respectively. Indeed, as reviewed by Engel et al. (2013) 5-HT_3 receptors expressed on cortical interneurons and Cajal-Retzius cells regulate the morphology, positioning, and connectivity of the local microcircuitry during late embryogenesis. As the authors suggest, the 5-HT_3 receptor may play an important role in autism, given that mice lacking the 5-HT_3 receptor show social impairments and hypercomplexity of cortical layer 2/3 as is characteristic for autism. Furthermore, the $5HT_3$ receptor is a likely target of prenatal SSRI effects as neatly described by Olivier et al. (2013).

As reviewed by Kinast et al. (2013) the cognitive (PFC-dependent) and somatosensory phenotypes observed in 5-HTT knockout rodents as well as human subjects carrying the low activity variant of the serotonin transporter linked polymorphic region (5-HTTLPR) strikingly resemble those seen in autistic patients, rats prenatally treated with VPA (rat model for autism), and human and rodent subjects prenatally exposed to SSRIs (see

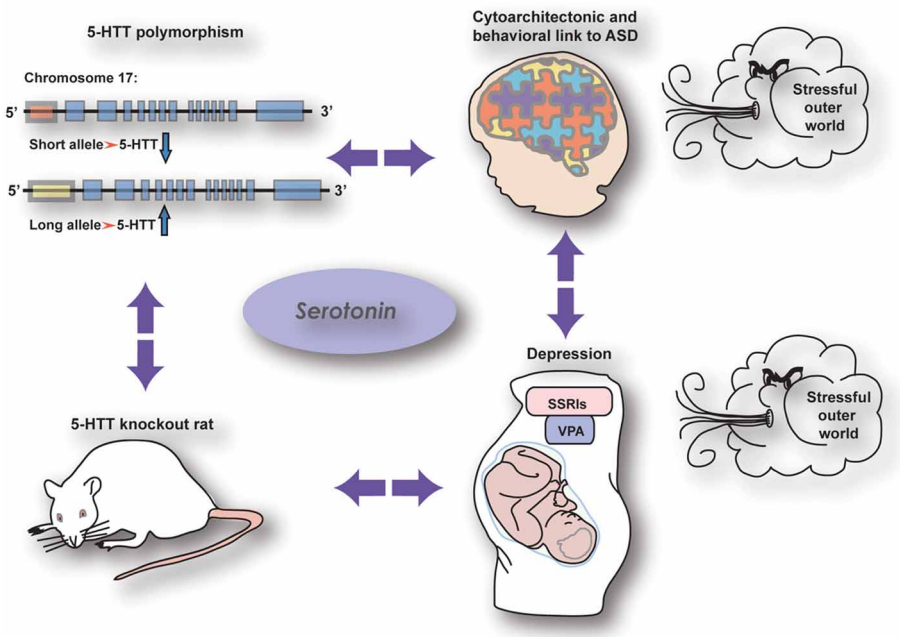

FIGURE 1 | The relationship between genetic and pharmacological manipulations affecting the serotonin system showing similar effects on brain wiring and behaviour (from Kinast et al., 2013), see below.

also Olivier et al., 2013) (**Figure 1**). However, these commonalities may be dependent on maternal depression, as executive function got worse in children being homozygous for the 5-HTTLPR long allele when the mother was depressed, whereas children prenatally exposed to SSRIs and carrying the 5-HTTLPR short allele were insensitive to maternal depression (Weikum et al., 2013). Nonetheless, loss of 5-HTT, prenatal SSRI exposure and autism may be interconnected by both showing a reduction in callosal-dependent intercortical connectivity, which—together with the finding of Witteveen et al. (2013) that callosal projection neurons seem to be altered in 5-HTT knockout rats—raise the possibility that serotonin affects the identity of projection neurons. Early serotonergic innervations may control laminar and cellular identities of cortical areas involved in complex behavior, possibly by acting on the reelin release by Cajal Retzius cells through the 5-HT_3 receptor. The results presented in this Research Topic demonstrate the crucial role of serotonin in neurodevelopment and thereby reveals itself as a key player in the onset of neuropsychiatric disorders like anxiety, depression, and autism.

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Received: 19 October 2013; accepted: 27 October 2013; published online: 18 November 2013.

Citation: Homberg JR, Kolk SM and Schubert D (2013) Editorial perspective of the Research Topic "Deciphering serotonin's role in neurodevelopment." Front. Cell. Neurosci. 7:212. doi: 10.3389/fncel.2013.00212

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 31 October 2013
doi: 10.3389/fncel.2013.00200

Subset specification of central serotonergic neurons

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The last decade the serotonin (5-hydroxytryptamine; 5-HT) system has received enormous attention due to its role in regulation of behavior, exemplified by the discovery that increased 5-HT tone in the central nervous system is able to alleviate affective disorders. Here, we review the developmental processes, with a special emphasis on subset specification, leading to the formation of the 5-HT system in the brain. Molecular classification of 5-HT neuronal groups leads to the definition of two independent rostral groups positioned in rhombomere 1 and 2/3 and a caudal group in rhombomere 5-8. In addition, more disperse refinement of these subsets is present as shown by the selective expression of the 5-HT1A autoreceptor, indicating functional diversity between 5-HT subsets. The functional significance of the molecular coding differences is not well known and the molecular basis of described specific connectivity patterns remain to be elucidated. Recent developments in genetic lineage tracing models will provide these data and form a major step-up toward the full understanding of the importance of developmental programming and function of 5-HT neuronal subsets.

Keywords: serotonin, dopamine, differentiation, prosomere, development

INTRODUCTION

Serotonin is one of the monoamine neurotransmitters in the brain and has a widespread innervation pattern. The importance of the serotonergic transmitter system is exemplified by the association of serotonergic activity with psychiatric disorders like depression, and specific drugs aimed at changing the serotonergic tone, as selective serotonin reuptake inhibitors (SSRIs), are widely used in the clinic to alleviate such psychiatric disorders. Serotonergic neurons are generated in the central nervous system (CNS), born between E10.5 and E12.5 in the mouse (Pattyn et al., 2004), and make up the anatomical locations designated as B1–B9 in the adult brain (reviewed in Goridis and Rohrer, 2002 and refined in Alonso et al., 2013). These neurons are identified by the enzymes that produce serotonin through the hydroxylation (Tryptophan hydroxylase, Tph2) of tryptophan to 5-hydroxytryptophan and the subsequent decarboxylation (L-Aromatic amino acid decarboxylase, Aadc) to produce serotonin. The latter enzyme is shared with dopaminergic, noradrenergic and adrenergic neurons, since it also catalyzes the decarboxylation of L-dopa to dopamine.

It has become apparent that prenatal and early postnatal exposure to SSRIs can have an important influence on the development of the CNS, which can result in lifelong modifications of behavior (Ansorge et al., 2004, 2008; Noorlander et al., 2008; Smit-Rigter et al., 2012). This influence seems a logical consequence of the fact that the pharmacological target, the serotonin reuptake transporter, Sert, is expressed during development (reviewed in Daws and Gould, 2011) and may therefore influence the 5-hydroxytryptamine (5-HT) tone during developmental processes. These data highlight the notion that 5-HT is not merely a classical neurotransmitter, but has neuromodulatory and trophic actions which are essential for proper brain development (reviewed in Daubert and Condron, 2010; Homberg et al., 2010). The essential role of 5-HT during development and in the adult has therefore raised a long-standing interest in the developmental programs that define 5-HT neurons (reviewed in Kiyasova and Gaspar, 2011; Deneris and Wyler, 2012). Interestingly, the data from genetic studies in mice have shown that the molecular programming over the rostral caudal axis, of the 5-HT neuronal containing regions in the hindbrain, is not equal. In this review we focus on these molecular distinctions and try to propose subset specific programs in the development of 5-HT neurons.

DEVELOPMENTAL PROCESSES THAT DETERMINE THE PERMISSIVE REGION FOR SEROTONERGIC NEURONAL DEVELOPMENT

An essential first step in providing cellular diversity is the subdivision of the developing CNS in longitudinal and transverse domains which are specified through specific gene expression patterns. The longitudinal domains are designated: floor plate, basal plate, alar plate, and roof plate. The transverse domains along the anterior/posterior (A/P) axis lead to the following domains in a rostral to caudal order: telencephalon, rostral diencephalon, prosomer 3-1, midbrain, and hindbrain (Puelles and Rubenstein, 2003). Since serotonergic neurons are born in the hindbrain rhombomere 1–8 region (reviewed in Deneris and Wyler, 2012) the anterior border of the permissive region is formed by the mid/hindbrain border; the isthmic organizer. The hindbrain A/P segmental origin is coded by a combinatorial code of Hox gene expression (reviewed in Alexander et al., 2009). The most rostral expression boundary is formed by the expression of Hoxa2 at the R1/R2 boundary, leaving the rhombomere 1 segment without influence of Hox gene expression. The caudal position of serotonergic permissiveness is coded by the presence of high local retinoic acid (RA) synthesis inducing Hoxa/b/d4 toward R7/8.

The origin of the 5-HT cell groups rely on floor plate signals as Shh. The different response of cells to go into serotonergic instead of other monoaminergic cell types differentiation relies on specific Fgf signals. The combination of early signaling of Shh and Fgf4 leads to the correct patterning of the region that later produces neurons with the rostral serotonergic identity (Ye et al., 1998). Moreover, Fgf4 and in addition Fgf2 were able to ectopically induce the 5-HT phenotype. Through elegant *ex vivo* experiments in rat and chicken, Farkas et al. (2003) have shown that Tgf-β is an additional signaling component, essential for the early Shh signaling and subsequent induction of floor plate derived neuronal systems. This signaling positions Tgf-β together with Fgfs and Shh (Hynes et al., 1997; Ye et al., 1998) central in defining the local molecular signaling in order to enable the progress of serotonergic differentiation within the permissive region.

Early molecular coding of the ventricular zone influences the fate of newborn neurons at specific dorsal/ventral positions along the A/P axis (Craven et al., 2004). The coding is generated by early players that are involved in early instructive signals during CNS patterning. Therefore, specific transcription factors expressed in the ventricular zone instruct newborn neurons for their early differentiation steps into the serotonergic phenotype. In the following sections we will discuss the general and subset specific transcriptional programs that define and generate specific serotonergic neuronal clusters.

SEROTONERGIC NEURONAL SPECIFICATION

GENERAL MOLECULAR PROGRAMMING OF SEROTONERGIC NEURONS

The genetic network of gene activation leading to the appearance of 5-HT neuronal groups has been studied in detail and have led to the definition of a specific transcription factor program (recently reviewed in Kiyasova and Gaspar, 2011; Deneris and Wyler, 2012). Here we will recapitulate the most important events that lead to the generation of 5-HT neurons (**Figure 1A**; for a complete model see Deneris and Wyler, 2012) as parts of this programming is also used to generate 5-HT neuronal diversity among the different 5-HT subsets (see below). As mentioned above, in a permissive region in the rostral hindbain region signaling events lead to the induction of critical early activators as Foxa2, Ascl1, Nkx2.2, and Nkx6.1.

The early expression of Foxa2 is required to suppress the expression of Phox2b and thereby initiate a switch from visceral motor neuron (VMN) programming toward 5-HT programming. Importantly, conditional deletion of Foxa2 *in ovo* in the posterior hindbrain did show an equal distributed diminishing of all 5-HT neurons without affecting the expression of Nkx2.2 (Jacob et al., 2007), suggesting that Foxa2 acts as a separate essential activator for the serotonergic cell-fate in all 5-HT neuronal clusters.

As a result of the presence of high concentration of the signaling molecule Shh (reviewed in Tannahill et al., 2005), a medial ventral domain in the rostral hindbrain starts to express the critical activator of the 5-HT lineage, Nkx2.2. Analysis of Nkx2.2 ablated mutants showed that serotonergic neurons at rhombomere2 level are ablated (R1 level is spared) as a consequence of a ventral to dorsal shift of programming at this A/P position (Briscoe et al., 1999). In addition, the essential role for Nkx2.2 in specifying the 5-HT phenotype was underscored by the fact that ectopic 5-HT neurons could be detected in the midbrain in Otx2 mutants where, as a consequence of changed Otx2 dose, Nkx2.2 is ectopically upregulated (Vernay et al., 2005). These data suggest that other essential activators for the 5-HT lineage are present in the midbrain and that Nkx2.2 upregulation is enough to initiate 5-HT specification programs.

It was shown that Nkx2.2 has to work together with other factors as Ascl/Mash1 in programming neurons toward the 5-HT phenotype. Analysis of Ascl1/Mash1 mutants has indicated that Ascl1 together with Nkx2-2 is involved in activating the 5-HT specification factors Lmx1b, Pet1, and Gata3 (Pattyn et al., 2004). Moreover, it has been suggested that Ascl1 binds the promoter of Insm1 and thereby activates this factor which was shown to be essential for the activation of the 5-HT synthesis gene Tph2. In Isnm1 ablated mice, the 5-HT neurons are generated but show lower expression of the 5-HT differentiation factors Pet1, Lmx1b, and Gata2, in addition to the failed Tph2 presence (Jacob et al., 2009). These data underscore the importance of Insm1 and suggest that its activation is downstream of Ascl1, besides the cooperation with Ascl1, in activating the full 5-HT phenotype.

In parallel to the above described activation of Gata2/3 by the Nkx2.2/Ascl1/Insm1 program, is the activation of the Lim homeobox gene Lmx1b, which is co-expressed in all 5-HT neurons (Ding et al., 2003). It has been suggested that Lmx1b acts as an intermediate step in the terminal differentiation between Nkx2.2 activity and activation of Pet1. Interestingly, Lmx1b ablation studies have shown that all 5-HT neurons rely on this transcription factor for their normal developmental program (Cheng et al., 2003). In these Lmx1b mutants the expression of Pet1, driving the 5-HT terminal differentiation markers, is eventually lost, leading to the absence of 5-HT phenotypic characteristics. Moreover, in Pet1 mutants the expression of Lmx1b is unaffected, suggesting that Lmx1b is positioned upstream of Pet1 in the genetic cascade. However, close examination of the developmental program in Lmx1b mutants suggest that the initial activation of Pet1 (E11.5-E12.5) in these mutants is unaffected (Cheng et al., 2003). This indicates that Lmx1b is not required for initial Pet1 activation, but in maintaining the Pet1 expression. Conditionally ablated Lmx1b mice [driven by Cre under the control of Pet1; (Zhao et al., 2006)] displayed a normal initial generation of 5-HT neurons. At E12.5 the amount of 5-HT neurons was markedly decreased and by E14.5 almost all neurons are lost as measured by 5-HT markers as Tph2 and Sert. These data suggest that Lmx1b is essential for maintaining the expression of Pet1 and therefore the 5-HT phenotype. The essential step in terminal differentiation, inducing the 5-HT phenotype, is established, by the action of Pet1 (Hendricks and Francis, 1999; Hendricks et al., 2003; Liu et al., 2010). This Ets factor is expressed just before the cells are starting to display 5-HT production. In ablation studies it was shown that Pet1 is essential, since in its absence most 5-HT precursors fail to develop (see also below) and the cells lack the genes for synthesis, uptake, storage, and signaling of 5-HT. Besides these most general 5-HT neuronal specification programs, it has become apparent that molecular differences exist within the 5-HT neuronal population, indicating that subsets specific programming might exist which we will discuss in more detail below.

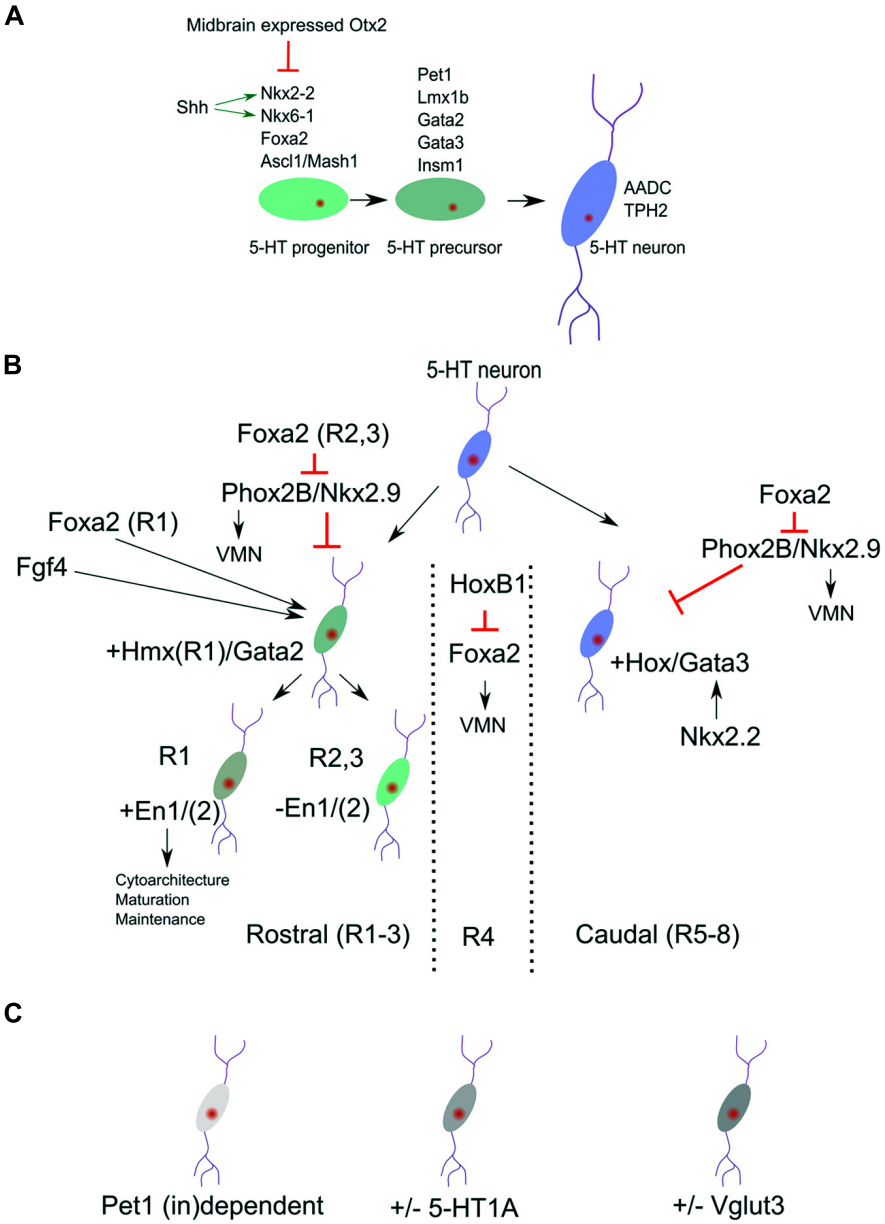

FIGURE 1 | Molecular identity of 5-HT neuronal subgroups. (A) Definition of broads programs of 5-HT neuronal specification. **(B)** The general pool of 5-HT neurons is molecularly divided in a rostral and caudal subgroup. These subgroups are identified by the presence of *Hmx/Gata2* and *Hox/Gata3* genes. The most rostral group can be subdivided by the presence or absence of the homeodomain genes Engrailed 1 and 2 (En1/2). **(C)** Next to the position related programming differences **(B)** more disperse transcription factors dependence (Pet1) and 5-HT subset markers exist in the 5-HT neuronal cluster.

PONTINE VERSUS MEDULLAR CODING OF 5-HT NEURONAL GROUPS

In most of the studies described above it was clear that not always all 5-HT neuronal groups were affected equally by ablation of essential transcription factors. This suggests that next to the overall existence of a general genetic program toward 5-HT neurons, other specific programming should be present that defines such distinctions.

Among the first emergence of subset specification is the division of rostral (pontine, R1 and R2/3) and caudal (medullar, R5-8) 5-HT clusters. The rostral cell group are located close to the caudal edge of the isthmus and the caudal cell group in the caudal myelencephalon which are divided by a region that does not contain any 5-HT neurons [branchial motor area; (Pattyn et al., 2004)]. This space devoid of 5-HT neurons located around rhombomere4 could be initiated through the activation of the 5-HT-program repressive transcription factor Phox2b, which is activated in this region through the local activation of Hoxb1 (Jacob et al., 2007 and reviewed in Alexander et al., 2009). This initial diversity is mimicked by the selective dependence of developing 5-HT neurons on the presence of the transcription factor Nkx2.2.

In classic Nkx2.2 knock-out animals large groups of 5-HT neurons are ablated. However, a small dorsal group remains in these animals suggesting that they are not depending on Nkx2.2 activity for the presence and survival (Briscoe et al., 1999; Hendricks et al., 2003; Jensen et al., 2008). The activity of Nkx2.2 is combined with the presence of the forkhead box protein a2 (Foxa2) and repression of the VMN activators, paired like homeobox 2b (Phox2b) and Nkx2.9. This combinatorial activation leads to the repression of the VMN fate and appearance of 5-HT neurons in R2/3 and R5-8. The activation of 5-HT neurons in of rhombomere 1 seems to follow a direct activation pattern, instead of fate change from VMN to 5-HT, as exemplified by their 1 day earlier appearance during development (Jacob et al., 2007). These data suggest that Foxa2 has a dual function in 5-HT specification, one direct acting as an intrinsic determinant for 5-HT neuronal differentiation (all 5-HT groups) and second, indirect, through repression of the VMN fate in R2/3 and R5-8. In the region where no 5-HT neurons are generated, R4, the presence of HoxB1 represses the dorsal expansion of Foxa2 en thereby maintains the VMN fate in this region (Jacob et al., 2007; **Figure 1B**). It is currently unknown how exactly the selective independence of R1 5-HT neurons on Nkx2.2 function is programmed. It seems likely that this independence is linked to the difference between direct activation of the 5-HT program as present in R1 compared to the indirect activation via the VMN program as present in R2/3 and R5-8, and exemplified by the R1 absence of Hox activity (Wylie et al., 2010 and reviewed in Alexander et al., 2009). The specific presence of the homeobox gene, En1, in the 5-HT region of R1 (see below) might be another determining difference (Fox and Deneris, 2012). Finally, it was shown that Nkx2.2 deletion leads to a ventral medullar specific ablation of the GATA binding factor 3, Gata3 (Cheng et al., 2003). This specific Gata3 ablation in these 5-HT groups suggest that another regional specific Nkx2.2 dependence exists in this area.

Gata2 and Gata3 have essential function in the rostral and caudal 5-HT cell groups, respectively. In the rostral hindbrain region the combinatorial action of the Shh responsive genes Nkx2.2 and Nkx6.1 induce Gata2 and Gata3. In rhombomere1 (the rostral 5-HT neuronal group) Gata2 is sufficient to induce Lmx1b and Pet1 in the programming toward the full 5-HT phenotype. Interestingly, Gata3 is not required at this position and is unable to rescue the ablation of Gata2 (Craven et al., 2004). On the other hand, Gata3 is required for the proper specification of caudal 5-HT neurons (Van Doorninck et al., 1999; Pattyn et al., 2004). In chimeric mice of the Gata3−/− phenotype the rostral cell population seems unaltered, whereas the caudal group (mostly the nucleus raphe obscurus (ROb) group) is severely affected in terms of cytoarchitecture. Taken together, these two distinct groups of 5-HT neurons rely on two different Gata factors which suggests that these neurons rely on analogous but dissimilar gene activation programs to create the same transmitter phenotype (**Figure 1B**).

Through an elegant series of experiments involving fluorescence activated cell sorting (FACS) of 5-HT neurons and subsequent transcriptome analysis, the rostral and caudal subgroup were identified and marked by the expression of Hmx homeodomains and Hox genes respectively. The rostral groups is further divided in two by the specific expression of Engrailed (En1/2) genes in one rostral subset (R1) (Wylie et al., 2010; Fox and Deneris, 2012). With the use of a Pet1-Cre driver, postmitotic 5-HT neurons lacking En1, En2, or En1/2 were generated which showed that En1 is functionally dominant and functions in phenotypic maintenance, survival, and cytoarchitecture (Fox and Deneris, 2012). Genetic lineage analysis indicated that the rostral En1 positive group may comprise only the 5-HT cells derived from rhombomere 1 (Jensen et al., 2008). These neurons move toward more caudal positions (along the B4-9 regions in a dorsal to ventral gradient of neuronal density) and therefore 5-HT neurons along the rostral caudal axes can form homogenous genetic groups next to each other linked to their respective origin. In the same study it was shown that R2 derived 5-HT neurons, driven through the combinatorial activation of Pet1-Flp, Rse2-Cre, and the Rosa26-dual recombinase reporter (Jensen et al., 2008), comprise the B5/8/9 regions. Using Egr2-Cre as subsets driver in similar combinations they mapped the R3/5 population toward the B5/8/9 group. Interestingly, through usage of this lineage tracing approach coupled to Nkx2.2 mutants it was shown that in Nkx2.2 mutants some cells of the B1/3 group were spared in addition to R1 derived 5-HT neurons as described earlier. This excellent lineage tracing approach has led to the definition of a spatial map 5-HT neuronal origin toward the position in the adult stage and has defined some of the molecular programming of the subsets. It might be worth while to use this setup in a combination with cell sorting and RNA-sequencing to define the exact molecular makeup of these subsets.

CROSS DOMAIN SUBSET SPECIFICATION AND SUBSET MARKERS

The above describe subsets specification could be coupled to an anatomical location as the rostral and caudal 5-HT neurons with accompanying molecular programming. In reality there are more levels of subsets specification that do not follow these domains (**Figure 1C**), although this might in part be due to the fact that most rostral 5-HT neurons migrate to more caudal positions causing a positional mix of 5-HT subsets in the adult stage (Jensen et al., 2008). First, the 5-HT phenotype defining factor Pet1 is not essential for full differentiation of all 5-HT neurons of the raphe nucleus (Hendricks et al., 2003). In these classic Pet1-KO animals a small group of 5-HT neurons (∼30%; 10–15% transmitter level) survive this ablation, almost equally distributed over the B1–B9 5-HT groups. Importantly, of these remaining 5-HT positive neurons, Aadc and 5-HT are present whereas the levels of Tph, Vmat, and Sert are diminished. In a recent study the functionality of these spared 5-HT neurons was confirmed as well as the presence of Tph2 in these neurons (Kiyasova et al., 2011). The data suggest that within the current distinction of 5-HT neuronal programming, specific differences must exist that define the specific requirement toward Pet1. An indication for differences in Pet1 dependence was found by genetic mapping of the Pet1 promoter region (Scott et al., 2005). In these experiments it was found that Pet1 acts as a maintenance factor for its own expression in a subset of 5-HT neurons, again evenly distributed for all B groups. So the activation and maintenance of Pet1 and its downstream targets, as described above, show specific dependence on Pet1.

A second not-region-defined subset refinement could be described by the selective absence of the 5-HT1A autoreceptor (Kiyasova et al., 2013). In these experiments using two reporter lines, ePet1-Gfp and 5-HT1A-iCre/R26R, they established through immunohistochemical techniques and single cell PCR that about 16% of cells measured over all 5-HT neuronal domains did not express this 5-HT1A autoreceptor. Taken together, these data suggest that another layer of molecular programming exists that defines Pet1 dependency and 5-HT1A autoreceptor presence. Although the regulation of the 5-HT1A autoreceptor has been described with some detail (reviewed in Albert, 2012), including activation by Pet-1 (Jacobsen et al., 2011), it is not clear how these processes act in concert to drive the 5-HT1A molecular distinctions. Finally, it has been described that the vesicular glutamate transporter 3 gene, Vglut3, is expressed in most but not all 5-HT neurons, located in the medial region of the dorsal (B4, B6-7) and medial (B5/B8) raphe nuclei (Herzog et al., 2004). This subset specific localization of Vglut3 was confirmed in a later, more in-depth study, which showed that co-localization (fluorescent double in situ hybridization) with Tph2 was about 80% for the dorsal raphe nucleus, with the exception of the lateral wings were the co-localization dropped to about 6% (Hioki et al., 2010). This expression pattern seems mostly limited to the rostral 5-HT cluster, suggesting that within this rostral domain additional distinctions should be present in terms of molecular programming. It might be interesting to analyze the possible link toward the presence of En1 in specific rostral subsets (**Figure 1B** and see above) and the presence of Vglut3.

Analogous immunohistochemical mapping of neuropeptides within 5-HT neurons suggested that in mice a very small number of 5-HT neurons express substance P or galanin (Fu et al., 2010), although this analysis should be completed by other approaches showing that the transcripts are present in 5-HT neurons. Taken together, these data suggest that besides the programming distinction between rostral and caudal clusters, more refinement over and within these two clusters may be present, either depending on Pet1 or on other 5-HT subset regulators.

INNERVATION REPERTOIRE OF 5-HT NEURONAL SUBSETS

It is well known that 5-HT neuronal projections are present in almost all areas of the brain without a real specific profile. The above mentioned molecular subset specification of 5-HT neurons would suggest that specific innervation programs are present and are guided by unique programs of guidance molecule expression. Analysis of different guidance-factor mutants have shown the involvement of the frizzled (Fzd3), vang-like (Vangl2), cadherin, EGF LAG seven-pass G-type receptor (Celsr3) and Slit homolog (Slit1/2) family members (reviewed in Kiyasova and Gaspar, 2011). In Fzd3 mutants both the rostral and caudal 5-HT neurons lose their normal polarity (rostral group in rostral direction, caudal group in caudal direction) in axon outgrowth. The resulting axons are growing in all directions except for the midline. Interestingly, in Celsr3 mutants only the rostral groups seems affected in a similar manner as in the Fzd3 mutant. The Vangl2 mutant mimics the Fzd3 mutant with exception of the lateral outgrowth of the rostral 5-HT axons (Fenstermaker et al., 2010). Another study described the effects of Slit mutants (Slit2 and Slit1/2 double mutants) on the trajectory of 5-HT neurons in the brain (Bagri et al., 2002). Here, it was shown that both dopaminergic and 5-HT fibers were displaced ventrally in respect to the normal main forebrain bundle path in the Slit2 mutant. This defect is more severe in the Slit1/2 double mutant where fibers aberrantly cross the midline in ventral regions of the diencephalon and in addition the main forebrain bundle splits in two parts where some fibers run into the ventral hypothalamic area. Interestingly, in transcriptome and expression verified data of 5-HT neurons (Supplemental Table 1 in Wylie et al., 2010), Slit1 gene expression was identified, indicating that at least Slit1 is a functional component of the 5-HT neuronal axon guidance instructions set. The other above mentioned axon guidance components were not found in these data and if these data are correct it suggests that these components may not themselves be involved in 5-HT neurons, but maybe their signaling counterparts. Recently, a paper was published that described in more detail, through a lineage tracing setup (Jensen et al., 2008) the connectivity of R1, R2, and R3/5 originating 5-HT neuronal clusters (Bang et al., 2012). Although some areas like the hypothalamic region are evenly innervated by all these subsets, some areas show significant specificity in innervation pattern. The amygdala and subregion, basolateral amygdala, receive selective R1 derived innervation and R1/2 derived innervation respectively. Furthermore, the parietal cortex only receives 5-HT input from the R2 derived cluster. Also, lateral striatal areas receive selective input from the R1 cluster only. These examples show that selective axon guidance, synapse formation and pruning mechanism exist in these 5-HT subsets. What the molecular determinants are beneath this selectivity remains to be elucidated. The combination of lineage tracing techniques with FACS approaches and RNA-sequencing will in the near future solve this question.

CONCLUDING REMARKS

The development of the 5-HT system depends on a variety of molecular programs that determine the synthesis, re-uptake and signaling of 5-HT in the central nervous system. Recent developments in this field have elucidated the core genetic program in the development of the 5-HT neurons and latest data suggest that a subdivision can be made resulting in molecular distinction between 5-HT nuclei over the rostral to caudal axis (**Figure 1B**). In the rostral cluster it is clear that the programming of the R1 sets can be distinguished from R2/3 at different levels: (1) direct 5-HT neuronal developmental programming versus fate switch from initial VMN neurons toward 5-HT neurons; (2) Influence of the Hox cluster (R2/3) compared to Hmx genes (R1) and (3) selective influence of En1 in R1 versus R2/3. The gap between 5-HT clusters in R4 is maintained through HoxB1 expression, that is important in repressing Foxa2 and thereby maintaining the VMN lineage. Finally the caudal R5-8 clusters is defined by a specific dependence on Gata3 activity suggested to be activated by Nkx2.2 in this region.

The above described subset specificity is not well understood in terms of specific connectivity. Although some studies do report initial guidance codes and specific connectivity patterns for 5-HT neuronal subsets, the exact molecular guidance code in the described subsets remain to be elucidated. The most promising

data, in our view, are presented by the recent transcriptome data (Wylie et al., 2010) together with the described advanced cell-lineage analysis tools (Jensen et al., 2008). Meta analysis of these data sets might shed light on the presence of subset specific guidance codes in relation to subsets specific connectivity.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 February 2013; accepted: 11 October 2013; published online: 31 October 2013.

*Citation: Smidt MP and van Hooft JA (2013) Subset specification of central serotonergic neurons. Front. Cell. Neurosci. **7**:200. doi: 10.3389/fncel.2013.00200*

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 23 April 2013
doi: 10.3389/fncel.2013.00047

Placental serotonin: implications for the developmental effects of SSRIs and maternal depression

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In addition to its role in the pathophysiology of numerous psychiatric disorders, increasing evidence points to serotonin (5-HT) as a crucial molecule for the modulation of neurodevelopmental processes. Recent evidence indicates that the placenta is involved in the synthesis of 5-HT from maternally derived tryptophan (TRP). This gives rise to the possibility that genetic and environmental perturbations directly affecting placental TRP metabolism may lead to abnormal brain circuit wiring in the developing embryo, and therefore contribute to the developmental origin of psychiatric disorders. In this review, we discuss how perturbations of the placental TRP metabolic pathway may lead to abnormal brain development and function throughout life. Of particular interest is prenatal exposure to maternal depression and antidepressants, both known to alter fetal development. We review existing evidence on how antidepressants can alter placental physiology in its key function of maintaining fetal homeostasis and have long-term effects on fetal forebrain development.

Keywords: placenta, serotonin, SSRI, tryptophan, depression, fetal programming, fetal brain, serotonin transporter

INTRODUCTION

There is a wealth of evidence suggesting that serotonin (5-HT) plays a critical role in many neurodevelopmental processes. Basic and epidemiological studies link disruption of the 5-HT pathway to a host of developmental and functional disorders, yet direct evidence of the molecular mechanisms underlying these perturbations remains lacking, especially in humans. Studies in animal models have indicated that 5-HT is a key modulator of neuronal cell proliferation, migration, and brain wiring during fetal and early postnatal development (Brezun and Daszuta, 1999, 2000, 2008; Azmitia, 2001; Kindt et al., 2002; Banasr et al., 2004; Bonnin et al., 2007). Furthermore, genetic and environmental disruption of 5-HT receptor function during critical periods of fetal brain development in mice lead to behavioral abnormalities throughout life, such as adult anxiety disorders (Gaspar et al., 2003; Holmes et al., 2003a,b; Ansorge et al., 2004; Nordquist and Oreland, 2010; Morelli et al., 2011; Garbett et al., 2012; Malkova et al., 2012). Interestingly however, there is sparse evidence of specific associations between 5-HT receptor gene mutation or dysfunction and mental illness in humans (Gingrich and Hen, 2001; Gaspar et al., 2003; Segman et al., 2003).

Generally weak phenotypes in single receptor knockout mice and the existence of 15 different receptor subtypes for 5-HT suggest that genetic alteration of one specific subtype may be compensated for by the presence of other pharmacologically and functionally similar receptors (e.g., 5-HT1B and 5-HT1D receptors; see Van Kleef et al., 2012). Basic studies were able to alter function of several receptors simultaneously during restricted, critical time periods, thus potentially preventing compensatory signaling through other receptors and leading to clear phenotypes (Ansorge et al., 2004; Bonnin et al., 2007).

What is common to all receptor subtypes is their endogenous ligand, 5-HT. Therefore, altered 5-HT tissue concentration may lead to generalized disruption of signaling through more than one receptor type simultaneously. This possibility is supported by dramatic effects from the pharmacological disruption of 5-HT synthesis in early experiments, contrasting with mild effects of single receptor knockout models (Van Kleef et al., 2012).

Recent results show that 5-HT signaling, and thus extracellular levels of 5-HT, play a crucial role in the thalamocortical wiring of the fetal forebrain by affecting netrin-1 mediated axonal guidance (Bonnin et al., 2007, 2011). Thus, altered 5-HT concentration in the fetal brain tissue, in addition to signal/receptor interaction, may have far-reaching developmental and functional consequences (Bonnin and Levitt, 2012). A recent study showed that the fetal forebrain accumulates placentally derived serotonin during early pregnancy (Bonnin et al., 2011), a period during which axons are experiencing active outgrowth and guidance. The role of placental metabolism of 5-HT from maternally derived TRP, its potential genetic and environmental perturbations, and their downstream consequences are currently under intense investigation.

5-HT AND FETAL BRAIN DEVELOPMENT

Serotonergic neurons are one of the most ubiquitous circuits in the mammalian brain, forming early during fetal development, and innervating essentially the entire central nervous system. The early presence of 5-HT, as well as the proposed maternal origin of 5-HT, has led to the hypothesis that 5-HT may be an essential growth and regulatory factor for the fetal brain during critical periods of development (Lauder and Krebs, 1976; Lidov and Molliver, 1982; Gaspar et al., 2003; Bayard et al., 2007;

Bonnin et al., 2011; Migliarini et al., 2012). This is supported by the idea that disruption of the 5-HT signaling system is a key developmental component for a number of neuropsychiatric disorders, such as schizophrenia, affective disorders, anxiety, and autism (Chugani et al., 1999; Whitaker-Azmitia, 2001; Sodhi and Sanders-Bush, 2004; Bonnin and Levitt, 2012). Genetic mouse models have shown that excess levels of 5-HT in the brain, obtained by knocking out the transporter (SERT; *Slc6a4*) or monoamine oxidase-A *(MAO-A)* genes, which are involved in the re-uptake and degradation of 5-HT, respectively, lead to abnormal development of topographically organized whisker-barrel fields in the somatosensory cortex (Cases et al., 1996; Persico et al., 2001). Furthermore, recent studies have shown that increased activity of the serotonergic pathway may lead to abnormal cortical development and neuronal migration (Janusonis et al., 2004; Riccio et al., 2009). On the other hand, 5-HT depletion through the use of *Pet1* knockout mice, in which there is a dramatic reduction of serotonergic neuron number and differentiation, shows no identifiable gross brain malformations, despite evidence of later behavioral and functional deficits (Hendricks et al., 2003; Liu et al., 2010). Similarly, targeted inactivation of *tryptophan hydroxylase 2 (Tph2)*, the rate-limiting enzyme for the synthesis of 5-HT specifically in the brain, in the mouse model has been demonstrated to produce behavioral and functional deficits. However, lack of 5-HT did not lead to obvious cellular or histological abnormalities in the brain (Savelieva et al., 2008; Alenina et al., 2009; Yadav et al., 2009). Nevertheless, the more recent analysis of a knock-in mouse line, in which the brain-specific *Tph2* gene was replaced by an eGFP reporter, showed significant abnormalities in serotonergic innervation in several regions of the rostral brain (Migliarini et al., 2012). Combined, these data suggest that specific circuits are finely tuned to 5-HT during their initial formation, including the serotonergic system itself. The next logical question is to determine if, and how, 5-HT signaling during development is impacted by genetic and environmental perturbations shown to be associated with increased risk of neuropsychiatric disorders.

Recent work suggests that the maternal and placental source of 5-HT may be a critical link between early genetic and environmental perturbations and their impact on fetal brain development. Consequently, exposure to pharmacological or environmental insults, combined with genetic factors that disrupt maternal or placentally derived 5-HT may have profound and long-lasting consequences on the developing brain, leading to a host of neuropsychiatric disorders thought to have developmental origins.

In the next section, we discuss how particular environmental and pharmacological insults such as exposure to maternal depression and antidepressants during pregnancy may impact fetal brain development, taking into account the potential effects on the maternal-fetal interface function.

PRENATAL EXPOSURE TO MATERNAL DEPRESSION AND ANTIDEPRESSANTS, EFFECTS ON FETAL BRAIN DEVELOPMENT AND LONG-TERM CONSEQUENCES

Major Depression Disorder (MDD) is a devastating mood disorder that indiscriminately affects individuals of all backgrounds and ages, and is common even in women during gestation. In fact, the prevalence of MDD is about 15% during pregnancy, and Selective Serotonin Reuptake Inhibitors (SSRIs) are the primary pharmacologic intervention (Oberlander et al., 2006). Despite an unclear safety profile and a lack of well-controlled safety studies, an estimated 13% of pregnant women are prescribed an SSRI antidepressant during all or part of their pregnancy (Cooper et al., 2007). This common off-label use is warranted for its beneficial effects of improving maternal mood and relieving symptoms of depression, which presumably lead to better pregnancy outcomes. Due to their high use and unknown safety, there is high surveillance of SSRIs by the US Food and Drug Administration, which has placed some SSRIs in Pregnancy Category D, indicating demonstrated risks to the fetus (Greene, 2007).

Recent epidemiological studies suggest that fetal exposure to maternal SSRI therapy is implicated in disturbing several physiological and cognitive domains during fetal development. Their prescribed use is associated with increased prevalence of preterm delivery, intrauterine growth restriction, and neurobehavioral disturbances in infants (Oberlander et al., 2009). Additionally, fetal SSRI exposure has been shown to increase risks of Postnatal Adaptation Syndrome, low Apgar scores, Persistent Pulmonary Hypertension of the Newborn, long-term changes in cardiac morphology and physiology, gastrointestinal abnormalities, Autism Spectrum Disorders, and postnatal language learning deficits in humans (**Figure 1**). (Cohen et al., 2000; Simon, 2002; Laine et al., 2003; Källén, 2004; Chambers et al., 2006; Levinson-Castiel et al., 2006; Oberlander et al., 2006; Cooper et al., 2007; Louik et al., 2007; Talge et al., 2007; Calderon-Margalit et al., 2009; Lund et al., 2009; Merlob et al., 2009; Pedersen et al., 2009, 2010; Hadjikhani, 2010; Kornum et al., 2010; Reis and Källén, 2010; Croen et al., 2011; Haskell et al., 2012; Jimenez-Solem et al., 2012; Nijenhuis et al., 2012a,b; Nordeng et al., 2012; Weikum et al., 2012; Yonkers et al., 2012).

Leaving maternal MDD untreated to avoid the potential teratogenicity of SSRIs also poses significant risks. The anguish and psychological distress accompanied by untreated MDD induces considerable maternal stress, one of the earliest adverse experiences with long-term effects on the offspring. Several animal and human studies show that maternal stress or depression disrupt fetal neurobehavioral development and affect cognitive, emotional and behavioral outcomes throughout childhood (Peters, 1990; Hayashi et al., 1998; Talge et al., 2007; Homberg et al., 2010). Children exposed to the stress induced by depressed mothers are also at increased risk of developmental delay, impaired language development, and even low IQ scores (**Figure 1**) (Deave et al., 2008; Paulson et al., 2009). The impact of maternal depression on newborns has effects that last beyond infancy, as one-third of school-aged children of depressed mothers suffer from depression and anxiety disorders (Pilowsky et al., 2006). Beyond childhood, animal studies have shown that neonatal SSRI exposure suppresses adult serotonergic signaling and elicits depressive- and anxiety-like behaviors in adulthood (Ansorge et al., 2008; Shanahan et al., 2009).

Maternal depressive states and prenatal exposure to SSRIs both alter fetal health. For the developing fetus, associated risks

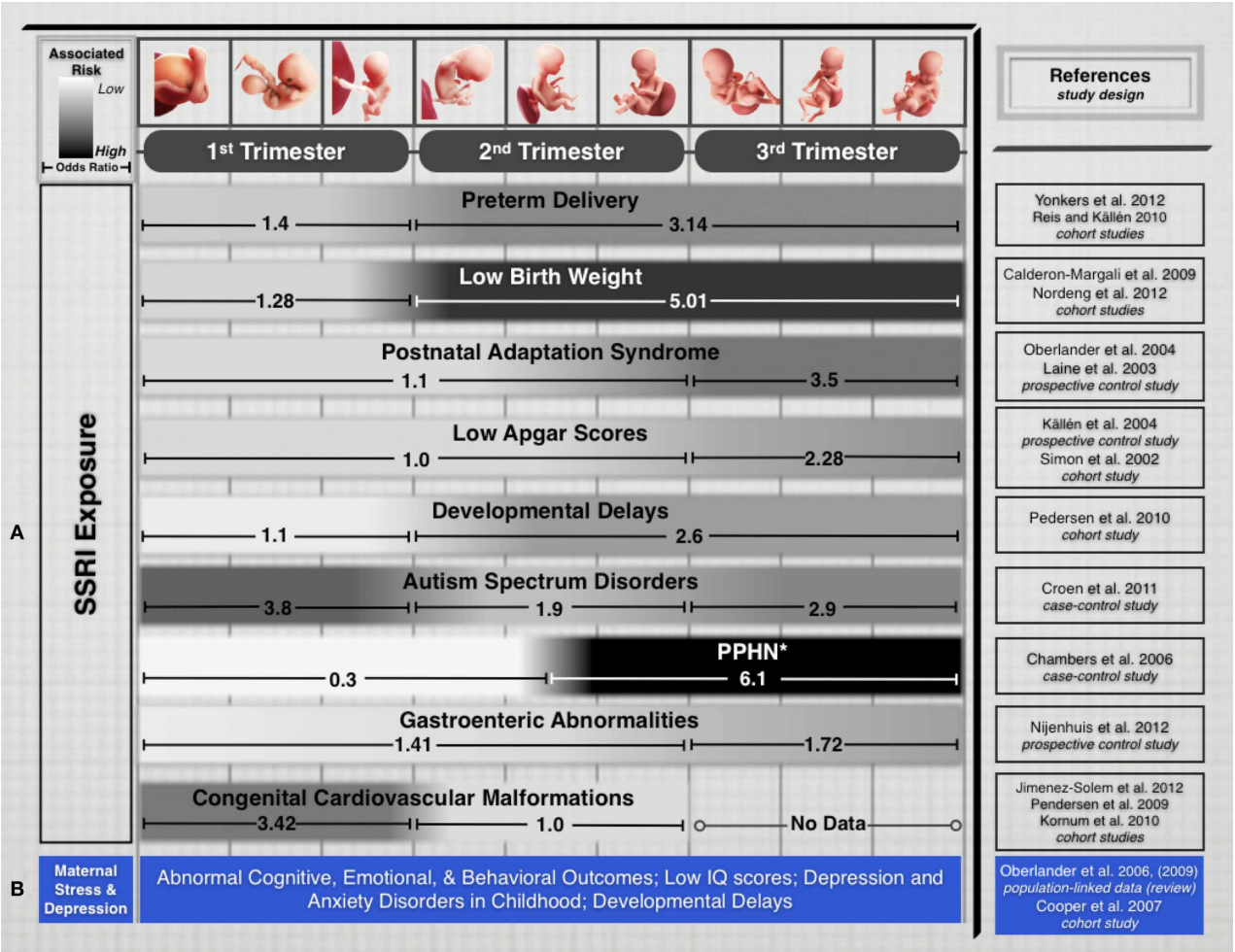

FIGURE 1 | (A) Treatment of maternal depression with SSRIs is associated with varying pregnancy outcomes. While every gestational stage of SSRI exposure has been implicated in increased risks for cognitive, physiological, or developmental teratogenicity, the period of exposure is an important factor that appears to influence clinical outcomes in the offspring. We limited this list to outcomes that have been the focus of several epidemiological studies in recent years and for which differential exposure data during pregnancy was available. **(B)** Untreated maternal depression and stress have been associated with several risks that affect cognitive and developmental outcomes. While associations are not generally correlated to specific trimesters, exposure to untreated maternal depression or stress during pregnancy pose adverse risks to fetal health and development. *Study Selection and Data Extraction* Studies were selected if they had clearly identified maternal SSRI exposure for specific trimesters of pregnancy and assessed neonatal outcomes. Epidemiological studies that included medium-to-large number samples exposed to different SSRI drugs were selected. Direct comparison of absolute odds ratio values across these studies is not possible due to varying specific study designs, adjustments for level of maternal depression and various sociodemographic and lifestyle factors, drug dosages, length of exposure, and SSRI treatment options. *PPHN, Persistent pulmonary hypertension of the newborn.

stem from both the untreated illness and the treatment itself, underscoring a therapeutic risk-benefit dilemma: SSRI treatments that safeguard maternal health have adverse effects on the developing fetus, but leaving maternal depression untreated also poses various significant, adverse risks.

Several perspectives have been offered to account for how some psychiatric disorders may arise from the disruption of particular neurotransmitter systems during development. Disruption during sensitive developmental periods may have lasting effects expressed during adulthood, and since 5-HT signaling participates in several developmental programs (see above), dysfunction of the 5-HT system may be implicated in the etiology of several mental disorders in humans, particularly in MDD. Genetic studies in mice show that transient developmental disruption the 5-HT system by exposure to SSRIs results in long-term behavioral abnormalities and increased anxiety in adult offspring (Ansorge et al., 2004, 2008; Maciag et al., 2006; Oberlander et al., 2008). Not only does neonatal SSRI exposure reduce serotonergic signaling, but also elicits a down regulation in midbrain expression of *Tph2*, an essential enzyme in the serotonin synthesis pathway (see above, Maciag et al., 2006).

As mentioned above, studies in animal models point to evidence that 5-HT influences mammalian nervous system development. Disruption of 5-HT signaling has several important

implications, namely in the modulation of axonal guidance mechanisms that establish precise fetal brain circuits (Gross et al., 2002; Bonnin et al., 2006, 2007). Because embryonic thalamocortical axons (TCAs) accumulate 5-HT and express a range of 5-HT receptors as well as SERT, serotonin is able to shape the outgrowth and synaptic connectivity of their projections (Bonnin et al., 2012). SSRIs target and block SERT with high affinity, and have been shown to directly affect serotonergic modulation of TCA responses to the guidance cue netrin-1 *in vitro*. The presence of the SSRI citalopram (R/S enantiomers mixture) switched TCA response to netrin-1 from attraction to repulsion, impacting the direction of their projections (Bonnin et al., 2012). Moreover, mice with genetically disrupted SERT function, which may serve as a model for chronic SSRI exposure, display changes in neuronal cytoarchitecture, 5-HT function and neurobehaviors, all components that have developmental origins (Oberlander et al., 2009). In addition, genetic studies in mice show that disruption of 5-HT receptors expression during a restricted period of pre- and postnatal development results in long-term behavioral abnormalities (Gross et al., 2002). Taken together, these results suggest that SSRIs could induce topographical shifts in important circuits of the fetal brain, thus constituting a possible mechanism that gives rise to certain mental illnesses by altering circuit-formation and ultimately, proper brain function later in life.

IMPACT OF SSRIs ON FETAL DEVELOPMENT MAY DEPEND ON ROUTES OF EXPOSURE DURING PREGNANCY

The placenta is essential for ensuring the growth and survival of the fetus during development. Not only does it support fetal homeostatic functions, but also serves as the essential source of 5-HT for the fetal forebrain during a transient, critical period of development (Bonnin et al., 2011; Bonnin and Levitt, 2012). The placenta is able to synthesize 5-HT from a maternal TRP precursor in both mice and humans (Bonnin et al., 2011; Bonnin and Levitt, 2012; Goeden and Bonnin, 2013). This exogenous source of 5-HT is available to the fetal brain during developmental milestones including cortical neurogenesis, cell migration, and circuit formation (Bonnin et al., 2011). Therefore, proper placental function during gestation may be essential for the 5-HT modulation of neurodevelopment.

The placenta may play a major role between SSRIs exposure and their associated teratogenicity during gestation. Since the fetal brain acquires placenta-derived 5-HT during a critical period of widespread axonal outgrowth, the effects of SSRIs on fetal brain development may be through an indirect pathway that affects proper placental physiology, resulting in indirect, downstream effects on the fetus. Although it is not clear whether SSRI exposure induces physiological changes in the placenta, its high expression of SERT support the notion that SSRIs would retain their high binding affinity in this organ (Ganapathy et al., 1993; Yavarone et al., 1993; Shearman et al., 1998; Verhaagh et al., 2001). If blocking SERT function alters placental 5-HT synthesis and/or transport to the fetus, or maternal 5-HT degradation, SSRI treatments could be teratogenic primarily by altering placental physiology. The placenta's key function of maintaining fetal homeostasis may thus be compromised and have long-term effects on fetal forebrain development.

Alternatively, SSRIs may be able to readily cross the placenta and enter the fetal circulation, where they could directly target the developing brain's serotonergic system. While there is some evidence of SSRIs crossing the placenta, studies have focused on umbilical cord concentrations at birth in humans (Hostetter et al., 2000; Hendrick, 2003; Sit et al., 2011). Several commonly used SSRIs such as Citalopram, Fluoxetine, and Paroxetine were shown to cross the placental barrier at term, with various efficiencies (e.g., mean ratios of umbilical cord to maternal serum concentrations ranged from 0.29 to 0.89) (Hendrick, 2003). These studies give a snapshot of maternal-fetal SSRI transplacental transport at birth; however, there is no data earlier in gestation, particularly when the fetal brain may be most susceptible to disruptions of 5-HT signaling. Such data is difficult to obtain in humans, rendering studies in animal models as crucial and necessary to providing key insights.

The impact of SSRIs on fetal brain development may therefore result from direct actions on the fetal brain, indirect actions on placental or maternal physiology or, more likely, a combination of all these routes (**Figure 2**). Ongoing efforts to measure transplacental transfer and effects on placental physiology of SSRIs throughout the course of pregnancy in mice, and to determine the drugs biodistribution in the fetus, will help determining precisely how they affect fetal brain development.

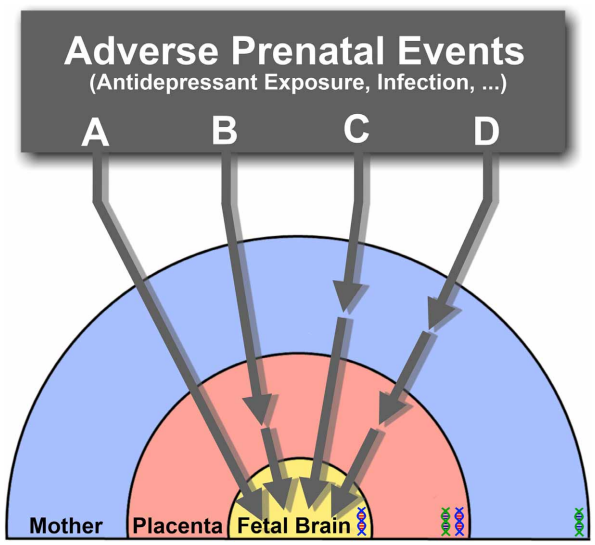

FIGURE 2 | The effects of SSRIs on fetal brain development may be through direct (A) or indirect pathways that affect placental (B), maternal (C), or both maternal and placental physiology (D), ultimately resulting in downstream effects on the fetus. Direct effects **(A)** suggest that SSRIs readily cross the placenta and enter the fetal circulation, where they would directly target the developing brain's serotonergic system. Alternatively, physiological changes in the placenta **(B)**, or delivery of maternal factors essential for the developing fetal brain **(C)** may be affected through indirect pathways. The combination of both direct and indirect pathways inducing adverse effects on the fetal brain may also be possible **(D)**. Under the influence of varying s maternal, fetal and placental (maternal-fetal combination) genetic susceptibilities (DNA double helix), the effects of SSRI exposure at different pregnancy stages may lead to diverse developmental outcomes.

A CONCLUDING PERSPECTIVE ON THE ROLE OF 5-HT ON THE NEURODEVELOPMENTAL PROGRAMMING OF MENTAL DISEASES

Transient disruption of essential signaling events during critical developmental periods may have lasting effects that are expressed throughout life. The serotonergic system steers neurodevelopment through the key modulation of neurogenesis, cell migration, and brain wiring that give rise to proper brain function. With a diversity of molecular targets on which to focus, it makes sense that perturbations of 5-HT signaling have been implicated in the pathogenesis of diverse neurodevelopmental disorders. The perturbations of the 5-HT neurotransmitter system during development, whether directly on the fetal brain or on its placental modulation during early gestation, may have long-lasting developmental and physiological consequences. Risk factors, both genetic and environmental, that alter 5-HT concentration in the fetal brain tissue may thus ultimately pose far-reaching functional consequences throughout life.

Fetal exposures to SSRIs and maternal stress induced by MDD are early exposures that have been associated with various diseases affecting physiological and cognitive domains. The heterogeneity and diversity of different disease outcomes is informed by the length and developmental period of adverse exposures, in addition to fetal genetic susceptibilities. Together with the altered fetal brain 5-HT signaling caused by SSRI exposure in different stages, the influence of maternal, fetal and placental (maternal-fetal; see **Figure 2**) genetics could possibly lead to different disease states. The manifestation of several mental disorders associated with serotonin dysfunction, namely MDD, ASD, and other psychiatric illnesses may thus require multiple events of environmental, genetic, and their interactions, to occur.

While the associated risks from fetal SSRI exposure continue to be elucidated, the mechanisms of 5-HT neurodevelopmental disruptions, and how they ultimately lead to adult-onset disorders need further study. There is also a clinical demand for effective and safe treatment of maternal MDD, taking into consideration the effects of drug therapy on the safety of the developing fetus.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 February 2013; accepted: 03 April 2013; published online: 23 April 2013.

Citation: Velasquez JC, Goeden N and Bonnin A (2013) Placental serotonin: implications for the developmental effects of SSRIs and maternal depression. Front. Cell. Neurosci. 7:47. doi: 10.3389/fncel. 2013.00047

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 04 October 2013
doi: 10.3389/fncel.2013.00143

Lack of serotonin reuptake during brain development alters rostral raphe-prefrontal network formation

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Besides its "classical" neurotransmitter function, serotonin (5-HT) has been found to also act as a neurodevelopmental signal. During development, the 5-HT projection system, besides an external placental source, represents one of the earliest neurotransmitter systems to innervate the brain. One of the targets of the 5-HT projection system, originating in the brainstem raphe nuclei, is the medial prefrontal cortex (mPFC), an area involved in higher cognitive functions and important in the etiology of many neurodevelopmental disorders. Little is known, however, about the exact role of 5-HT and its signaling molecules in the formation of the raphe-prefrontal network. Using explant essays, we here studied the role of the 5-HT transporter (5-HTT), an important modulator of the 5-HT signal, in rostral raphe-prefrontal network formation. We found that the chemotrophic nature of the interaction between the origin (rostral raphe cluster) and a target (mPFC) of the 5-HT projection system was affected in rats lacking the 5-HTT (5-HTT$^{-/-}$). While 5-HTT deficiency did not affect the dorsal raphe 5-HT-positive outgrowing neurites, the median raphe 5-HT neurites switched from a strong repulsive to an attractive interaction when co-cultured with the mPFC. Furthermore, the fasciculation of the mPFC outgrowing neurites was dependent on the amount of 5-HTT. In the mPFC of 5-HTT$^{-/-}$ pups, we observed clear differences in 5-HT innervation and the identity of a class of projection neurons of the mPFC. In the absence of the 5-HTT, the 5-HT innervation in all subareas of the early postnatal mPFC increased dramatically and the number of Satb2-positive callosal projection neurons was decreased. Together, these results suggest a 5-HTT dependency during early development of these brain areas and in the formation of the raphe-prefrontal network. The tremendous complexity of the 5-HT projection system and its role in several neurodevelopmental disorders highlights the need for further research in this largely unexplored area.

Keywords: explant assays, prefrontal cortex, serotonin transporter, microdissection, axon guidance, depression, SERT, autism

INTRODUCTION

It has become increasingly clear that several "classical" neurotransmitters, such as serotonin (5-HT), additionally act as neurodevelopmental signals to direct the assembly of the developing brain (Lauder, 1990; Whitaker-Azmitia et al., 1996; Buznikov et al., 2001; Sodhi and Sanders-Bush, 2004; Cunningham et al., 2005; Riccio et al., 2009; Souza and Tropepe, 2011; Bonnin and Levitt, 2012; Migliarini et al., 2012). Even before the raphe-derived neurites start extending, there is an external placental source of 5-HT (Bonnin et al., 2011). Furthermore, 5-HT signaling molecules such as enzymes responsible for 5-HT synthesis and breakdown, 5-HT receptors and the 5-HT transporter (5-HTT) are already expressed in the brain before 5-HT neurons are born (Bruning et al., 1997; Zhou et al., 2000; Cote et al., 2007; Bonnin et al., 2011; Bonnin and Levitt, 2012). The role of 5-HT and its signaling molecules during development is especially important in the light of recent discussions on the effect of serotonin-reuptake inhibitors (SSRIs) during pregnancy (Vitalis et al., 2007; Alwan and Friedman, 2009; Oberlander et al., 2009; Gentile and Galbally, 2011; Simpson et al., 2011). SSRIs given to the pregnant mother to treat depression, will increase the extracellular 5-HT in not only the mother but also in the brains of the unborn child (Rampono et al., 2004; Gentile and Galbally, 2011). These children acquire an increased risk to develop reduced somatosensory responses (Oberlander et al., 2009) and/or psychomotor control (Casper et al., 2011), and appear to have a higher risk to develop autism-like symptoms (Croen et al., 2011).

The 5-HT projection system is one of the earliest neurotransmitter systems to develop and send out its projections to distant targets (Whitaker-Azmitia, 2001; Homberg et al., 2010). Specifically, the 5-HT neurons located in the rostral raphe cluster extend profuse axon tracts into the fore- and midbrain (Gaspar

et al., 2003; Bang et al., 2012). A distant target of the ascending 5-HT projection system within the forebrain is the medial prefrontal cortex (mPFC) (Del Cid-Pellitero and Garzon, 2011; Waselus et al., 2011). The mPFC is the seat of our highest cognitive abilities and known to be involved in attentional processes, working memory and behavioral flexibility (Miller and Cohen, 2001; Heidbreder and Groenewegen, 2003). In rodents, the developing 5-HT-positive fibers reach the mPFC around embryonic day 16–17 (E16 in mouse and E17 in rats), where they initially innervate the marginal zone and the subplate, before massively innervating the cortical plate proper (Janusonis et al., 2004). The 5-HT fibers, found within the marginal zone of the mPFC, are thought to contact Cajal-Retzius (CR) cells, cortical layer I cells secreting the glycoprotein reelin crucial for the correct layering of the cortex (Janusonis et al., 2004; Leemhuis et al., 2010). These CR cells express 5-HT_{1A} and 5-HT_{3A} receptors (Janusonis et al., 2004; Chameau et al., 2009; Vucurovic et al., 2010) and differences in 5-HT input onto the latter could result in an altered reelin release, cortical layering and ultimately, PFC-mediated cognitive functioning. Indeed, altered 5-HT innervations of the mPFC have been implicated in the etiology of neurodevelopmental disorders such as schizophrenia, autism spectrum disorders (ASD) and intellectual disability (Gurevich and Joyce, 1997; Chugani et al., 1999; Whitaker-Azmitia, 2001, 2005; Canli et al., 2005; Canli and Lesch, 2007; Robbins and Arnsten, 2009; Costa et al., 2012; Mann, 2013).

There are indications that 5-HT acts as a soluble cue and modulates the response of targeting axons to guidance cues (Petit et al., 2005; Bonnin et al., 2007). Due to the important role of 5-HT in neurodevelopment, factors that influence 5-HT signaling may also have profound effects on the correct development of the brain. The presynaptically located 5-HTT is the primary regulator of 5-HT signaling, terminating the 5-HT signal by allowing reuptake for recycling or degradation (Homberg et al., 2007a, 2010; Neumann et al., 2011). Apart from being expressed in 5-HT neurons, the 5-HTT is also transiently expressed in non-aminergic neurons, belonging to many topographically distinct brain areas (Lebrand et al., 1998; Zhou et al., 2000; Narboux-Neme et al., 2008; Kalueff et al., 2010). In humans, the 5-HTT gene-linked polymorphic region (5-HTTLPR), composed of a short and a long version (Canli and Lesch, 2007; Neumann et al., 2011; Haddley et al., 2012), affects 5-HTT expression and function. The short (s) variant has been associated with robust neurodevelopmental changes in corticolimbic structures, and increased risk for depression in the context of stress (Canli and Lesch, 2007). The 5-$HTT^{-/-}$ rat model is also known to display anxiety- and depression-related responses to stressors (Homberg et al., 2007b; Kalueff et al., 2010). Extracellular levels of 5-HT are increased throughout the brain of the 5-$HTT^{-/-}$ rodent, and affect 5-HT receptor expression, where the 5-HT_{1A} is known to be down-regulated in both 5-$HTT^{-/-}$ rodents and s-variant carriers (David et al., 2005; Riccio et al., 2009; Kalueff et al., 2010). Also, due to 5-HTT deficiency, increased activity at the 5-HT_6 receptor affects proper cortical cytoarchitecture and interneuron migration (Riccio et al., 2009, 2011). The mechanisms by which a reduced 5-HTT function in humans, or reduction/deficiency of 5-HTT in rodents, and consequent increased 5-HT levels, affects areal maturation, guidance and network formation are still not fully understood.

Here we report the results of our study of the chemotropic nature of the interaction between the origin (rostral raphe cluster) and a target (mPFC) of the 5-HT projection system using the 5-HTT knockout rat model. Additionally, we have examined the ability of the outgrowing neurites to form fascicles, and whether differences in fasciculation could be due to 5-HTT deficiency. Moreover, in order to determine whether the early lack of the 5-HTT also affected the maturation of the 5-HT raphe-mPFC projection system, we examined the 5-HT innervation within various subareas of the mPFC in 5-$HTT^{-/-}$ and 5-$HTT^{+/+}$ pups. Using the transcription factor Satb2 (special AT-rich sequence binding protein 2) as a marker for callosal projection neurons in cortical layers II-VI, we analyzed the number of Satb2-positive neurons in 5-HTT-deficient pups.

MATERIALS AND METHODS

ANIMALS

All animal use and care were performed in accordance with the institutional and national guidelines and regulations of the Committee for Animal Experiments of the Radboud University Nijmegen, The Netherlands. All animal experiments conformed to the relevant regulatory standards. The 5-HTT mutant rats ($Slc6a4^{1Hubr}$) were generated in a Wistar background by target-selected ENU-induced mutagenesis (for detailed description, see Smits et al., 2006). Timed-pregnant rats were individually housed in macrolon cages in a temperature- and humidity-controlled room (21 ± 1°C and 60% relative humidity, respectively). The rats had *ad-libitum* access to food and water and a normal light-dark cycle was maintained. Timed-pregnant rats were sacrificed by means of CO_2/O_2. The morning on which a vaginal plug was detected is considered E0.5. Genotyping of the embryos and pups was performed by KBioscience (Hoddesdon, United Kingdom).

EXPLANT CULTURES

Three-dimensional collagen matrix explant assays were performed as described previously (Kolk et al., 2009). Embryonic day 16.5 (E16.5) rat embryos were collected in ice-cold L15 medium (Leibovitz with L-glutamine, PAA, Austria) and brains were rapidly dissected. Explants (<300 μm) were microdissected from (1) the rostral cluster of raphe nuclei, in a rostral-to-caudal direction dividing it in a rostral, intermediate and caudal subarea, bisected along the midline; and (2) the mPFC. Rostral and intermediate subareas correspond to the dorsal raphe nucleus, and the caudal subarea corresponds to the median raphe nucleus (MnR; **Figures 1A,B**; **Supplemental Figure 1A**). The explants were collected in ice-cold L15 medium containing 10% fetal calf serum (FCS).

Combinations of the various raphe subareas and the mPFC were embedded in close proximity (∼300 μm apart) in a collagen matrix (10% 10X MEM, Invitrogen; and 10% $NaHCO_3$ in diluted rat tail collagen, Invitrogen) in four-well culture dishes (Nunclon surface, Nunc, ThermoScientific). As controls, the various raphe subareas and the mPFC explants were cultured individually to check for their radial growth. Explants were cultured in growth medium (DMEM-F12 with 10% glutamine and

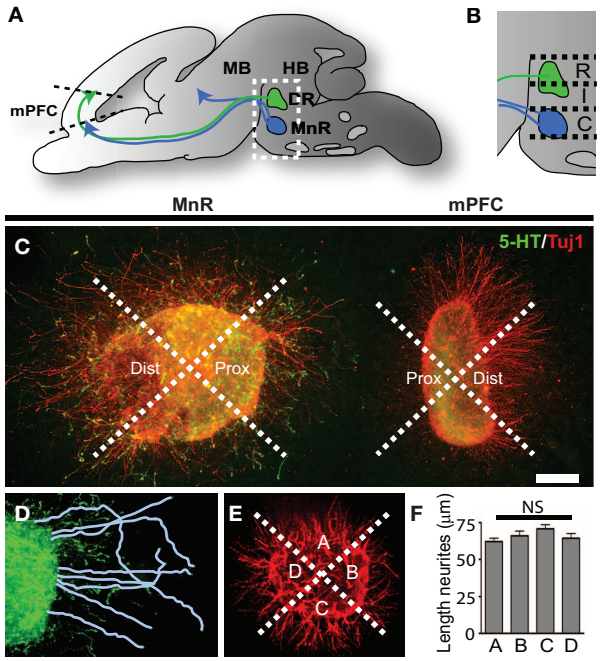

FIGURE 1 | Three-dimensional collagen co-cultures of explants taken from the mPFC, DR, and MnR show trophic responses. (A) Schematic of an embryonic brain showing the position of the 5-HT-positive rostral cluster of raphe nuclei projecting to the mid- and forebrain. The dorsal raphe nucleus (DR) projects to forebrain regions (green arrow) including the prefrontal cortex (mPFC). The median raphe nucleus (MnR) projects (blue arrows) to fore-and midbrain regions. **(B)** Enlargement of the boxed area in **(A)**. The rostral (R) and intermediate (I) subarea correspond to the DR and the caudal (C) subarea corresponds to the MnR. **(C)** Example of a 5-HTT$^{+/-}$ caudal subarea (MnR) co-cultured with mPFC, divided in proximal and distal quadrants and stained for 5-HT (5-HT neurites, green) and Tuj1 (β-III tubulin, all outgrowing neurites, red). **(D)** In the proximal (and distal, not shown) quadrants the neurites are traced and measured. **(E)** Control explants were cultured separately and neurite outgrowth was measured in the 4 quadrants (example of WT mPFC). **(F)** The average length of the neurites in quadrants A, B, C, or D showed no significant (NS) difference. HB, hindbrain; MB, midbrain. Scale bar represents 80 μm.

antibiotics, 6% 1,7M glucose, and 10% FCS) in a humidified incubator at 37°C with 5% CO_2 for 4 days. Growth medium was renewed after 24 h. For each of the combinations of co-cultures mentioned above, at least four independent experiments were performed.

IMMUNOHISTOCHEMISTRY

Brains were rapidly dissected from E16.5 embryos and postnatal day 6 (P6) pups, fixed by immersion for 90 min. in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), washed in PBS and cryoprotected in 30% sucrose in PBS. Brains were frozen in M-1 embedding matrix (Thermo Fisher Scientific) on dry ice in a plastic cup and stored at −80°C. Cryostat sections were cut at 16 μm, mounted on Superfrost Plus slides (Thermo Fisher Scientific), air-dried, and stored desiccated at −20°C.

Cryosections were stained immunohistochemically as described previously (Kolk et al., 2006). Rabbit anti-5-HT (Sigma, 1:5000) and mouse anti-Satb2 (Abcam, 1:500) were diluted in blocking buffer (BB, 1.7% normal donkey serum, 1.7% normal goat serum, 1.7% normal horse serum, 1% BSA, 1% glycine, 0.1% lysine, 0.4% Triton X-100, in PBS) and incubated overnight at 4°C. Sections were incubated in species-specific Alexa-conjugated secondary antibody (Molecular Probes) generated in goat and diluted 1:500 in BB for 30 min. at RT. After washing in PBS, sections were counterstained with fluorescent Nissl stain (NeuroTrace; Invitrogen; 1:500), washed extensively in PBS, and embedded in 90% glycerol. Antibody specificity was tested by omitting the primary antibody resulting in no positive signal (negative control) and careful comparison of immuno-positive brain areas with the areas described before (Riccio et al., 2009; Balamotis et al., 2012) (positive control). The nomenclature to describe 5-HT-positive cells and fibers within various brain areas is as described by (Abrams et al., 2004; Kolk et al., 2009).

The explants in their collagen matrix were quickly washed in PBS, fixed in buffered 4% PFA for 1.5 h, and washed extensively o/n at 4°C before performing immunocytochemistry. Explants were incubated in BB for 6–8 hr at room temperature (RT). The explants were incubated with primary antibody diluted in BB o/n at 4°C. Rabbit anti-5-HT (Sigma, 1:5000) and mouse anti-Tuj1 (β-III tubulin, Covance, 1:1000) were used to visualize 5-HT or all outgrowing neurites, respectively. On the second day, explants were washed 4 times for a total of 4–5 h at RT. They were then incubated with species-specific Alexa-conjugated secondary antibody (Molecular Probes) generated in goat and diluted 1:500 in BB for 1 h at RT. After washing extensively in PBS o/n at 4°C, the explants were embedded (Prolong Gold, antifade reagent, Invitrogen). For visualization, a Leica DMRA Fluorescence microscope with DFC340FX camera and LASAF software was used.

DATA ANALYSIS

All data analysis was performed in a double-blind fashion. For quantification of the explants assays, the explants were divided in a proximal and distal quadrant of which images were captured as described by (Kolk et al., 2009). The length of the 20 longest neurites was measured in both the proximal and distal quadrants of the culture using Neuron J (Image J plug-in) with an average of 5 explants per condition. The average value of length of each explant in both proximal and distal quadrants was used to determine the proximal/distal ratio (P/D ratio) per explant (Kolk et al., 2009). The number, width, and length of the neurite fascicles were analyzed in the proximal and distal quadrants of the culture using NeuronJ by tracing across and along the fascicle.

For assessing 5-HT fiber length and number of Satb2-positive neurons in the various subareas of the mPFC of 5-HTT$^{+/+}$, 5-HTT$^{+/-}$ and 5-HTT$^{-/-}$ rats, three to five pups of each genotype of at least three independent litters were analyzed and two to four well-spaced (120 μm) sections at the same neuroanatomical level were imaged. A 0.1-mm-wide rectangle spanning the cerebral wall was placed over the center of the subarea (either infralimbic, IL, prelimbic, PL or cingulate cortex, Cg) of the mPFC. The overall cortical width of a subarea was divided into 10 equal bins [bin 1

within the deep cortical zone and bin 10 within the presumptive layer I] within this rectangle, and 5-HT-positive fiber length or Satb2/Nissl-positive neuron number was measured within each bin using ImageJ software (NIH, Bethesda, USA). Data were normalized to total length or number per square micrometer and averaged for each pup. To better visualize and compare 5-HT innervation of wild-type and mutant mPFC, reconstructions of the individual fibers were obtained using NeuronJ from two to three consecutive sections, bilaterally. Data were statistically analyzed by one-way ANOVA ($\alpha = 5\%$) and expressed as means ± SEM.

RESULTS

PRESENCE OF 5-HTT DURING EARLY DEVELOPMENT MODULATES ROSTRAL RAPHE-mPFC DIRECTIONAL RESPONSES *In vitro*

The rostral cluster of raphe nuclei forms projections toward their targets in the fore- and midbrain (Dahlstrom and Fuxe, 1964; Van Bockstaele et al., 1993; Waselus et al., 2011; Bang et al., 2012). One of the targets within the forebrain is the mPFC (Wilson and Molliver, 1991; Verney et al., 2002; Del Cid-Pellitero and Garzon, 2011; Puig and Gulledge, 2011). The 5-HT projections are guided along the way to their target by various cues, either soluble or membrane-bound, as they develop (Petit et al., 2005; Anitha et al., 2008; Lee et al., 2010).

To identify the chemotropic nature of the interaction between the rostral cluster of raphe nuclei and the mPFC and to evaluate possible changes in the 5-HTT knockout model, we performed three-dimensional collagen co-cultures of the rostral cluster of raphe nuclei and the mPFC (**Figure 1**; **Supplemental Figures 1A,B,D**). Brain areas were microdissected from E16.5 5-HTT$^{+/+}$, 5-HTT$^{-/-}$ and 5-HTT$^{+/-}$ embryonic brains (**Supplemental Figure 1A**). Explants were taken from the rostral cluster of raphe nuclei and were divided into three subareas; rostral, intermediate and caudal (**Figures 1A,B**; **Supplemental Figure 1A**). The rostral and intermediate subareas correspond to the dorsal raphe nucleus (DR) which mainly projects to the forebrain, including the mPFC (Van Bockstaele et al., 1993; Waselus et al., 2011). The caudal subarea corresponds to the median raphe nucleus (MnR) which innervates both the fore- and midbrain (**Figures 1A,B**; **Supplemental Figures 1A–C**) (Puig and Gulledge, 2011). The explants from the mPFC were co-cultured with one of the subareas of the raphe in a collagen hill for 4 days (**Figure 1**; **Supplemental Figures 1A,B,D**). After 4 days, the explants were fixed and immunostained for 5-HT and Tuj1 (β-III tubulin, a marker for outgrowing neurites). To measure the extent of attraction or repulsion of outgrowing neurites, revealing the chemotrophic nature of the interaction between the two areas, the explants were divided into a proximal and a distal quadrant, with the proximal quadrant facing the co-cultured explant (**Figure 1C**). Within the proximal and distal quadrant the lengths of the longest neurites were measured and averaged (**Figure 1D**). The average length of the neurites on the proximal site was then divided by the average length of the distal site neurites giving the proximal/distal-ratio (P/D ratio). A P/D ratio above 1 indicates an attractive interaction, whereas a P/D ratio less than 1 denotes repulsion (Pasterkamp et al., 2003; Kolk et al., 2009; Fenstermaker et al., 2010). As a control, explants of the various brain areas were cultured individually and divided into four quadrants (**Figure 1E**). The lengths of the longest neurites were measured in each quadrant and statistical analysis revealed no significant differences between the 4 quadrants, indicating a radial neurite outgrowth when cultured individually (**Figure 1F**).

Figures 2A–F show examples of the proximal and distal side of explants of subareas of the rostral raphe and the mPFC co-cultured together (arrowheads above the schematic indicate the displayed explant). P/D ratios of the 5-HT neurite outgrowth and the overall neurite outgrowth (Tuj1-positive) of subareas of the raphe co-cultured with the mPFC (examples in **Figures 2A–D**) were calculated and depicted in **Figures 2G,H**. The P/D ratios of 5-HT neurite outgrowth of the DR (rostral and intermediate subarea) indicated little attraction toward the mPFC (**Figure 2G**). Lack of 5-HTT had no effect on the targeted neurite outgrowth from the DR (**Figure 2G**). The P/D ratios of the 5-HT neurite outgrowth of the MnR (caudal subarea) showed significant differences due to 5-HTT deficiency (**Figures 2A,B,G**). In the wild-type situation a repulsive interaction toward the mPFC was observed (P/D ratio, 0.53; **Figures 2A,G**). However, the reduction or lack of the 5-HTT caused a significant attractive interaction (P/D ratios, 1.52 and 1.20, respectively (resp.); $p = 0.0018$ and 0.033, resp.; **Figures 2B,G**). Since the fibers are no longer repulsed, we can speculate that an increased number of 5-HT fibers may now target the mPFC. The observed switch to an attractive interaction was not found in the overall neurite outgrowth (Tuj1) from the MnR, here the interaction remained repulsive (P/D ratios, 0.59, 0.49, 0.47 for HTT$^{+/+}$, 5-HTT$^{+/-}$, and 5-HTT$^{-/-}$, resp.; **Figure 2H**). In the wild-type situation, the overall neurite outgrowth of the DR (rostral and intermediate explants) seemed to be slightly attracted by the mPFC (P/D ratio, 1.10 and 1.01; **Figures 2C,H**). However, the 5-HTT$^{+/-}$ situation resulted in a switch to significant repulsion, although with the complete lack of 5-HTT this repulsion became less obvious (P/D ratios, 0.57 and 0.74, resp.; $p = 0.012$ and 0.022, resp.), especially in the more caudal (intermediate subarea) part of the DR (P/D ratios, 0.58 and 0.94, resp.; $p = 0.022$ between HTT$^{+/+}$and 5-HTT$^{+/-}$; **Figures 2D**,**H**).

Figures 2D,**I** show the P/D ratios of overall neurite outgrowth from the mPFC which was co-cultured with either the rostral, intermediate, or caudal subarea of the raphe. The mPFC neurite outgrowth in the wild-type and 5-HTT$^{-/-}$ situation was attractive toward all subareas of the rostral raphe (P/D ratios, 1.19, 1.01 and 1.29, resp.; **Figures 2E,F,I**). However, for the 5-HTT$^{+/-}$ explants this interaction of the mPFC with all subareas of the rostral raphe switched to repulsive (P/D ratios, 0.81, 0.72 and 0.58, resp.). This phenomenon was most prominent when the mPFC was co-cultured with the MnR (caudal subarea) as shown in **Figures 2E,F,I** ($p = 0.017$ between HTT$^{+/+}$and 5-HTT$^{+/-}$). In absence of the 5-HTT, the P/D ratios were comparable with the wild type situation (P/D ratios, 1.23, 1.06, and 1.22, resp.; **Figure 2I**).

Taken together, these data show that the outgrowing neurites of the DR/MnR and from the mPFC, show directional responses

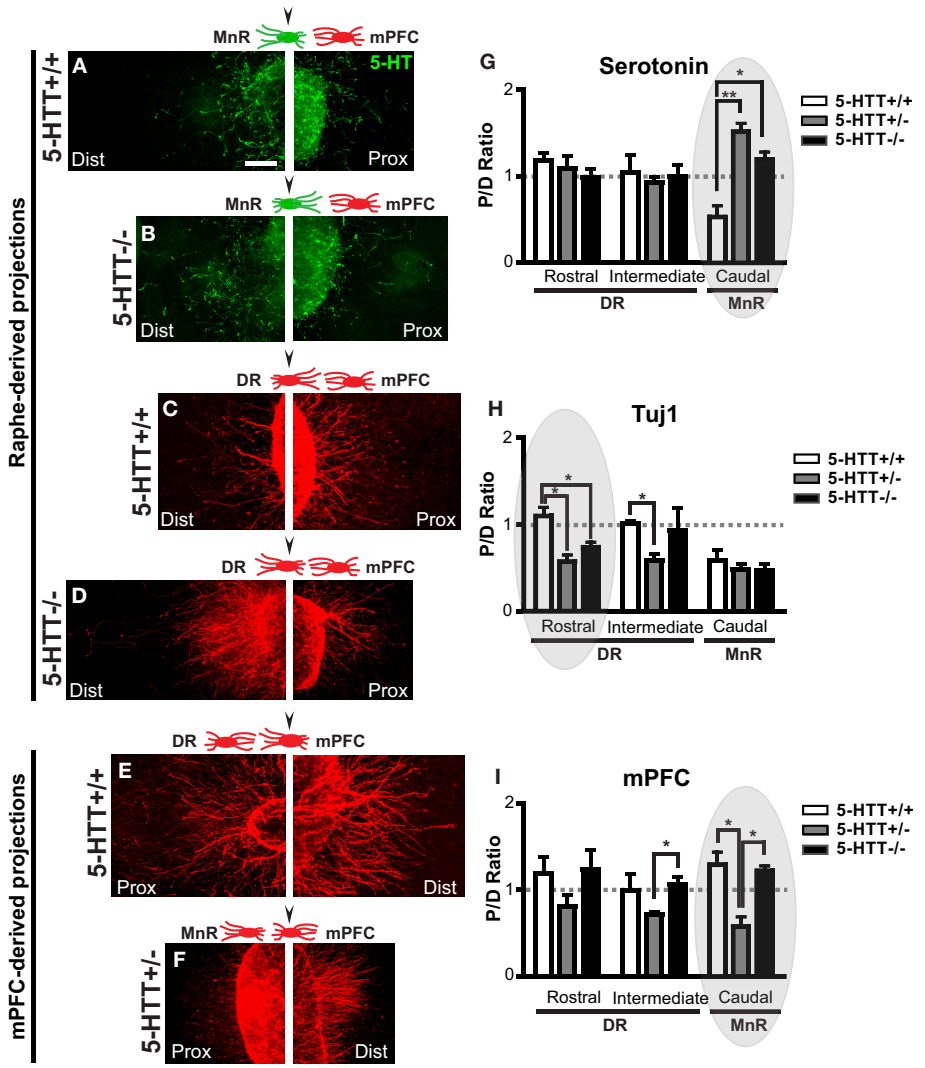

FIGURE 2 | The chemotrophic nature of the interaction between the mPFC and the DR and MnR depends on 5-HTT during development. **(A-F)** High magnification photographs of proximal and distal quadrants of the raphe (positive for 5-HT, green and Tuj1, red) and the mPFC (Tuj1, red). Arrowhead in schematic representations indicate the example given. **(G,H)** Quantification of the length of 5-HT-positive and Tuj1-positive neurites in the proximal and distal quadrants of the DR and the MnR, co-cultured with mPFC. Graphs show average P/D ratios ± SEM. **(I)** Quantification of the length of Tuj1-positive neurites in the proximal and distal quadrants of the mPFC co-cultured with either the DR or MnR. Graph shows average P/D ratios ± SEM. One-Way ANOVA ($\alpha = 0.05$), $^{*}p < 0.05$, $^{**}p < 0.01$. Gray ovals in G-I indicate the example given on the left. Dist, distal quadrant; DR, dorsal raphe nucleus; mPFC, medial prefrontal cortex; MnR, median raphe nucleus; Prox, proximal quadrant.

when cultured together. The nature of this response is different for DR compared to MnR, and is affected by the level of 5-HTT expression.

PRESENCE OF 5-HTT DURING EARLY DEVELOPMENT MODERATES FASCICULATION OF OUTGROWING mPFC NEURITES TOWARD ROSTRAL RAPHE

During the formation of neuronal projection systems, outgrowing axons are guided to their distant targets by a variety of guidance cues (Tessier-Lavigne and Goodman, 1996; Dickson, 2002). Most axons grow alongside other axons for much of their lengths as pioneer axons create the first scaffold for the different projection pathways. Subsequent axons may associate in specific bundles or fascicles, and grow alongside this scaffold in order to reach their proper targets (Van Vactor, 1998; Jaworski and Tessier-Lavigne, 2012). The process of fasciculation of axons requires internal membrane-bound cues, such as members of the neuronal cell adhesion molecule (NCAM) or semaphorin family (Barry et al., 2010). However, soluble guidance cues secreted from (intermediate) targets also modulate fasciculation (Jaworski and Tessier-Lavigne, 2012).

When examining the outgrowing neurites of the explants from the various subareas and genotypes, we noticed differences in the number of outgrowing fascicles, especially from the mPFC (**Figure 3**; **Supplemental Figure 2A**). Therefore, we first determined in how many of the explants

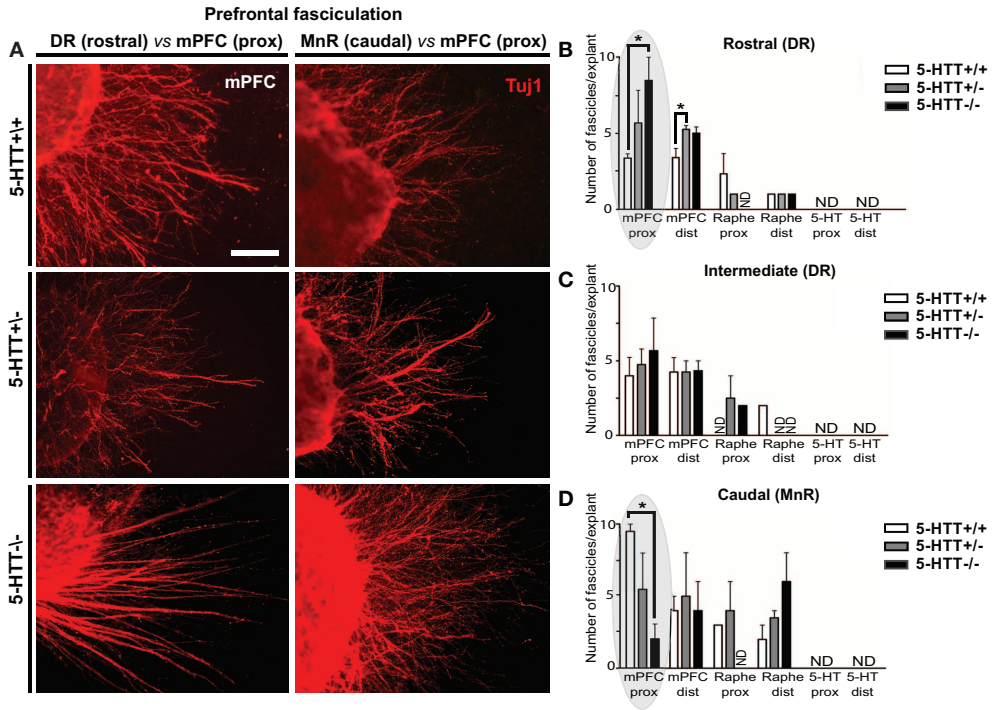

FIGURE 3 | The amount of 5-HTT during development influences mPFC fasciculation. (A) Examples of differences in number of fascicles per explant in WT, 5-HTT$^{+/-}$ and 5-HTT$^{-/-}$ mPFC (proximal quadrant) co-cultured with the rostral (DR, left panels) or caudal subarea (MnR, right panels). Scale bar represents 50 μm. **(B–D)** Quantification of the number of mPFC fascicles (>5 μm) per explant, for the different subareas and genotypes in both the proximal (prox) and distal (dist) quadrant. Gray ovals in **(B,D)** indicate the example given on the left. Graphs show average number of fascicles (>5 μm) per explant ± SEM. One-Way ANOVA ($\alpha = 0.05$), $^*p < 0.05$.

(proximal quadrant) of the different subareas (both raphe as well as mPFC), fascicles with a minimum width of 5 μm, were formed (**Supplemental Figures 2C–E**). It became obvious that most of the mPFC explants exhibited fasciculation. Considering exclusively the 5-HT-positive neurites, no fascicles were formed in explants either from the DR or the MnR (**Supplemental Figures 2C–F**). Lack of 5-HTT did not affect this deficient 5-HT fasciculation. However, in some cases, the 5-HT-positive neurites did grow alongside Tuj1-positive fascicles (**Supplemental Figure 1D**). In most explants of the mPFC, fascicles were formed, although differences were found when co-cultured with the rostral raphe subareas and across genotypes (**Supplemental Figures 2C–E**). For example, the percentage of mPFC explants co-cultured with the rostral subarea (DR) showing fascicles was increased in 5-HTT$^{+/-}$ and 5-HTT$^{-/-}$ as compared to the 5-HTT$^{+/+}$ mPFC (**Supplemental Figure 2C**). All mPFC explants of wild-type and 5-HTT$^{-/-}$ animals co-cultured with the intermediate (DR) and caudal subarea (MnR) had formed fascicles. Notably, the 5-HTT$^{+/-}$ situation resulted in a reduced number of mPFC explants with fascicles co-cultured with the intermediate (DR) and caudal subarea (MnR) (**Supplemental Figures 2D,E**).

Furthermore, we noticed that the number of fascicles formed per explant varied and depended on the genotype. We therefore quantified the average number of fascicles per mPFC explant. **Figure 3A** shows the number of fascicles in wild-type (upper panels) as compared to 5-HTT$^{+/-}$ (middle panels) and 5-HTT$^{-/-}$ (lower panels) in the proximal quadrants of mPFC explants co-cultured with either the DR (left panels) or the MnR (right panels). Lack of 5-HTT resulted in a significant increase in the number of fascicles in the proximal quadrant of the mPFC co-cultured with the rostral subarea (DR) of the raphe (average number is 3.33, 6.25 and 7.33 fascicles for HTT$^{+/+}$, 5-HTT$^{+/-}$, and 5-HTT$^{-/-}$, resp.; $p = 0.023$; **Figures 3A–D**, left) and a significant decrease when co-cultured with the caudal subarea (MnR, **Figures 3A,D**, right; average number is 9.50, 4.33, and 2.67 fascicles for HTT$^{+/+}$, 5-HTT$^{+/-}$, and 5-HTT$^{-/-}$, resp.; $p = 0.022$). An increase in the number of fascicles per mPFC explant was also observed in the distal quadrant of the mPFC that was co-cultured with the rostral part of the DR upon (partial) lack of the 5-HTT (average number is 3.40, 4.40, and 5.40 fascicles for HTT$^{+/+}$, 5-HTT$^{+/-}$, and 5-HTT$^{-/-}$, resp.; $p = 0.036$; **Figure 3B** and data not shown).

The length and width of a fascicle may provide information about the number of neurites bundled together, the nature of internal membrane-bound cues, and the nature of (soluble) environmental cues. For example, a repulsive interaction between axon and environment may favor fasciculation by channeling axons in a common path (Van Vactor, 1998; Jaworski and Tessier-Lavigne, 2012).

To measure the length and width, we traced along and across the fascicle, resp. (**Supplemental Figure 2B**). We found

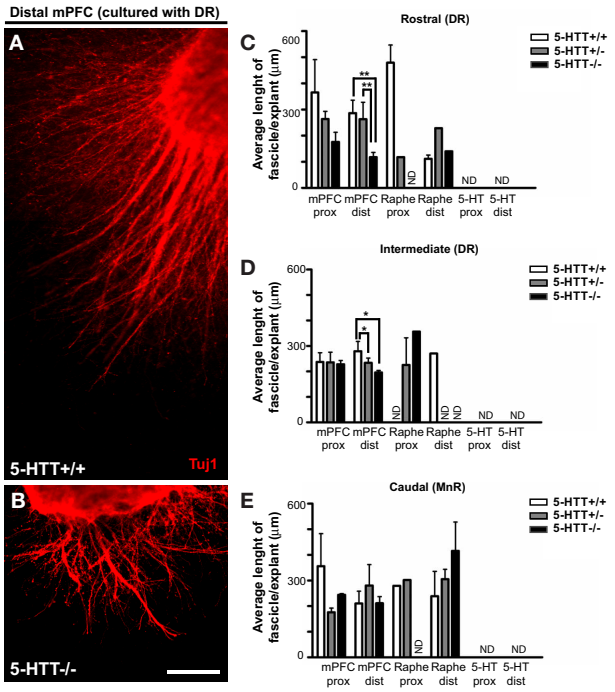

FIGURE 4 | Differences in length of fascicles from the mPFC controlled by the presence of 5-HTT during development. (A,B) Example of differences in fascicle length in the distal quadrant of 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mPFC co-cultured with the rostral subarea. Scale bar represents 100 µm. **(C–E)** Quantification of the length of fascicles in the proximal and distal quadrants of a subarea cocultured with mPFC. Graphs show average length of fascicles per explant ± SEM. One-Way ANOVA ($\alpha = 0.05$), $^{*}p < 0.05$, $^{**}p < 0.01$.

differences in fascicle length growing from the mPFC when co-cultured with either the DR or the MnR, which depended on the genotype. Although not significant in the proximal quadrant of the mPFC, **Figures 4A–D** shows that in the distal quadrant of a wild-type or 5-HTT$^{+/-}$ mPFC co-cultured with DR significantly longer fascicles were formed compared to the 5-HTT deficient situation (average length of fascicles, 286.3, 234.8 and 137.3 µm for HTT$^{+/+}$, 5-HTT$^{+/-}$, and 5-HTT$^{-/-}$, resp.; $p = 0.0097$ and 0.0012, resp. for the rostral subarea and the average length of fascicles, 279.4, 221.9 and 195.5 µm for HTT$^{+/+}$, 5-HTT$^{+/-}$, and 5-HTT$^{-/-}$, resp.; $p = 0.049$ and 0.012, resp. for the intermediate subarea). Small differences between DR and MnR were observed as well. For example, when comparing the distal quadrant of the rostral subarea of the DR (**Figure 4C**) with the MnR (**Figure 4E**) immunostained for β-III tubulin (all outgrowing neurites), the average fascicle length of fascicles from the MnR was longer.

Quantification of the average fascicle width among the various subareas and genotypes showed little significant differences, except for the proximal quadrant of the mPFC co-cultured with the rostral subarea of the DR where the width of the fascicles was significantly lower in the 5-HTT$^{-/-}$ compared to fascicles of 5-HTT$^{+/-}$ explants (average fascicle width is 12.03, 14.61, and 9.65 µm for HTT$^{+/+}$, 5-HTT$^{+/-}$, and 5-HTT$^{-/-}$, resp.; $p = 0.034$; **Supplemental Figure 3C**). **Supplemental Figures 3A,B** illustrate the fascicle width of mPFC co-cultured with the MnR in 5-HTT$^{-/-}$ compared to wild-type explants which was increased, although not significantly.

Taken together these data suggest that there are differences in the formation of fascicles by outgrowing neurites from the DR/MnR and from the mPFC, which seems to be affected by the (partial) lack of 5-HTT.

SEROTONERGIC INNERVATION OF VARIOUS SUBAREAS OF THE mPFC IS MODULATED BY 5-HTT EXPRESSION DURING DEVELOPMENT

The ability of 5-HTT to regulate 5-HT levels during development (Buznikov et al., 2001; Narboux-Neme et al., 2008; Wiggins et al., 2012) together with the different directional responses of both raphe- as well as mPFC-derived projections raises the possibility that 5-HT and its signaling molecules can play an important role in axon guidance events navigating 5-HT axons to their forebrain targets. Therefore, we studied 5-HT innervation of the mPFC *in vivo* in 6 days old 5-HTT$^{-/-}$ pups (P6) and compared that to wild-type innervation. Coronal sections of P6 5-HTT$^{-/-}$ rats ($n = 3$) and wild-type littermate controls ($n = 4$) were stained for 5-HT to visualize raphe-derived projections and the length of the 5-HT innervation within the various subareas of the mPFC was measured. The length of the projections was quantified in 10 bins comprising the cerebral gray matter width (**Figure 5**). To better visualize 5-HT-positive fibers over the cerebral swatch containing the gray matter which included the deeper and most superficial cortical layers, we made camera lucida drawings (**Figures 5B–D**). In 5-HTT$^{-/-}$ rats compared to control littermates, the drawings showed a clear increase in the amount of prefrontal 5-HT innervation in all subareas (**Figures 5B–D**). The average 5-HT-positive fiber length in the 5-HTT$^{-/-}$ mPFC was higher as compared to the wild-type mPFC in every bin, except for bin 8 (IL) and bin 8 and 9 (PL), likely to represent layers II and III (**Figures 5B,C,E,F**). Remarkably, the cingulate mPFC showed a higher 5-HT fiber density in the deeper layers in absence of 5-HTT, whereas reduced levels were found in the more superficial layers (**Figures 5D,G**), suggesting another raphe-derived route of the developing 5-HT projections.

Overall, these results indicate a crucial developmental role for 5-HTT in the guidance of 5-HT projections to their targets in the mPFC.

ABSENCE OF 5-HTT DURING DEVELOPMENT ALTERS THE IDENTITY OF PREFRONTAL PROJECTION NEURONS

Cortical neurons migrate to the proper location within the cortical plate (CP) through cell-autonomous and non-autonomous mechanisms (Kolk et al., 2009; Manent et al., 2011; Molnar and Clowry, 2012). To assess whether 5-HTT, either directly or indirectly, influences the identity and/or migration of prefrontal cortical neurons, we performed immunocytochemistry for both 5-HT and Satb2, a marker for upper-layer neurons (Dobreva et al., 2006; Alcamo et al., 2008; Britanova et al., 2008). Although the total number of neurons within the different subareas of the mPFC did not differ, we observed a remarkable reduction of Satb2-positive cells in all subareas of the mPFC in 5-HTT$^{-/-}$

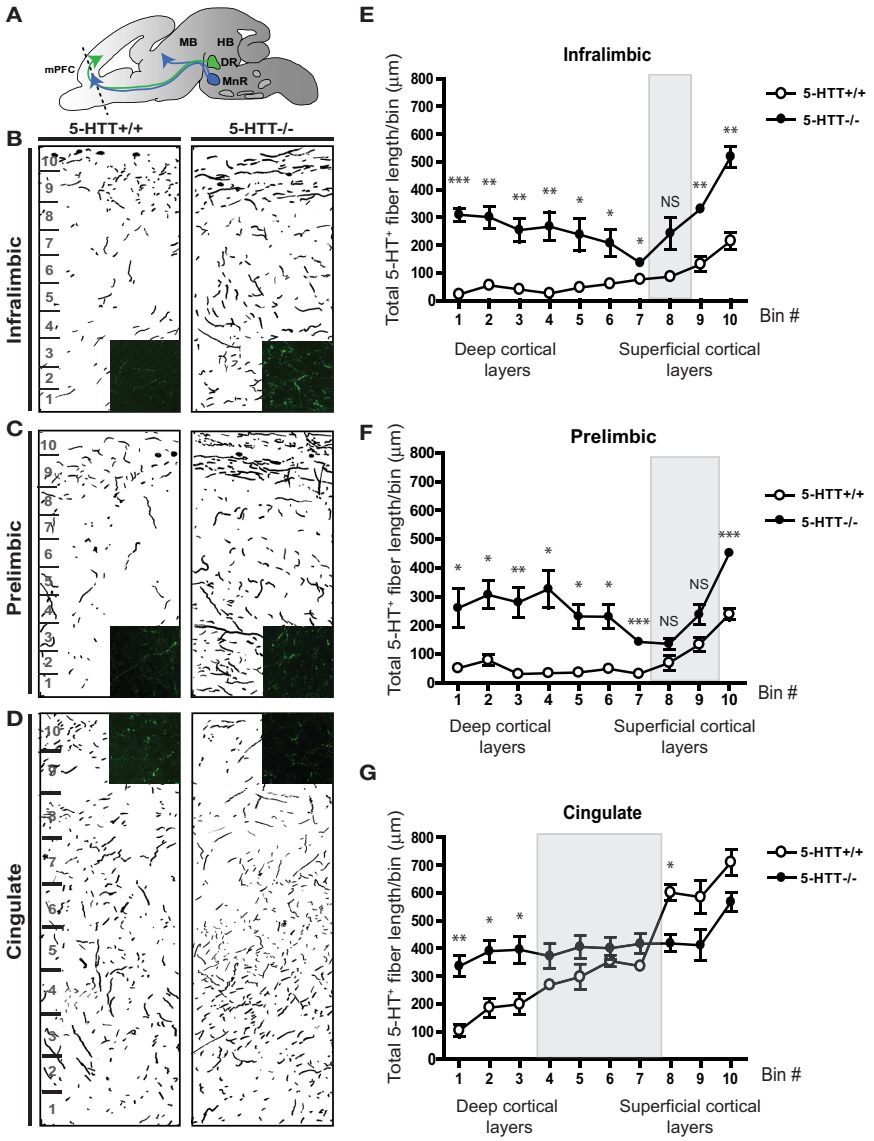

FIGURE 5 | Serotonergic innervation of the mPFC is increased in absence of the 5-HTT during development. (A) Schematic showing the level of coronal sectioning (dotted line) within the mPFC comprised of the Infralimbic (IL, **B**), Prelimbic (PL, **C**) and Cingulate cortex (Cg, **D**). **(B)** Camera lucida drawings of 5-HT-positive fibers within the IL of 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mPFC. Inset shows 5-HT fiber density (green) in the deeper layers of the mPFC. **(C)** Camera lucida drawings of 5-HT-positive fibers within the PL of 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mPFC. Inset shows 5-HT fiber density (green) in the deeper layers of the mPFC. **(D)** Camera lucida drawings of 5-HT-positive fibers within the Cg of 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mPFC. Inset shows 5-HT fiber density (green) in the superficial layers of the mPFC. **(E)** Quantification of the total 5-HT fiber length in μm in 10 bins dividing the IL as indicated in **(B)** confirming the qualitative observations as seen in **(B)**. **(F)** Quantification of the total 5-HT fiber length in μm in 10 bins dividing the PL as indicated in **(C)** confirming the qualitative observations as seen in **(C)**. **(G)** Quantification of the total 5-HT fiber length in μm in 10 bins dividing the Cg as indicated in **(D)** confirming the qualitative observations as seen in **(D)**. There is a significant increase in the most deep cortical layers of the IL, PL as well as the Cg of 5-HTT$^{-/-}$ compared to 5-HTT$^{+/+}$ pups ($p < 0.05$–0.001). The gray boxes indicate the non-significant bins in the more superficial layers. Graphs show average length of 5-HT-positive fibers per bin ± SEM. One-Way ANOVA ($\alpha = 0.05$), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

animals ($n = 3$) as compared to their wild-type littermates ($n = 4$) (**Supplemental Figure 4**). Surprisingly, Satb2-positive cells were no longer homogeneously restricted to layers II-VI, where a small percentage was scattered over the cerebral swatch in 5-HTT$^{-/-}$ as compared to control mPFC (**Figures 6A–F**). Furthermore, it appeared that in the prelimbic but especially in the cingulate cortex of 5-HTT$^{-/-}$ animals the Satb2-positive cells were often positioned in patches (**Figures 6B,C**, arrowheads). It remains to be established whether this reduction of Satb2-positive cells in the mPFC of rats lacking 5-HTT is cell-autonomous or due to increased 5-HT innervation, or both.

In sum, these results indicate that appropriate 5-HTT levels during early brain development are important for proper maturation of the raphe-prefrontal projection system (**Figure 7**).

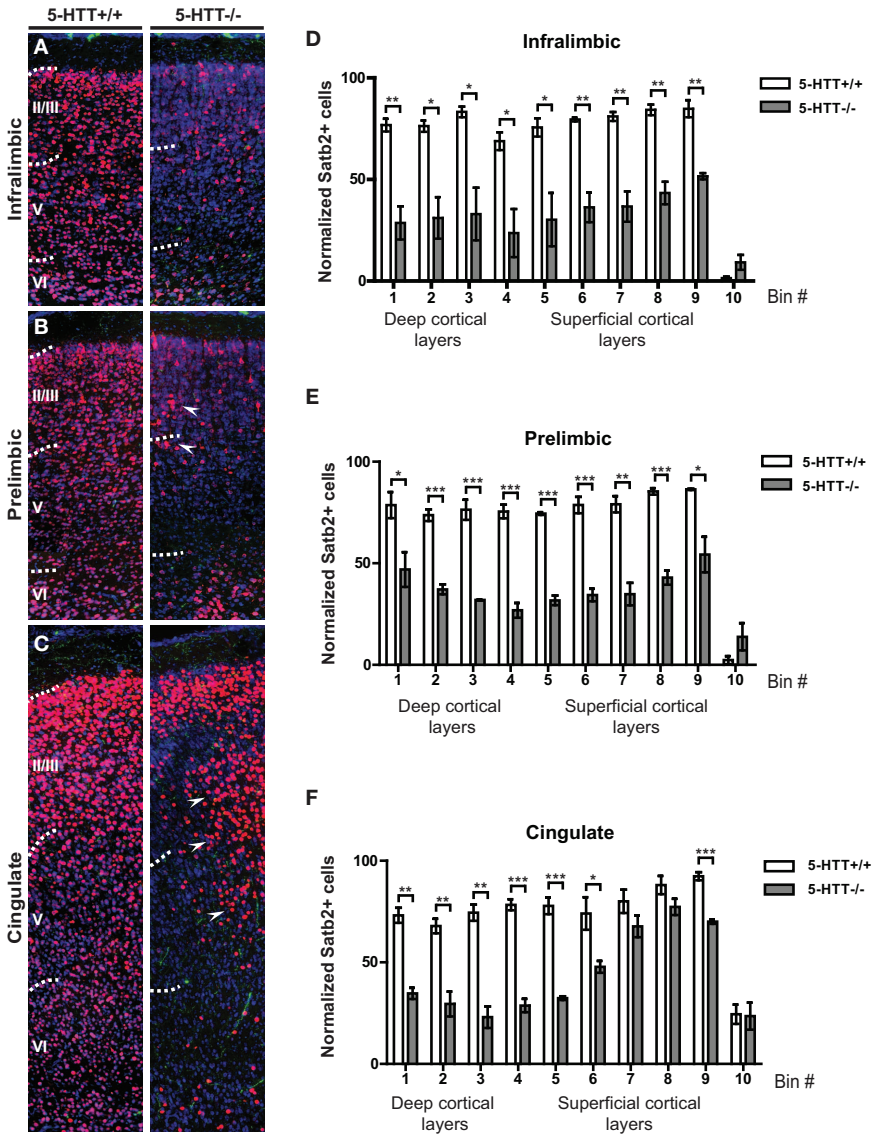

FIGURE 6 | Projection neuron identity is altered in absence of the 5-HTT during development. **(A)** Coronal sections immunostained for 5-HT (green), Satb2 (red) and counterstained with fluorescent Nissl (blue) of 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ IL showing the position of callosal projection neurons (presumptive layer II-V). **(B)** Coronal sections of the PL as in **(A)**. **(C)** Coronal sections of the Cg as in **(A)**. Arrowheads indicate clusters of misplaced neurons. **(D)** Quantification of the number of Satb2-positive cells in 10 bins dividing the IL confirming the qualitative observations as seen in **(A)**. **(E)** Quantification of the number of Satb2-positive cells in 10 bins dividing the PL confirming the qualitative observations as seen in **(B)**. **(F)** Quantification of the number of Satb2-positive cells in 10 bins dividing the Cg confirming the qualitative observations as seen in **(C)**. There is a significant decrease in the number of Satb2-positive cells in the IL, PL, and Cg of 5-HTT$^{-/-}$ compared to 5-HTT$^{+/+}$ ($p < 0.05$–0.001). Graphs show average number of Satb2-positive cells per bin ± SEM. One-Way ANOVA ($\alpha = 0.05$), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

DISCUSSION

Our data show that the 5-HTT knockout rat represents an excellent model to investigate the role of 5-HTT in the development of the rostral raphe-prefrontal network formation. The present study evaluates the trophic nature of the interaction between the origin (rostral raphe cluster) and a target (mPFC) of the 5-HT projection system and how this interaction is modulated by the lack of the 5-HTT during development. Furthermore, we observed the ability of outgrowing neurites originating from the DR or MnR and mPFC to form fascicles, and once formed, we quantified the number, length, and width of the fascicles to evaluate the effect of 5-HTT expression during outgrowth. The nature of the interaction appears to depend on (1) the origin of 5-HT-positive projections within the rostral raphe cluster and (2) the presence of 5-HTT during development. In wild-type explants, the 5-HT fibers of the DR have a slightly attractive interaction with the mPFC, although not significant, while the 5-HT neurites of the MnR are repulsed by the mPFC. The most striking finding of this study shows that the 5-HT projections from the MnR become strongly attracted by the mPFC instead of being

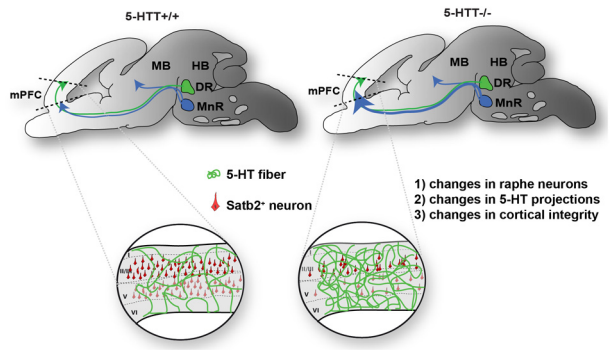

FIGURE 7 | Proposed model of the effect of 5-HTT deficiency on DR and MnR serotonergic neurite outgrowth during development. Cg, cingulate cortex; DR, dorsal raphe nucleus; HB, hindbrain; IL, infralimbic; MB, midbrain; MnR, median raphe nucleus; PL, prelimbic.

repelled in the absence of 5-HTT during development. *In vivo*, this is paralleled by the fact that in the 5-HTT$^{-/-}$ mPFC the 5-HT innervation was significantly increased as compared to the wild-type situation. In addition we show that the number of Satb2-positive callosal projection neurons is reduced in absence of the 5-HTT. Together, these results lead us to hypothesize, as depicted in **Figure 7**, that due to lack of 5-HTT throughout development (1) the characteristics of the raphe neurons might have changed, (2) raphe neurons can have an altered guidance of their outgrowing neurites as well as (3) the identity of the neurons within the mPFC, which send out projections in their turn, may have changed.

THE ROLE OF 5-HTT IN THE DEVELOPMENT OF THE RAPHE NUCLEI

The 5-HTT is expressed in serotonergic neurons of the rostral raphe cluster as early as E11.5 (mouse) and E12.5 (rat) but also in non-serotonergic fibers such as thalamocortical projections (Schroeter and Blakely, 1996; Bengel et al., 1997; Bruning and Liangos, 1997; Bruning et al., 1997; Hansson et al., 1998; Zhou et al., 2000; Galineau et al., 2004; Narboux-Neme et al., 2008; Daws and Gould, 2011). One possibility to explain the observed results is that the characteristics of the 5-HT neurons within the rostral raphe have changed because of altered 5-HTT expression. Indeed, in absence of the 5-HTT, the DR neurons are fewer in number which could have an effect on the organization of the MnR and its projections (Lira et al., 2003). The 5-HT$_{1A}$ of the DR neurons shows furthermore a marked desensitization when 5-HTT is lacking which could lead to functional consequences for the areas these DR axons innervate (Li et al., 2000; Mannoury La Cour et al., 2001; Holmes et al., 2003; Bose et al., 2011).

Three-dimensional collagen explant assays, are an excellent way to study interactions between areas within a particular network (Bonnin et al., 2007; Bozkurt et al., 2007; Kolk et al., 2009; Schmidt et al., 2012). When cultured alone, the various explants of the raphe and mPFC showed radial growth, indicating optimal growth conditions for the outgrowing neurites. Furthermore, we found specific and consistent outgrowth read-outs demonstrating the validity of the assay. Yet, there are several points of discussion when using sensitive *in vitro* assays like the explant assay. Although it was not measured in this particular study, we can assume that extracellular levels of 5-HT were elevated when the 5-HTT was absent (Gaspar et al., 2003; Riccio et al., 2009, 2011; Haenisch and Bonisch, 2011; Van Kleef et al., 2012). Even though the extrasynaptic concentration of 5-HT can reach the millimolar range (Bruns et al., 2000), the concentration and/or clearance of the released 5-HT within the medium was not measured over time in this experiment which could have had an effect on the observed results. In addition, the fact that a reduced 5-HT reuptake results in an increased 5-HT synthesis has been well documented (Kim et al., 2005; Haenisch and Bonisch, 2011). Although raphe explants from 5-HTT$^{-/-}$ /5-HTT$^{+/-}$ animals showed 5-HT-positive outgrowing neurites, it is to be investigated how much 5-HT, or other soluble cues, is actually secreted by these neurons *in vitro*.

AXONAL GUIDANCE OF SEROTONERGIC PROJECTIONS

The 5-HT projection system is one of the earliest neurotransmitter systems to innervate the brain, but the last to innervate the hippocampus and the cortex (Wilson and Molliver, 1991). During development, 5-HT may complete the maturation of a variety of neuronal projections systems, including its own, and the progression of interneuronal contacts (Lidov and Molliver, 1982; Daubert and Condron, 2010; Puig and Gulledge, 2011). The 5-HT neurons of the DR and MnR are known to innervate various subareas of the cortex including the mPFC (Bennett-Clarke et al., 1991; Del Cid-Pellitero and Garzon, 2011; Waselus et al., 2011; Celada et al., 2013; Chandler et al., 2013). While it cannot be excluded that the 5-HT raphe neurons are functionally different due to lack of 5-HTT, guidance of these and other (e.g., thalamocortical) projections might have been affected as well in absence of 5-HTT during development of the raphe-prefrontal network.

The observed interaction of the DR when co-cultured with the mPFC being neutral was unexpected since the DR is known to strongly innervate the cortical regions including the mPFC (Wilson and Molliver, 1991; Puig and Gulledge, 2011). Lack of 5-HTT did not affect the chemotropic nature of this interaction. This may indicate that another target of the 5-HT fibers lies beyond the mPFC and may encounter the mPFC as an intermediate target or needs an older mPFC to become attracted. Another possibility can be that the growing 5-HT projections need intermediate targets along its projection (e.g., thalamic regions) which is first encountered before projecting toward the cortical areas. An interesting question would be to evaluate whether the responses of the outgrowing neurites would change when E16.5 raphe tissue would be co-cultured with older mPFC explants or with other (intermediate) targets such as thalamus or hippocampus.

Interestingly, the interaction of the 5-HT fibers of the MnR toward the mPFC switched from a fairly strong repulsive to an attractive interaction in the absence of 5-HTT. *In vivo*, this could result in an increased innervation of the mPFC by the MnR. MnR-derived varicose M-fibers, which are believed to not express 5-HTT (Amilhon et al., 2010), mainly target layers II and III in the frontal parts of the cortex (Hensler et al., 2007), layers where we found the smallest differences in 5-HT innervation. In

order to exclude the possibility that the switch of MnR neurites to attraction is due to an altered mPFC, it would be of interest to culture wild-type cortex together with DR/MnR from 5-HTT$^{+/-}$ or 5-HTT$^{-/-}$ animals.

Furthermore, 5-HT has been shown to have a modulatory effect on outgrowing axons by affecting their response to classical guidance cues such as netrin-1 (Bonnin et al., 2007). This means that in absence of the 5-HTT during development, the responses of the outgrowing neurites from either the raphe and/or the mPFC to guidance molecules may have altered. This would furthermore explain the *in vivo* results in that the 5-HT projections coming from the MnR are highly attracted by the mPFC when 5-HTT is lacking during development. It is plausible that because of the diminished expression of Satb2 (and maybe other transcription factors as well such as Ctip2), the expression of guidance cues is influenced. Indeed, it has been shown that the expression of a variety of axonal guidance molecules is amended in the absent expression of Satb2 (Alcamo et al., 2008). It would therefore be of interest to look for aberrant guidance cue expression in the 5-HTT$^{-/-}$ animals and co-culture either the various subareas of the rostral raphe cluster, intermediate targets or the mPFC with HEK cells secreting an axonal guidance cue and to see whether the responsiveness of the outgrowing neurites would change in the absence of 5-HTT and/or could be modulated by 5-HT.

It has been shown that 5-HT plays a major role in the plasticity of the various other projection systems by modulating the guidance of their projections (Martin-Ruiz et al., 2001; Cunningham et al., 2005; Zhong et al., 2008). Since the overall outgrowth of neurites from the DR and MnR is affected by a (partial) lack of the 5-HTT, neurotransmitter systems other than the 5-HT system may be affected as well. Future experiments with 5-HTT$^{-/-}$ three-dimensional explants of nuclei from other neurotransmitter systems such as the dopaminergic or noradrenergic system and their targets may give insight into such additional modulatory actions of 5-HT during development. While the total (Tuj1-positive) raphe and mPFC neurite outgrowth in the wild-type and 5-HTT$^{-/-}$ situation appeared to be attractive by nature, this interaction becomes repulsive in the heterozygous animal. Perhaps, *in vitro*, either too high or too low concentrations of 5-HT permit an attractive response of both raphe as well as mPFC outgrowing neurites due to counteracting effects of different subtypes of 5-HT receptors expressed by the respective neurons (Celada et al., 2001; Martin-Ruiz et al., 2001; Ferezou et al., 2002; Santana et al., 2004; David et al., 2005; Alexandre et al., 2006; Riccio et al., 2009; Puig et al., 2010; Vucurovic et al., 2010), while intermediate 5-HT levels might elicit effects only via the more sensitive of the receptors producing a repulsive interaction. These results are intriguing considering that the s-allele carriers of the human 5-HTT polymorphism have a reduction in the amount of 5-HTT but never a complete lack (Lesch et al., 1996; Champoux et al., 2002; Pezawas et al., 2005; Homberg and Lesch, 2011).

THE FUNCTION OF 5-HTT IN CORTICAL INTEGRITY

A distant target of the ascending 5-HT projection system is the mPFC (D'Amato et al., 1987), which is involved in working memory and behavioral flexibility (Miller and Cohen, 2001) and is also one of the last brain areas to mature (Molnar and Clowry, 2012). The 5-HTT is the primary regulator of the 5-HT signal and may therefore affect the role of 5-HT in correct development of the brain. The presence of 5-HTT itself within the developing mPFC already starts from E14.5 onwards with 5-HTT-positive cells and later fibers innervating the mPFC in two parallel paths contacting many other cells (e.g., Cajal-Retzius cells) important for its correct development and layering (Lebrand et al., 1998; Zhou et al., 2000). Lack of 5-HTT during development could therefore have profound effects on the integrity of the mPFC itself.

The transcription factor Satb2 (special AT-rich sequence binding protein 2) is a marker exclusively expressed by callosal projection neurons from E13.5 onwards and Satb2-positive cells reside in cortical layers II-V and in subsets of neurons in layer VI (Dobreva et al., 2006; Alcamo et al., 2008; Britanova et al., 2008; Zhang et al., 2012). Satb2-positive callosal projection neurons were also present in the mPFC in which they most likely need selective 5-HT excitation to communicate (Avesar and Gulledge, 2012). In absence of the 5-HT transporter, the number of Satb2-positive neurons in the mPFC was decreased in all cortical layers, suggesting an altered identity of a set of prefrontal callosal projection neurons. Interestingly, there are indications that SSRIs given for the treatment of depression have an effect on the anatomy of the corpus callosum (Djavadian et al., 2003; Reyes-Haro et al., 2003; Simpson et al., 2011). In absence of Satb2, many downstream targets (e.g., guidance cues) are either up- or down-regulated in expression which can have an effect on the identity of a class of mPFC neurons themselves and/or have an effect on incoming projection systems (Alcamo et al., 2008). Since the 5-HT fibers are thought to contact the Cajal-Retzius (CR) cells in the marginal zone (presumptive cortical layer I) of the mPFC [(Janusonis et al., 2004; Chameau et al., 2009) and unpublished data], an increase in axonal innervation could result in an altered 5-HT signal onto the CR cells. The subsequent altered reelin release could have profound effects on the cortical layering resulting in a modified mPFC-mediated behavioral control. Therefore, we cannot rule out the possibility that during development, the migration of various classes of cortical projection neurons is also affected. To verify whether the callosal trajectory and/or identity of other projection neurons is affected in the absence of 5-HTT further experiments using various layer- and cell type-specific markers combined with electrophysiolological recordings are needed.

The excessive 5-HT fibers seen in the 5-HTT$^{-/-}$ cortical plate can also have an effect on the maturity and dendritic arborization of the pyramidal neurons it harbors (**Figure 6** and Miceli et al., 2013). Therefore it is likely that subsets of mPFC projections neurons have an altered identity due to altered expression levels of transcription factors (such as Satb2) in absence of 5-HTT during development. This can have consequences for later innervations of either serotonergic or other transmitter projections because of an altered permissive environment of the mPFC.

During the formation of long projections systems, scaffolds are formed by pioneer axons. The following axons can now trail these scaffolds toward their proper targets and associate together

in fascicles (Van Vactor, 1998; Jaworski and Tessier-Lavigne, 2012). This is modulated by intrinsic membrane-bound cues and extrinsic diffusible cues. The number of mPFC explants of which neurites had formed fascicles was affected in the 5-HTT$^{-/-}$ animals. This could indicate that the altered 5-HT signal influences the response of the mPFC neurites to the intrinsic membrane-bound and soluble cues influencing fasciculation. Additionally, the altered 5-HT signal could alter the actual cues, and thereby influence fasciculation (Petit et al., 2005). Altered fasciculation in the brain could have profound effects. For example in the case of defasciculation, fibers would be less densely packed and spread out over a larger area, with possible projections into surrounding areas that normally are not innervated. For future experiments, co-culturing mPFC of the different genotypes with wild-type raphe would shed some light on the role of an altered mPFC due to absence of 5-HTT in the development of the raphe-prefrontal network.

Because of the altered developmental 5-HT levels, one may wonder whether the expression of particular 5-HT receptors within the mPFC is altered (Li et al., 2003; Chameau et al., 2009; Riccio et al., 2009; Vucurovic et al., 2010), including 5-HT$_{1A}$ and 5-HT$_{3A}$ that are expressed by the CR cells themselves (Janusonis et al., 2004). The prefrontal pyramidal neurons express 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_6$ receptors (Celada et al., 2001; Martin-Ruiz et al., 2001; Santana et al., 2004; Riccio et al., 2009) and by changing the level of extracellular 5-HT, the conveyed signals differ and can result in an altered cell identity. The prefrontal neurons can show altered characteristics because of altered expression of 5-HTT in the mPFC itself (cell autonomous effect) or, due to the increased 5-HT innervation in the mPFC of 5-HTT$^{-/-}$ animals (non-autonomous effect) this can lead to differences in cortical identity. For example, the amount of brain-derived-neurotrophic factor (BDNF) is dramatically decreased in the mPFC of animals lacking the 5-HTT (Mallo et al., 2008; Molteni et al., 2010), thereby perhaps changing the permissiveness of the mPFC environment to incoming projections. Therefore, experiments involving manipulations of 5-HT levels in the culture medium of the explants using receptor agonists (e.g., Flesinoxan or mCPBG), antagonists (eg WAY 100635 or tropisetron) or 5-HT itself, may provide additional information about the role of the 5-HT signal and the regulation hereof by the 5-HTT on the 5-HT neurite targeting and interaction with the mPFC (Ferezou et al., 2002; Alexandre et al., 2006; Chameau et al., 2009).

In sum, we conclude that the 5-HT projections arising from the rostral cluster of raphe and which innervate the mPFC seem to depend on the presence of 5-HTT during development. These results indicate that appropriate 5-HTT levels are required for proper 5-HT guidance, fasciculation, and innervation of the mPFC and that appropriate 5-HTT levels are important for proper development of the raphe-prefrontal projection system (**Figure 7**). Nevertheless, decoding the molecular program of 5-HT neurons and their projections toward the mPFC is a challenging task, and additional research is necessary. Given that 5-HTT$^{+/-}$ animal models share many behavioral aspects with those seen in human 5-HTTLPR s-allele carriers, our data may help to understand the neurodevelopmental foundations of 5-HT associated behavioral phenotypes.

ACKNOWLEDGMENTS

This work was supported by the Dutch Organization of Scientific Research (NWO grant # 433-09-311) awarded to J. Homberg. We thank Stephanie Miceli and the reviewers for critically reading this manuscript and members of the Martens, Kolk, and Homberg labs for helpful editing and discussions. We thank the NCMLS microscopy platform (http://ncmls.nl/technology-platform/microscope-imaging-centre/) for excellent support and maintenance of the equipment.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Cellular_Neuroscience/10.3389/fncel.2013.00143/abstract

Supplemental Figure 1 | Explant assays as a tool to study raphe-prefrontal network formation *in vitro*. **(A)** Schematic representation showing the explant microdissection of the subareas of the rostral raphe cluster and the mPFC. The rostral raphe cluster was divided in a rostral (R), intermediate (I) and caudal (C) subarea and duplicated across the midline. Explants in a collagen hill were co-cultured at approximately 300 μm distance from each other. **(B)** Dorsal view of the dorsal raphe (DR) in a large explant showing 5-HT-positive neurons (green). **(B')** Enlargement of the boxed area in **(B)** showing the midline (dashed line) and individual 5-HT neurons sending out their projections. **(C)** Coronal cryosection showing 5-HT-positive neurons (green) in both the DR and MnR costained with Satb2 (red) and counterstained with fluorescent Nissl (blue). **(D)** DR explant stained for 5-HT (green) and Tuj1 (red) showing healthy axonal growth cones (asterisk and arrowheads).

Supplemental Figure 2 | Number of fascicles from the mPFC is moderated by the presence of 5-HTT during development. **(A)** Fascicles coming from the mPFC and immunostained for Tuj1 (β-III tubulin) with a minimum width of 5 μm (arrows) the length and width were measured. Scale bar represents 50 μm. **(B)** Enlargement of the boxed area in **(A)**. To measure the length and width, we traced along and across the fascicle. **(C–E)** The percentage of explants with fascicles measured in the proximal quadrant per subarea and per genotype. **(F)** Examples of the proximal quadrant of the rostral subarea (DR) of the genotypes (5-HTT$^{+/+}$, 5-HTT$^{+/-}$and 5-HTT$^{-/-}$) immunostained for 5-HT (green). No fascicles were formed by 5-HT-positive neurites growing from the DR.

Supplemental Figure 3 | Width of fascicles from the mPFC is modulated by the presence of 5-HTT during development. **(A,B)** Examples of fascicles with a certain width in the distal quadrant of the MnR (caudal subarea) of wild-type and 5-HTT$^{-/-}$ animals. Scale bar represents 20 μm. **(C–E)** Quantification of fascicle width for the different subareas and genotypes. Graphs show average length of fascicles per explant ± SEM. One-Way ANOVA ($\alpha = 0.05$), $^{*}p < 0.05$.

Supplemental Figure 4 | Number of Satb2-positive neurons in the mPFC decrease in contrast to total number of cells in absence of 5-HTT during development. **(A)** Schematic representation of the various subareas of the mPFC as quantified in **(B)**. **(B)** Quantification of the total number of cells (Nissl-positive) and the Satb2-positive cells in the various subareas of the mPFC over a swatch of 100 μm in width. Cg, cingulate cortex; IL, infralimbic; PL, prelimbic. Graphs show average number of cells per cortical swatch ± SEM. One-Way ANOVA ($\alpha = 0.05$), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 March 2013; accepted: 15 August 2013; published online: 04 October 2013.

Citation: Witteveen JS, Middelman A, van Hulten JA, Martens GJM, Homberg JR and Kolk SM (2013) Lack of serotonin reuptake during brain development alters rostral raphe-prefrontal network formation. Front. Cell. Neurosci. ***7****:143. doi: 10.3389/fncel.2013.00143*

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 07 June 2013
doi: 10.3389/fncel.2013.00088

High serotonin levels during brain development alter the structural input-output connectivity of neural networks in the rat somatosensory layer IV

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Homeostatic regulation of serotonin (5-HT) concentration is critical for "normal" topographical organization and development of thalamocortical (TC) afferent circuits. Down-regulation of the serotonin transporter (SERT) and the consequent impaired reuptake of 5-HT at the synapse, results in a reduced terminal branching of developing TC afferents within the primary somatosensory cortex (S1). Despite the presence of multiple genetic models, the effect of high extracellular 5-HT levels on the structure and function of developing intracortical neural networks is far from being understood. Here, using juvenile SERT knockout ($SERT^{-/-}$) rats we investigated, *in vitro*, the effect of increased 5-HT levels on the structural organization of (i) the TC projections of the ventroposteromedial thalamic nucleus toward S1, (ii) the general barrel-field pattern, and (iii) the electrophysiological and morphological properties of the excitatory cell population in layer IV of S1 [spiny stellate (SpSt) and pyramidal cells]. Our results confirmed previous findings that high levels of 5-HT during development lead to a reduction of the topographical precision of TCA projections toward the barrel cortex. Also, the barrel pattern was altered but not abolished in $SERT^{-/-}$ rats. In layer IV, both excitatory SpSt and pyramidal cells showed a significantly reduced intracolumnar organization of their axonal projections. In addition, the layer IV SpSt cells gave rise to a prominent projection toward the infragranular layer Vb. Our findings point to a structural and functional reorganization of TCAs, as well as early stage intracortical microcircuitry, following the disruption of 5-HT reuptake during critical developmental periods. The increased projection pattern of the layer IV neurons suggests that the intracortical network changes are not limited to the main entry layer IV but may also affect the subsequent stages of the canonical circuits of the barrel cortex.

Keywords: serotonin, SERT, barrel cortex, somatosensory, columnar circuitry, pyramidal cell, spiny stellate cell, morphology

INTRODUCTION

Serotonin (5-hydroxytryptamine; 5-HT) modulates key processes of mammalian brain development (Gaspar et al., 2003; Daubert and Condron, 2011; van Kleef et al., 2012). Throughout critical periods of neural development, extracellular 5-HT homeostasis is maintained by the serotonin transporter (SERT), responsible for the reuptake of 5-HT at the synapse (Blakely et al., 1991). SERT function can be modulated by the common 5-HTTLPR polymorphism in humans (Lesch et al., 1996; Champoux et al., 2002) or by a mutation of the SLC6SA4 gene in rodents (Olivier et al., 2008) which both leads to a general increase in extracellular 5-HT brain levels. Elevated 5-HT levels during critical developmental stages have been associated with changes in cognitive function, emotional processing as well as sensory perception (Canli and Lesch, 2007; Homberg et al., 2010). Previous studies in rodent models have shown that important structural changes in the neural circuitry underlie the observed behavioral phenotypes that result from a disrupted 5-HT homeostasis (Persico et al., 2001; Salichon et al., 2001; Esaki et al., 2005; Canli and Lesch, 2007; Wellman et al., 2007; Lee, 2009; Riccio et al., 2011).

Perceiving and correctly interpreting sensory information in the mature brain requires a high degree of precision in the topographical organization of the sensory pathways. Because of the strong topographic relationship of the whisker receptive fields mapped onto layer IV barrels (Woolsey and Van der Loos, 1970; Diamond, 1995), the rodent primary somatosensory cortex (S1) represents a unique structure for the study of cortical development. Following whisker deflection, the spatio-temporal information concerning the stimulus is transmitted along the lemniscal pathway via the brainstem and thalamus before reaching S1. During neural development, afferent axonal projections from neurons in the ventroposteromedial nucleus of the thalamus (VPM) grow by relying on the temporal expression of a series of guidance cues eventually resulting in a topographic innervation of the input layers of S1 (López-Bendito and Molnár, 2003; Bonnin et al., 2007). The development of primary sensory cortices

is also strongly dependent on a tight regulation of extracellular 5-HT concentrations regulated by SERT, transiently expressed on growing thalamocortical afferents (TCAs) from E11 to P7 in rats (Bennett-Clarke et al., 1996; Lebrand et al., 1996, 1998; Rebsam et al., 2002; Gaspar et al., 2003). Increasing extracellular 5-HT levels, either by blocking its reuptake or its degradation, have been shown to cause a deregulation of guidance cues resulting in a reduction of axon terminals innervating S1 (Cases et al., 1996; Salichon et al., 2001; Lee, 2009). Also, presynaptic glutamatergic release from the TCAs instructs the neuronal clustering of postsynaptic excitatory cells in the typical barrel formation (Narboux-Neme et al., 2012). Serotonin (5-HT1-B) receptor is co-expressed on TCAs during development, and its activation has been shown to negatively regulate glutamatergic vesicle release at the TC synapse (Laurent et al., 2002). A reduced thalamic innervation accompanied with a decreased synaptic transmission has been shown to produce an alteration in the topography of the layer IV barrel field pattern (Cases et al., 1996; Narboux-Neme et al., 2012; van Kleef et al., 2012). The main excitatory target neurons of the TCA projections are the pyramidal and spiny stellate (SpSt) cells in layer IV, whose dendritic fields orient and cluster around the incoming thalamic input (Staiger et al., 1996; Datwani, 2002). Interestingly, Lee (2009) have demonstrated morphological changes in dendritic organization following a pharmacological increase of 5-HT. The efferent axonal projection from layer IV toward the associative supragranular layers differs greatly between both classes of excitatory cells (Staiger et al., 2004; Feldmeyer, 2012). Whereas SpSt cells are considered to be the neuron type that most distinctively reflects the columnar organization of S1, both on the structural and functional level by projecting within the home column (HC) and keeping information segregated, pyramidal cells project to neighboring columns (NCs) to allow integration of information between multiple columns (Schubert et al., 2003). Changes in the input-output microcircuitry of these layer IV neurons could considerably alter the intracortical processing of somatosensory information (Feldmeyer et al., 2013).

In order to study the effect of increased developmental 5-HT levels on both the afferent and efferent connectivity of the excitatory layer IV cell population, we used juvenile SERT knockout ($SERT^{-/-}$) rats, known to exhibit 9-fold increases in brain 5-HT levels (Homberg et al., 2007). We first performed a classical characterization of our model by examining the posteromedial barrel subfield (PMBSF) anatomy of S1 using a cytochrome oxidase staining. We then validated the effect of high 5-HT on the anatomical organization of TCAs by analyzing the morphology of thalamic afferents arising from the VPM nucleus to the input layer IV using *in vitro* biocytin tracing. Our main goal was to quantify the structural changes in the dendritic and axonal arborizations of pyramidal and SpSt cells and to further analyze their intrinsic electrophysiological properties using the whole cell patch clamp technique. A changed organization of the thalamic afferents, combined with elevated extracellular 5-HT levels could alter the cortical microcircuitry as well as the physiological mechanisms involved in the early intracortical signal processing of somatosensory information.

MATERIALS AND METHODS

ANIMALS

Experiments were performed on male juvenile (postnatal day 21–25) Wistar rats. $SERT^{-/-}$($Slc6a4^{1Hubr}$) rats were generated by ENU-induced mutagenesis (Smits et al., 2006). All animals were bred and reared in the Central Animal Laboratory of the Radboud University Nijmegen Medical Centre (Nijmegen, The Netherlands). Breeding animals were derived from outcrossing heterozygous ($SERT^{+/-}$) knockout rats for eight generations. Experimental animals were derived from homozygous breeding. We genotyped the animals routinely in order to confirm their genetic background. Animals were supplied with food and water *ad libitum* and were kept on a 12 h:12 h dark:light cycle (lights on at 0600 h). All experiments were approved by the Committee for Animal Experiments of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, and all efforts were made to minimize animal suffering and to reduce the number of animals used.

MORPHOLOGICAL ANALYSIS OF THE BARREL FIELD

Cytochrome oxidase staining and analysis

Animals were anesthetized with isofluorane and decapitated. The brains were extracted and cortices were dissected and placed between two glass slides compressed within a distance of 1.2 mm in a PFA 4% solution for 48 h. The glass was removed and the tissue was postfixed overnight before being transferred to PBS 0.1 M. The tissue was embedded in 2% agarose and cut at 60 μm with a vibratome (Leica). CO activity was revealed as described by Wong-Riley and Welt (1980). In brief, sections were incubated in phosphate buffer (0.1 M; pH 7.4) containing (mg/ml): 0.6 cytochrome *C*, 0.5 diaminobenzidine, 45 sucrose and 0.3% catalase for 4 h at 40 °C.

Morphometric quantification of the barrel field

The CO stained tissue including the PMBSF barrel field was photographed at 5× magnification and pictures were analyzed using NIH ImageJ software. The external contour of barrels was outlined and areas were measured. The width of the septa between adjacent barrels was defined by the length of the line segment between two barrel borders of a straight line connecting the two barrel centroids (**Figure 1**).

ELECTROPHYSIOLOGY AND THALAMOCORTICAL AXON TRACING

Acute thalamocortical (TC) slices from the rat S1 containing the pathway from the thalamus to the barrel cortex (Agmon and Connors, 1991) were used for both electrophysiology and TCA tracings. Following anesthesia and decapitation, brain tissue containing the barrel cortex was excised, quickly removed from the skull, and stored in ice-cold artificial cerebro-spinal fluid (ACSF) oxygenated with carbogen (95% O_2, 5% CO_2). ACSF consisted of (in mM): 124 NaCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 5 $MgCl_2$, 3 KCl, 10 glucose at pH 7.4. The hemispheres were separated and cut with a 55° angle from the midline according to the rat brain coordinates of Land and Kandler (2002). The tissue block containing the region of interest was glued to a chilled Vibratome platform (Microm HM 650 V, Microm, Germany) and slices (300 μm thickness for electrophysiology and 600 μm for

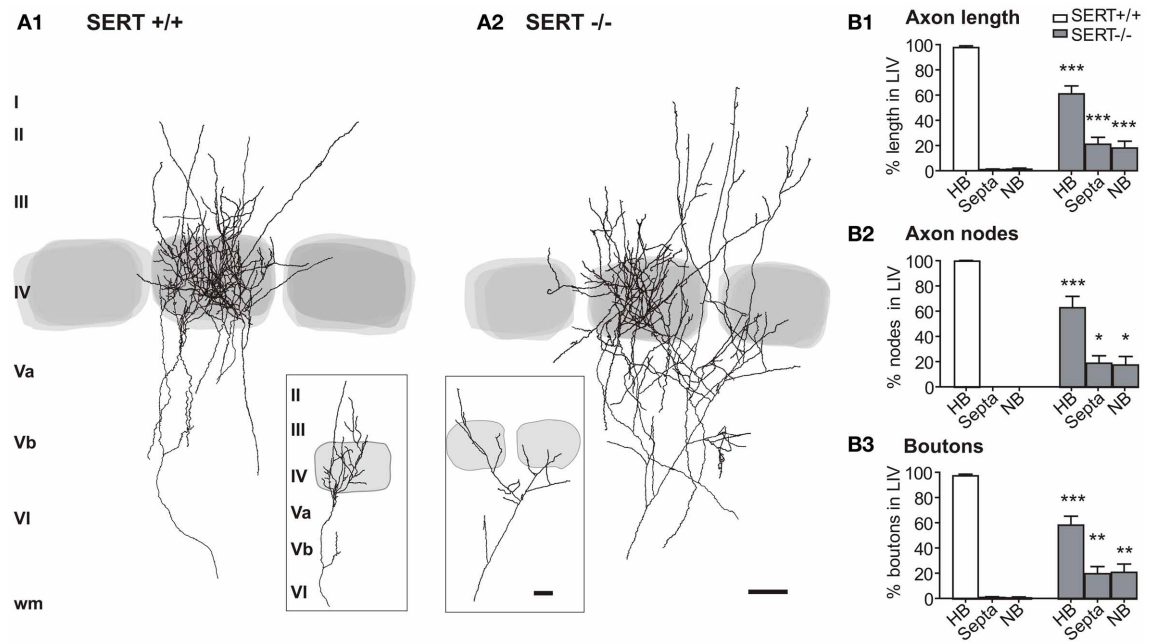

FIGURE 1 | Thalamocortical afferent projection pattern in the layer IV. Overlay of 5 reconstructions for the $SERT^{+/+}$ **(A1)** and $SERT^{-/-}$ **(A2)** rats, respectively, in reference to the barrel borders stained by cytochrome oxidase. Percentages are given for the total axon length **(B1)**, number of nodes **(B2)**, and number of boutons **(B3)** innervating the HB, S, and NB ($SERT^{+/+}$ $n = 11$; $SERT^{-/-}$ $n = 8$). Gray shaded areas indicate the position of the respective home and adjacent barrels. Data are mean ± SEM, asterisks mark significant differences between genotypes. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

TCA tracings) were cut. The slices were stored in an incubation chamber containing carbogenated ACSF at room temperature for at least 1 h, then transferred to the recording chamber and submerged in 32°C ACSF (Ca^{2+} 2 mM, Mg^{2+} 1.8 mM) at a flow rate of 1 ml/min.

Electrophysiology

Layer IV pyramidal cells were identified using an upright microscope (Axioskop FS, Carl Zeiss, Göttingen, Germany) fitted with 2.5× and 40× objectives. The barrel field was visualized at low magnification and the individual cells were selected within the barrels at high magnification using an infrared enhanced quarter-field illumination. Somatic whole-cell recordings were performed using borosilate glass pipettes with a tip resistance of 4–6 MΩ. Patch pipettes were filled with (in mM): 13 KCl, 117 K-gluconate, 10 K-HEPES, 2 Na_2ATP, 0.5 NaGTP, 1 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, and 1% biocytin. Membrane capacitance and series resistance were not compensated. Cells were selected at a minimum depth of −60 μm to retain the maximum network and minimize cutting artifacts. Each cell was characterized for its resting membrane potential and passive and active intrinsic membrane properties by injection of a series of depolarizing pulses until reaching action potential (AP) firing. Electrophysiological data was not corrected for a liquid junction potential of ca. −10 mV.

Signal acquisition and analysis

The signals were amplified (SEC-05L; npi-electronics, Tamm, Germany), filtered at 3 kHz, and digitized using an ITC-16 interface (Instrutech, Great Neck, NY). Data were recorded, stored, and analyzed with PC-based software (TIDA 4.1 for Windows; Heka Electronik, Lambrecht, Germany). After recording, the slices were photographed in the bath chamber to document the topography of barrel-related columns and laminae as well as the respective position of the patch electrode. The slices were then fixed in 4% buffered paraformaldehyde and stored at 4°C overnight.

TRACING OF THALAMOCORTICAL AXONS

Acute TC brain slices (600 μm thickness) were obtained in the same manner as for the electrophysiology slice preparation. Following the vibratome slicing, brain slices were positioned on an interface chamber (Harvard Apparatus) superfused with ACSF and maintained at 32°C. A maximum of 2 acute slices per hemisphere were used in order to preserve the complete VPM to S1 axonal pathway. The VPM nucleus of the thalamus was identified under binocular magnification and a biocytin crystal (Sigma) was positioned at the center. The slices were maintained in the chamber for a period of 6 h allowing active transport of the biocytin along the afferents (King et al., 1989). The slices were then fixed overnight in a 4% PFA solution and cryoprotected in 30% sucrose solution before cryostat reslicing to 300 μm thickness. The tissue was processed for ABC-DAB staining [adapted from Staiger et al. (2004)]. In short, slices were permeated with Triton-X (0.5%), endogenous peroxidase activity was blocked by 1 h incubation with H_2O_2 (1%) and the slices were incubated with ABC (1:400; Vector Laboratories, Burlingame, CA) for 48 h at 4°C. Peroxidase was revealed by incubation with DAB + H_2O_2, and the staining was intensified by brief (1–8 min) incubation

with $AgNO_3$, followed by 10 min AuCl for enhanced photoresistance. The sections were counter-stained for CO histochemistry to visualize the TCA position within the barrelfield using a protocol as described in section Morphological Analysis of the Barrel Field.

TCA AND SINGLE CELL *MORPHOMETRIC ANALYSIS*

Following the staining procedure, the brain slices containing labeled TCAs and single layer IV neurons, respectively, were analyzed using an upright brightfield microscope (Zeiss AxioImager A1) connected to the computerized reconstruction system Neurolucida (software Vers. 10, Microbrightfield Europe, Germany). For 3-dimensional reconstruction of both, TCA and single excitatory neurons in the layer IV, we used a 40× objective (Zeiss EC Plan-Neofluar, numerical aperture 0.75. The quantitative morphometrical analysis was performed by using NeuroExplorer software (software Vers. 10, Microbrightfield Europe, Germany). In order to allow layer and column specific quantitative analysis, we compartmentalized the tissue in terms of barrel/septum associated columns and the different layers under low magnification (2.5× or 5×). The borders and positions of the layer IV barrels was done based on the picture photographs of the respective acute slice preparation.

For the analysis of single neuron somatodendritic morphometric properties, we determined the largest size of the soma, the vertical and horizontal span of the dendritic field, the number of dendrites, the number of dendritic ends, the total dendritic length, the number of nodes, and the covered surface (calculated by a 2-dimensional convex hull estimation of the structure perimeter). For the axonal morphometric properties of TCAs and single neurons, we quantified the total length, the number of endings and nodes, the covered surface as well as the number and density of boutons. The boutons were identified as a distinct, point like swelling of minimum 2× the local axon thickness. The data were not corrected for the histochemistry related tissue shrinkage.

For the reconstruction of TCAs, we selected slices that contained distinguishable terminal clusters arising from a moderate amount of individual axon projections in the cortical layersVI/Vb. For single neuron reconstructions, we used the somatodendritic morphology of the biocytin stained neurons to discriminate between the two main classes of excitatory neurons in S1 layer IV, SpSt, and pyramidal neurons (Pyr). Our sample of somatodendritically reconstructed pyramidal neurons also contained a small number of star pyramidal cells which showed the typical non-skirt like organization of basal dendrites (2 out of 12 $SERT^{+/+}$; 2 out of 15 $SERT^{-/-}$). With no reports showing a cell class specific difference in the functional properties of these two cell types (Schubert et al., 2003), we pooled star pyramidal and pyramidal neurons. The axonally reconstructed neurons contained pyramidal neurons only. The low prevalence of star pyramidal cells in our sample can possibly be explained by a bias toward the triangular shaped somata during visual pre-selection for electrophysiological recording. The morphometric statistical analysis was carried out in SPSS (SPSS Inc., Chicago, IL).

STATISTICAL ANALYSIS

All data were acquired blindly and tested for normal distribution using a Shapiro–Wilk test. For the electrophysiological analyses, we used a multivariate analysis (MANOVA) to test for statistically significant differences between the two genotypes and a discriminant analysis to test for differences on the cellular population level. For the anatomical analyses, we performed a Two-Way ANOVA followed by a Bonferroni correction for *post-hoc* pairwise comparisons. All values are given as mean ± standard error of mean (SEM).

RESULTS

We investigated the effect of high 5-HT levels on the development of the afferent projections from the thalamus to the input layer of S1 in juvenile (P21-P25) $SERT^{-/-}$ rats, compared to $SERT^{+/+}$ rats with "normal" brain 5-HT levels. To this end, we first characterized the morphological properties of the terminal axonal projections of VPM neurons to the layer IV of S1. We then investigated the overall barrel field organization as well as the morphological and intrinsic functional properties of the main excitatory target neurons of VPM axons in the layer IV.

BLOCKING 5-HT REUPTAKE DURING DEVELOPMENT ALTERS THALAMOCORTICAL PROJECTIONS AND ORGANIZATION OF THE BARREL FIELD IN S1 CORTEX

Reduced "home" barrel specific innervation by TCAs in $SERT^{-/-}$ rats

The typical somatotopic axon patterning in S1 consists of segregated thalamic afferents synapsing onto the layer IV cell clusters of the barrels. In the "normally" developed rat somatosensory system, single TC afferents of the VPM predominantly form their terminal clusters in one individual barrel of the cortical layer IV (Jensen and Killackey, 1987; Diamond, 1995; Bureau et al., 2006; Meyer et al., 2010; Oberlaender et al., 2012a,b) and its respective barrel associated cortical column. In $SERT^{-/-}$ mice this topographical organization has been reported to be impaired (Persico et al., 2001). To evaluate the thalamic afferent distortion in our $SERT^{-/-}$ rat model, we evaluated differences between the organization of terminal projections of individual TCAs of $SERT^{+/+}$ ($n = 11$) and $SERT^{-/-}$ rats ($n = 8$) by means of their (i) total axon length found above the level of cortical layer Vb, (ii) their level of arborization, and (iii) the number of presynaptic boutons within the layer IV. Furthermore, we quantified the lateral axonal extensions in reference to the home barrel (HB), septa and neighboring barrels.

In both $SERT^{+/+}$ and $SERT^{-/-}$ rats, the VPM projections revealed distinguishable terminal axon clusters in S1 layer IV which were aligned with the CO staining of the large barrels. Within S1, between layers Vb and Va, TCAs gave rise to several side collaterals and continued toward layer IV where they formed extensively arborized axonal clusters. We classified the barrel that was vertically aligned with the arising TCA in the deeper infragranular layers as the HB. Besides the rich innervation of layer IV, in both genotypes the collaterals often extended into the supragranular layers II/III.

A striking difference between $SERT^{+/+}$ and $SERT^{-/-}$ rats was apparent with TCAs of the $SERT^{-/-}$ rats giving rise to

fewer axon collaterals within the HB Layer IV. Furthermore, the terminal fields were more widespread laterally and less confined to the HC (**Figures 1A,B**). Superimposing several of the reconstructed TCAs with respect to the position of their HB still revealed a dense and distinguishable cluster of axons within the HB and a more diffuse innervation of the septa and NC. For a more detailed study of TCAs, we performed a quantitative morphometric analysis of axonal parameters (axonal length, the number of nodes, and number of endings, bouton number and density; **Table 1**) in reference to the relevant compartments of the barrel field (HB, septa and NB). The mean bouton densities in the present study are comparable to previous findings (Lu and Lin, 1993) and in both genotypes, the boutons were evenly distributed over the axon branches within layer IV.

Axon length, nodes, and relative bouton distribution across the 3 compartments (HB, S, and NB) were significantly different for both genotypes ($p < 0.0001$; Two-Way ANOVA; **Figure 1B**). While in $SERT^{+/+}$ rats between 89.9 and 100% of the TC axon was restricted to the HB ($97.75 \pm 1.41\%$), $SERT^{-/-}$ rats showed only $60.91 \pm 6.4\%$ in the HB with an increased lateral innervation of the septa and the neighboring barrel ($p = 0.001$; **Figure 1B1**). In the latter, only 1 out of the 8 reconstructed TCAs remained exclusively within the barrel associated column. The remaining $SERT^{-/-}$ TCAs had between 41 and 67% of their axon restricted to their HB. Also, while the total number of nodes and boutons per TCA were similar (**Table 1**), they were both found in higher percentages in their neighboring compartments compared to $SERT^{+/+}$ TCAs (% nodes in HB: $SERT^{+/+}$ $100 \pm 0\%$, SERT $^{-/-}$ $63.2 \pm 8.9\%$, $p = 0.004$; % boutons in the HB: $SERT^{+/+}$ $97.7 \pm 1.3\%$, $SERT^{-/-}$ $58.6 \pm 7.1\%$, $p = 0.001$). Also, the range of reconstructed projections was broader in $SERT^{-/-}$ (2632–9346 μm) than in $SERT^{+/+}$ (1323–4890 μm). Taken together, TCAs of $SERT^{-/-}$ rats innervate a larger cortical surface and are less confined to their HB within S1.

Barrel field formation is distorted but not abolished in SERT$^{-/-}$

Synaptic transmission of peripheral input from the thalamus to the cortex is crucial for the development and refinement of layer IV topographic maps. Previous studies have shown a reduced definition of the barrel borders or in some cases, a complete abolishment of barrel field formation in rodents having been exposed to high 5-HT levels during early developmental periods (for review see van Kleef et al., 2012). In order to evaluate the general barrel field morphology, cytochrome oxidase staining was used to visualize the neuronal densities of the PMBSF centers in tangential slices of $SERT^{+/+}$ ($n = 6$) and $SERT^{-/-}$ ($n = 6$) rats. In contrast to previous reports where high levels of 5-HT during development have completely impaired barrel field formation, we could identify a complete barrel field containing distinct barrels in our $SERT^{-/-}$ rats (**Figure 2A**). However, compared to the barrel field of $SERT^{+/+}$ rats, the barrel borders in $SERT^{-/-}$ rats appeared less sharp and the septal areas more extensive. We quantified the area allocated to both, barrels and septa, respectively (**Figure 2B**). On average, $SERT^{-/-}$ rats had a more than 25% reduced barrel size ($25.3 \pm 1.4\%$, $p = 0.0075$; **Figure 2C**). These differences in size were most prominent in the large barrels of the D and E rows. In contrast, in $SERT^{-/-}$ rats, the width of the septa between the barrels was robustly increased almost 2-fold along the rows ($190.3 \pm 1.4\%$; $p = 0.01$; **Figure 2D**) and more than 2.5-fold along the arcs ($261.9 \pm 15\%$; $p = 0.0075$). The combination of decreased barrel size and increased septa width (see **Figure 2E**) resulted in a barrel field that, as a whole, remained similar in size for both genotypes.

In order to determine whether the wider septa and reduced barrels in $SERT^{-/-}$ rats were attributable to a broadening of the barrel borders, we measured the "steepness" of the barrel edges by evaluating the change in CO staining intensity between the barrel and the septa over distance. We calculated the slope of change of the gray level pixel values (Δ GL) over distance, between the minimal (barrel) and maximal brightness (septum; **Figure 2B**). In our data sample, CO labeling within barrels and

Table 1 | Structural organization of the thalamocortical afferent innervation of the barrel cortex.

		Length [μm]	Nodes	Boutons	Bouton density (*n*/100 μm)
Total	$SERT^{+/+}$	2976 ± 302	28.6 ± 3.5	632 ± 74	21.2 ± 1.2
	$SERT^{-/-}$	4903 ± 823**	31.6 ± 3.3	1137 ± 160*	24.2 ± 1.1
HC L4	$SERT^{+/+}$	1905 ± 155	24.6 ± 3.4	452 ± 44	23.8 ± 1.2
	$SERT^{-/-}$	1227 ± 211***	10.8 ± 3.3*	280 ± 41**	24.1 ± 2.2
NC L4	$SERT^{+/+}$	25.1 ± 17.3	0.0 ± 0.0	5.1 ± 3.5	22 ± 7.8
	$SERT^{-/-}$	354.1 ± 116.9***	1.8 ± 0.7*	93.6 ± 29.8*	27.1 ± 3.9
Septum	$SERT^{+/+}$	19.2 ± 9.6	0.0 ± 0.0	7.0 ± 3.8	32.9 ± 10.7
	$SERT^{-/-}$	415.8 ± 110.5***	3.0 ± 1.2	101.6 ± 31.9*	27.1 ± 3.9
HC L2/3	$SERT^{+/+}$	426.7 ± 127.8	0.1 ± 0.1	108.7 ± 37	24.6 ± 1.4
	$SERT^{-/-}$	536.8 ± 151.6	2.3 ± 0.7	144.6 ± 40.2	28 ± 1.7
NC L2/3	$SERT^{+/+}$	33.3 ± 33.3	0.0 ± 0.0	11.8 ± 11.8	–
	$SERT^{-/-}$	370.5 ± 181.7**	1.8 ± 2.2	111.8 ± 55.9	28.7 ± 1.8
Sept L2/3	$SERT^{+/+}$	8.2 ± 8.2	0.0 ± 0.0	2.4 ± 2.4	–
	$SERT^{-/-}$	297.8 ± 123.4***	1.1 ± 0.5	75.0 ± 31.0	23.7 ± 2.5

*HC, home column; NC, neighboring column. Data are means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.*

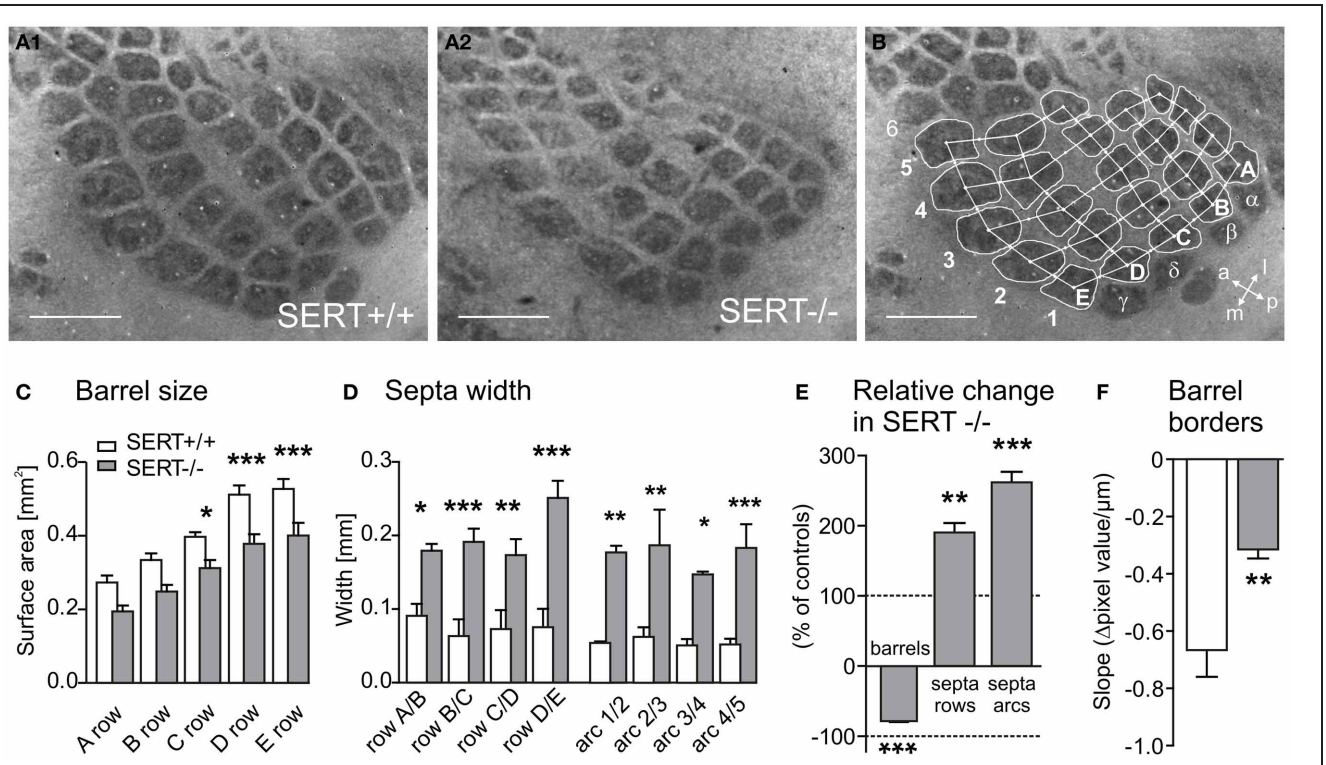

FIGURE 2 | Representative cytochrome oxidase stained tangential sections of layer IV SERT$^{+/+}$ (A1) and SERT$^{-/-}$ (A2) P21 rats (SERT$^{+/+}$ $n = 6$; SERT$^{-/-}$ $n = 6$). The sections were photographed and barrel areas (**C** and **E**) and septal distances (**D** and **E**) were quantitatively analyzed **(B)**. Barrel areas of SERT$^{-/-}$ rats were determined corresponding to rows A-B: 1–4; C-D-E: 1–5, septa were divided into rows A–E and Arcs 1–5. The edges of the barrel borders were evaluated in the gray level pixel values (ΔGL) over distance from the barrel to the septa **(D)** and showed a reduced steepness in SERT$^{-/-}$ compared to SERT$^{+/+}$ **(F)**. Scale bars = 1 mm. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

the respective septum was most reliable and homogeneous for the C1 and the C2 barrels. The slope of Δ GL across these barrel borders was significantly steeper in SERT$^{+/+}$ (Δ GL/mm: -666.3 ± 87.5) than in SERT$^{-/-}$ rats (Δ GL/mm: -314.4 ± 29.0; $p = 0.008$; **Figure 2F**), indicating less sharp borders between barrels and septa in SERT$^{-/-}$. In summary, CO staining of S1 layer IV revealed a slightly altered, but not abolished barrel pattern in SERT$^{-/-}$ rats, showing changes that indicate a spatially less organized neuronal clustering at the barrel borders within layer IV.

BLOCKING OF 5-HT REUPTAKE AFFECTS STRUCTURAL, BUT NOT INTRINSIC FUNCTIONAL PROPERTIES OF EXCITATORY NEURONS IN THE LAYER IV BARRELS

The main excitatory target neurons of TCAs originating from the VPM are SpSt and pyramidal neurons within the layer IV barrels. We investigated how the elevated 5-HT levels, changes in TCA projections and altered barrel field organization in SERT$^{-/-}$ rats affect the intrinsic electrophysiology properties of layer IV excitatory cells. We furthermore examined the afferent (somatodendritic) and efferent (axonal) organization of these layer IV neurons, which form a crucial backbone of the early intracortical signal processing of tactile sensory information. In TC slice preparations, we recorded from a total of 73 neurons which were classified according to their AP firing patterns as Regular Spiking (RS) or Intrinsic Bursting (IB) as well as their somatodendritic morphology as SpSt or pyramidal neurons (Pyr, including star pyramidal cells, see Materials and Methods). Following the recording, we determined the position of the neurons in respect to the layer IV barrel in the acute brain slice using bright field microscopy and confirmed with a subsequent CO staining.

Intrinsic electrophysiology of layer IV excitatory neurons

We investigated whether the anatomical changes in the afferent TC pathways were accompanied by changes in excitability of the excitatory layer IV neurons. We used whole cell patch clamp recordings to investigate both passive and active intrinsic membrane properties following a sustained current injection in cells of both genotype (SERT$^{+/+}$: $n = 32$, SERT$^{-/-}$: $n = 41$). A summary of the most relevant intrinsic electrophysiological properties recorded is given in **Table 2**. In agreement with previous studies, (Chagnac-Amitai and Connors, 1989; Feldmeyer et al., 1999; Schubert et al., 2003; Staiger et al., 2004) in SERT$^{+/+}$ rats, excitatory layer IV neurons were classified as being either regular spiking (RS; $n = 16$) or intrinsically burst spiking (IB; $n = 16$; **Figure 3A**). IB cells differed from RS cells most prominently in (i) eliciting an initial doublet or triplet of APs riding upon a depolarizing envelope at just suprathreshold stimulation, (ii) showing significantly shorter 1st inter-spike intervals (RS: 46.1 ± 2.1 ms; IB: 11.1 ± 4.3 ms; $p < 0.001$), and (iii) a reduced

Table 2 | Electrophysiological properties of Layer IV excitatory neurons.

	SERT$^{+/+}$		SERT$^{-/-}$	
	Pyramidal cell	**Spiny stellate cell**	**Pyramidal cell**	**Spiny stellate cell**
PASSIVE PROPERTIES	***n* = 22**	***n* = 10**	***n* = 30**	***n* = 11**
V_{rmp} [mV]	69.2 ± 1.4	−67.4 ± 1.9	−70.6 ± 1.4	−67.7 ± 2.1
R_m [MΩ]	142.5 ± 11.8	157.9 ± 23.4	128.0 ± 13.7	145.8 ± 17.6
τ_m [ms]	15.8 ± 1.3	15.8 ± 1.8	15.9 ± 1.2	16.1 ± 1.6
ACTIVE PROPERTIES				
AP threshold[1] [mV]	−37.4 ± 1.3	−31.6 ± 1.4	−37.0 ± 1.1	−36.8 ± 2.4
AP amplitude [mV]	77.6 ± 9.9	69.5 ± 5.0	78.2 ± 9.3	76.4 ± 12.7
AP halfwidth [ms]	1.3 ± 0.4	1.4 ± 0.2	1.4 ± 0.3	1.3 ± 0.3
1st ISI – weak[1] [ms]	27.2 ± 5.9	28.7 ± 8.4	43.6 ± 8.8	24.3 ± 8.9
1st ISI – strong[2] [ms]	9.0 ± 0.8	7.3 ± 1.0	10.3 ± 1.1	13.7 ± 3.7
AP FIRING PATTERN				
Regular spiking	54.5%	40%	73.3%	36.4%
Instrinsic bursting	45.5%	60%	26.7%	63.6%

Active properties were measured by [1]just suprathreshold stimulation, eliciting 2–4 APs, or [2]by stronger depolarizing current injection, eliciting 10–14 APs. No significant differences were found between SpSt and Pyr across both genotypes. Data are means ± SEM. Pyr, pyramidal cell; SpSt, spiny stellate cell; ISI, inter-stimulus interval.

2nd AP amplitude (RS: 69.2 ± 2.3; IB: 55.6 ± 2.3; $p < 0.001$). Across all neurons morphologically identified as SpSt cells or pyramidal cells, the two firing patterns were equally distributed (**Table 2**).

In SERT$^{-/-}$ rats we could identify RS and IB cells (RS: $n = 26$; $IB = 15$) in both morphological classes of excitatory layer IV neurons, the only difference being that RS cells were the prevalent class amongst pyramidal cells, whereas SpSt cells tended to be more of the IB type (**Table 2**). Apart from this, layer IV excitatory neurons from SERT$^{-/-}$ rats had similar membrane properties, including passive and active membrane properties (**Table 1**). We performed a discriminant analysis of layer IV electrophysiological properties of both SERT$^{+/+}$ and SERT$^{-/-}$ neurons to evaluate their relationship to either the morphological or the electrophysiological class. The results showed a clear segregation of both morphological and electrophysiological classes with both genotypes overlapping within these two groups (**Figure 3B**). These results indicate that the intrinsic electrophysiological properties of layer IV excitatory cells remain unchanged following exposure to high 5-HT concentrations during development.

Dendritic organization and axonal projections of layer IV excitatory cells

We next investigated the detailed dendritic and axonal morphology of both classes of layer IV excitatory cells using a quantitative morphometric analysis. In the normally developed barrel cortex, layer IV excitatory neurons, and in particular SpSt cells, the dendritic and axonal organization is strongly aligned with the respective HB and its associated cortical column. We performed 3-D reconstructions of a total of 50 electrophysiologically classified and biocytin labeled neurons for somatodendritic morphological quantification (SERT$^{+/+}$: SpSt: $n = 10$, Pyr: $n = 12$ and SERT$^{-/-}$: SpSt: $n = 13$, Pyr: $n = 15$). Additionally we reconstructed the axon in cells where (i) the collaterals were well preserved and stained and (ii) the main descending axon remained uncut at least until it reached the deeper layer Vb of the barrel cortex (SERT$^{+/+}$: SpSt: $n = 7$, Pyr: $n = 6$ and SERT$^{-/-}$:SpSt: $n = 5$, Pyr: $n = 9$). Representative reconstructions and overlays of SpSt and Pyr cells are shown in **Figures 4** and **5**.

One key dendritic property of SpSt cells in the "normal" rodent barrel cortex is that the primary dendrites that emerge from an ovoid or round soma stay within the borders of their HB (Staiger et al., 2004). We found that the dendrites of both SERT$^{+/+}$ and SERT$^{-/-}$ SpSt cells rarely extended beyond a barrel border. As a consequence, neurons of both genotypes that were located close to a barrel wall gave rise to an asymmetric dendritic arborization (**Figures 4A,C**). This indicates that, in SERT$^{-/-}$ rats, the somatodendritic organization is still well aligned with the general barrel pattern. Dendrites of pyramidal cells, however, typically extended into adjacent layers and into the septum, in particular the apical dendrite which in all cells of our sample reached the upper layer II/III or layer I where it terminated in a small unobtrusive tuft (**Figures 4B,D**).

For a more detailed quantitative evaluation of the dendritic organization in SERT$^{+/+}$ and in SERT$^{-/-}$ excitatory layer IV neurons, we characterized the morphometrical properties of basal and apical dendrites in terms of the total number of primary dendrites, the number of nodes, endings, dendritic span (covered surface area) and the complexity (number of endings/number primary dendrites; **Table 3**). Compared to SERT$^{+/+}$, SERT$^{-/-}$ SpSt cells showed no increase in length (SERT$^{+/+}$: 2585.1 ± 116 μm; SERT$^{-/-}$: 3800.3μm ± 483; $p = 0.039$) but did show an increased number of primary dendrites (SpSt: SERT$^{+/+}$: 3.6 ± 0.2; SERT$^{-/-}$: 5.2 ± 0.4; $p < 0.001$) and a decreased dendritic complexity (endings/ primary dendrites: SpSt: SERT$^{+/+}$:

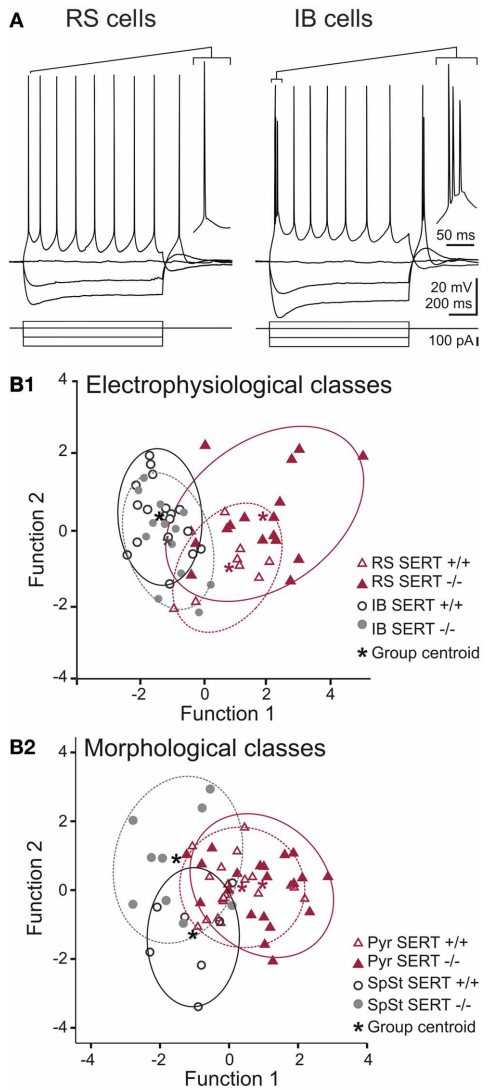

FIGURE 3 | Action potential firing pattern and discriminant analysis of electrophysiological and morphological properties of excitatory layer IV cells. (A) Representative whole cell current clamp recordings showing regular spiking (RS) and intrinsically bursting (IB) firing patterns in SERT$^{-/-}$ excitatory layer IV cells. Both firing patterns were observed in both genotypes (SERT$^{+/+}$ and SERT$^{-/-}$) as well as both morphological classes (spiny stellate cells and pyramidal cells). **(B)** Canonical score plots based on discriminant analysis of the genotype specific electrophysiological (upper panel) and morphological classes (lower panel) as *a*-priory groups. Plots were based on two functions which combined the best characteristics defining either the firing patterns (**B1**; function 1: high and low current 1st ISI; function 2: firing threshold, 2nd AP amplitude) and morphological classes (**B2**; function 1: Vrmp, high current 2nd ISI; function 2: high current 1st ISI, 2nd AP amplitude). Both analysis properties show no segregation of genotype specific populations.

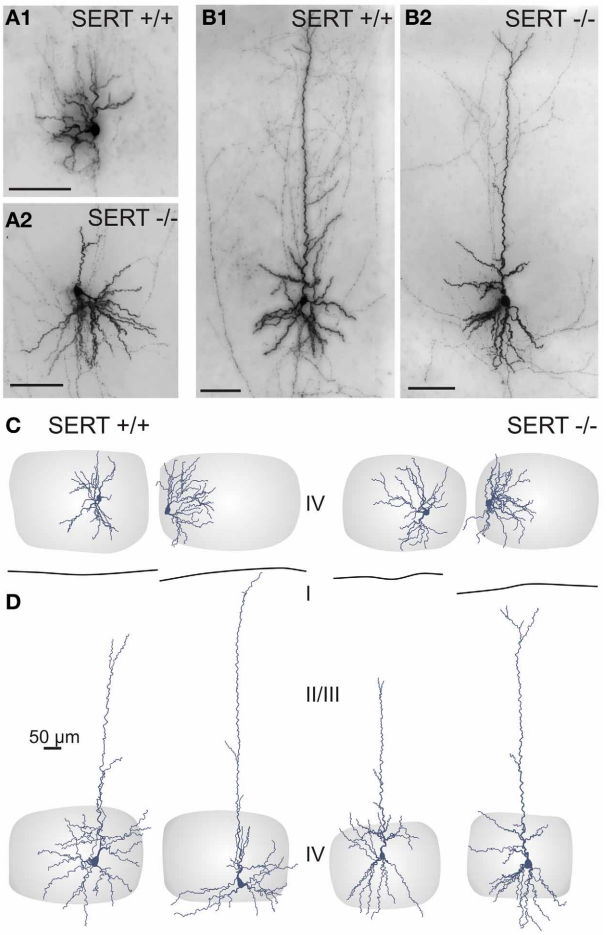

FIGURE 4 | Somatodendritic organization of excitatory spiny stellate (A,C) and pyramidal (B,D) cells in SERT$^{+/+}$ and SERT$^{-/-}$ layer IV barrel cortex. Micrographs of a biocytin stained spiny stellate **(A)** and pyramidal **(B)** cells in acute slice and representative morphological reconstructions; spiny stellate **(C)** and pyramidal **(D)** of both genotypes. Gray shaded areas indicate the position of the respective home barrel.

7.1 ± 0.6; SERT$^{-/-}$: 4.3 ± 0.3; $p = 0.001$). SERT$^{-/-}$ pyramidal cells also showed a significant increase in the number of branching dendrites (Pyr: SERT$^{+/+}$: 4 ± 0.2; SERT$^{-/-}$: 5.1 ± 0.4; $p = 0.05$) although no changes were found in their complexity (Pyr: SERT$^{+/+}$: 3.5 ± 0.3; SERT$^{-/-}$: 3.3 ± 0.4; $p = 0.574$).

The general axonal projection pattern of SERT$^{-/-}$ SpSt and pyramidal cells showed striking differences compared to that of SERT$^{+/+}$ cells, which was particularly obvious when superimposing reconstructed neurons in respect to their location within the HB (Bender et al., 2003; Lübke et al., 2003; **Figure 5**). SpSt cells in SERT$^{+/+}$ rats revealed, individually and as a population, a projection pattern that was almost exclusively restricted to their HC. While the main descending axon projected toward the white matter with few collaterals in the deeper infragranular layers Vb and VI, recurrent collaterals formed dense projection fields within the layer IV and II/III of the HC (**Figure 5A1**). SERT$^{+/+}$ pyramidal cells possessed more collaterals that projected into NCs, spanning up to 3 columns, but their main projection fields were still found within the layers IV and II/III of the HC (**Figure 5B1**). In contrast, in SpSt and pyramidal cells of SERT$^{-/-}$ rats, this prominent restriction to the HC was absent. Both morphological classes gave rise to numerous collaterals

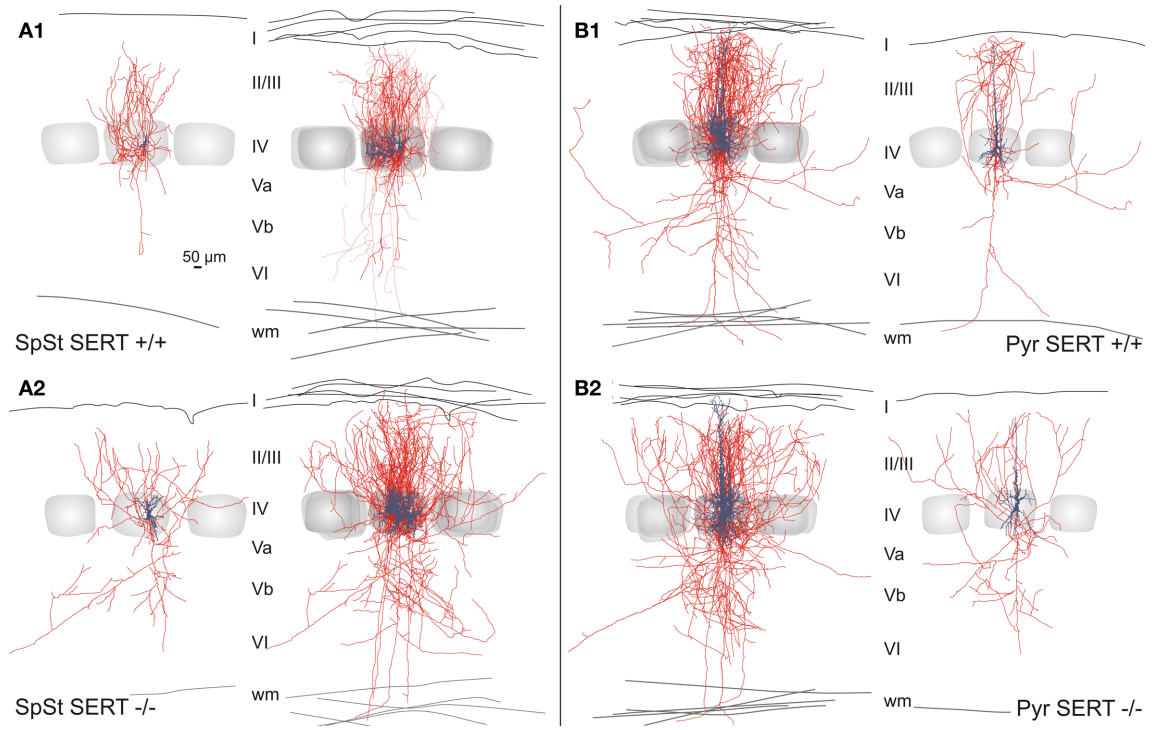

FIGURE 5 | Intracortical axonal projection pattern of excitatory cells in SERT$^{+/+}$ and SERT$^{-/-}$ layer IV of the barrel cortex. Representative morphological reconstruction(s) of the somatodendritic structure (blue) and axonal projections (red) of spiny stellate cells **(A1,B1)** and pyramidal cells **(A2,B2)** in SERT$^{+/+}$ and SERT$^{-/-}$ cortex. Overlay of 5 reconstructed neurons aligned to their position within their home barrels which illustrates the main axonal projection patterns on the population level. Gray shaded areas indicate the position of the respective home barrel (center) and adjacent barrels.

that projected into the septa and NCs within layer IV and II/III (**Figures 5A2**, **B2**). Furthermore, in particular SERT$^{-/-}$ SpSt cells possessed numerous projections into the deeper infragranular layers (**Figure 5A2**).

The detailed axonal properties were quantified on the basis of the axon length, number of nodes, endings, bouton number and density (for summary see **Table 3**). The layer and column specific properties of the axonal projections of layer IV SpSt and Pyr cells were evaluated by compartmentalized analysis, which included the HC, septal column (SC), and NC, as well as all cortical layers. Layer II/III of the home column was further subdivided into equally sized compartments reflecting the upper and lower layer II/III.

Our results show that while the length of the axonal projections as well as total number of boutons remained unchanged in SERT$^{-/-}$ SpSt and pyramidal cells, the axonal pattern underwent significant cell type specific redistributions ($p < 0.0001$, ANOVA; **Figures 6A,B**). Whereas in SERT$^{+/+}$ SpSt, 93% of the axon, 97% of the nodes and 94% of the boutons were found in the HC, in SERT$^{-/-}$ rat SpSt only 68% of the axon, 79% of the nodes and 69% of the boutons were found in this compartment ($p < 0.001$, $p = 0.002$, $p < 0.001$, respectively). These redistributions of the axon projections from the HC toward septal and NCs were most prominent in the granular and supragranular layers. There, the axon collaterals established significantly more boutons, while bouton numbers in the respective layers of the HC were reduced (**Figure 6A**). This reduction in HC layer II/III of SERT$^{-/-}$ rats mainly resulted from fewer axonal projections into the upper layer II/III, where axonal length and bouton numbers were halved ($p = 0.042$ and $p = 0.048$, respectively). Furthermore, we found that in SERT$^{-/-}$ SpSt cells, all parameters of axonal projections toward the infragranular layer Vb of the HC were significantly increased by 2–3-fold, i.e., axon length (SERT$^{+/+}$: $539 \pm 149\,\mu m$; SERT$^{-/-}$: $1805 \pm 254\,\mu m$; $p = 0.001$) arborizations (number of nodes; SERT$^{+/+}$: 1.7 ± 0.6; SERT$^{-/-}$: 13.0 ± 1.7; $p = 0.002$) and bouton numbers (SERT$^{+/+}$: 104 ± 32; SERT$^{-/-}$: 333 ± 71; $p = 0.009$).

Quantitative analysis of SERT$^{-/-}$ pyramidal cells revealed less prominent differences to those of SERT$^{+/+}$ pyramidal cells, partially due to a higher variability in the axon projections which were extensive in both genotypes. While the qualitatively observed increase in transcolumnar projections was not significant on the quantitative level, the bouton distribution in the HC supragranular layers was significantly reduced (% layer IV boutons within HC: SERT$^{+/+}$: $39.3 \pm 5.1\%$; SERT$^{-/-}$: $22.2 \pm 4.0\%$; $p = 0.02$; **Figure 6B**). As in SERT$^{-/-}$ SpSt cells, this reduction in HC associated projections was predominant for axon collaterals in the upper layers II/III (axon length: SERT$^{+/+}$: $2801 \pm 499\,\mu m$, SERT$^{-/-}$: $4713 \pm 602\,\mu m$, $p = 0.014$; number of nodes; SERT$^{+/+}$: 19.2 ± 0.6; SERT$^{-/-}$: 13.0 ± 1.7; $p = 0.002$) and bouton numbers (SERT$^{+/+}$: 104 ± 32; SERT$^{-/-}$: 333 ± 71; $p = 0.009$).

Table 3 | Morphological properties of Layer IV excitatory neurons.

	Spiny stellate cells		Pyramidal cells	
	$SERT^{+/+}$	$SERT^{-/-}$	$SERT^{+/+}$	$SERT^{-/-}$
SOMATODENDRITIC	***n* = 10**	***n* = 13**	***n* = 12**	***n* = 15**
Soma size (μm)	178.6 ± 18.3	151.1 ± 10.9	193.6 ± 12.9	197.5 ± 17.1
Primary dendrites (*n*)	3.6 ± 0.2	5.2 ± 0.4**	4.0 ± 0.2	5.1 ± 0.4*
Length (μm)	2427.8 ± 162	2459.2 ± 169	1802.9 ± 135	2439.8 ± 230
Nodes (*n*)	23.0 ± 1.4	21.9 ± 1.1	13.6 ± 1.1	15.8 ± 1.5
Nodes (*n*/mm)	9.6 ± 0.4	9.1 ± 0.3	7.6 ± 0.4	6.5 ± 0.3*
Endings (*n*)	26.7 ± 1.4	27.0 ± 1.1	17.5 ± 1.2	20.9 ± 1.7
Complexity	6.9 ± 0.8	4.6 ± 0.5*	3.5 ± 0.3	3.3 ± 0.4
Covered surface (μm^2)	86225 ± 6772	70814 ± 9898	62834 ± 6626	88721 ± 8623
APICAL DENDRITE				
Length (μm)	–	–	1988 ± 220	1874 ± 180
Nodes (*n*)	–	–	12.6 ± 1.7	14.7 ± 2
Nodes (*n*/mm)	–	–	6.4 ± 0.4	7.7 ± 1.4
Endings (*n*)	–	–	13.8 ± 1.8	15.8 ± 2
Covered surface (mm^2)	–	–	0.167 ± 0.029	0.142 ± 0.017
AXONAL	***n* = 7**	***n* = 5**	***n* = 6**	***n* = 9**
Axonal length (μm)	17201 ± 1872	18820 ± 1739	21253 ± 2046	18720 ± 1741
Axonal nodes (*n*)	79.4 ± 5.7	107 ± 4.6**	124.2 ± 17.7	100.7 ± 8.5
Axonal endings (*n*)	79.7 ± 5.7	108 ± 4.3**	123.8 ± 17.3	102.1 ± 8.6
Boutons (*n*)	3889 ± 447	3968 ± 335	4447 ± 465	4059 ± 443
Bouton density (/100μm)	21.5 ± 1.2	21.4 ± 1.3	20.9 ± 1.9	20.9 ± 0.8
Covered surface (mm^2)	1.0 ± 0.4	1.6 ± 0.3	2.2 ± 1.0	2.2 ± 0.7

*The covered surface of a structure was calculated by a 2-dimensional convex hull estimation of the structure perimeter. Dendritic complexity is calculated by the total number of nodes/endings. Data are means ± SEM. *$p < 0.05$; **$p < 0.01$.*

As a whole, the quantitative analysis of the morphological properties of layer IV SpSt and pyramidal cells in $SERT^{-/-}$ rats demonstrated a cell type specific loss of home column specific restriction of axonal projections and to increased descending projections toward the infragranular layers of the S1 cortical networks.

DISCUSSION

There is accumulating evidence showing that 5-HT plays a key role in the network formation within the developing brain, in particular for the correct establishment of the topographic organization of the somatosensory system (Cases et al., 1996; Persico et al., 2001; Salichon et al., 2001; Gaspar et al., 2003; Xu et al., 2004; van Kleef et al., 2012). The present study shows that increased 5-HT levels during critical neurodevelopmental stages not only distorts the topographic wiring between the thalamus and the individual barrels in the layer IV of the primary S1 but also affects the efferent axonal connectivity of layer IV excitatory networks. In these networks, which form the backbone of early intracortical processing of tactile sensory information (for review see Brecht, 2007; Petersen, 2007; Feldmeyer et al., 2013), we found (i) a reduced dendritic complexity of SpSt cells within the layer IV barrels, (ii) a decrease in the intracolumnar restrictions of projections toward the associative supragranular layers II/III, and (iii) increased axonal projections toward the infra-granular layer Vb.

REDUCED TOPOGRAPHICAL PRECISION OF THALAMOCORTICAL INNERVATION OF THE S1 CORTEX

During neural development, growing thalamic projections from the dorsal thalamus transiently express SERT (for review see Gaspar et al., 2003) and, guided by molecular cues that are modulated by extracellular 5-HT concentrations (Bonnin et al., 2007, 2012), reach the input layer IV of the barrel cortex around P7. Changes in 5-HT concentrations during this critical neurodevelopmental period (Erzurumlu and Kind, 2001) affects TC pathfinding and alters the barrel-like clusters of TC afferents (Bonnin et al., 2007, 2012; Li and Crair, 2011). Our analysis of individual TCAs projecting from the VPM to S1 showed that in both $SERT^{+/+}$ and $SERT^{-/-}$ rats, axonal clusters formed more densely in the input layer IV of S1. While normally individual thalamic axon mainly project to one barrel and its associated column (Jensen and Killackey, 1987; Diamond, 1995; Bureau et al., 2006; Meyer et al., 2010; Oberlaender et al., 2012a,b), TCAs of $SERT^{-/-}$ rats innervated a broader field within the layer IV. These results are in agreement with previous studies in $SERT^{-/-}$ and monoamine oxidase $(MAOA)^{-/-}$ mice (Cases et al., 1996; Young-Davies et al., 2000; Persico et al., 2001; Salichon et al., 2001; Rebsam et al., 2002). Interestingly, we observed a clear barrel pattern in both the acute brain slices and CO-processed tissue of the $SERT^{-/-}$ rats which allowed us to quantify the level of organization of TCA innervation in respect to the barrel associated cortical column. Our data show that in $SERT^{-/-}$ rats, the TC innervation

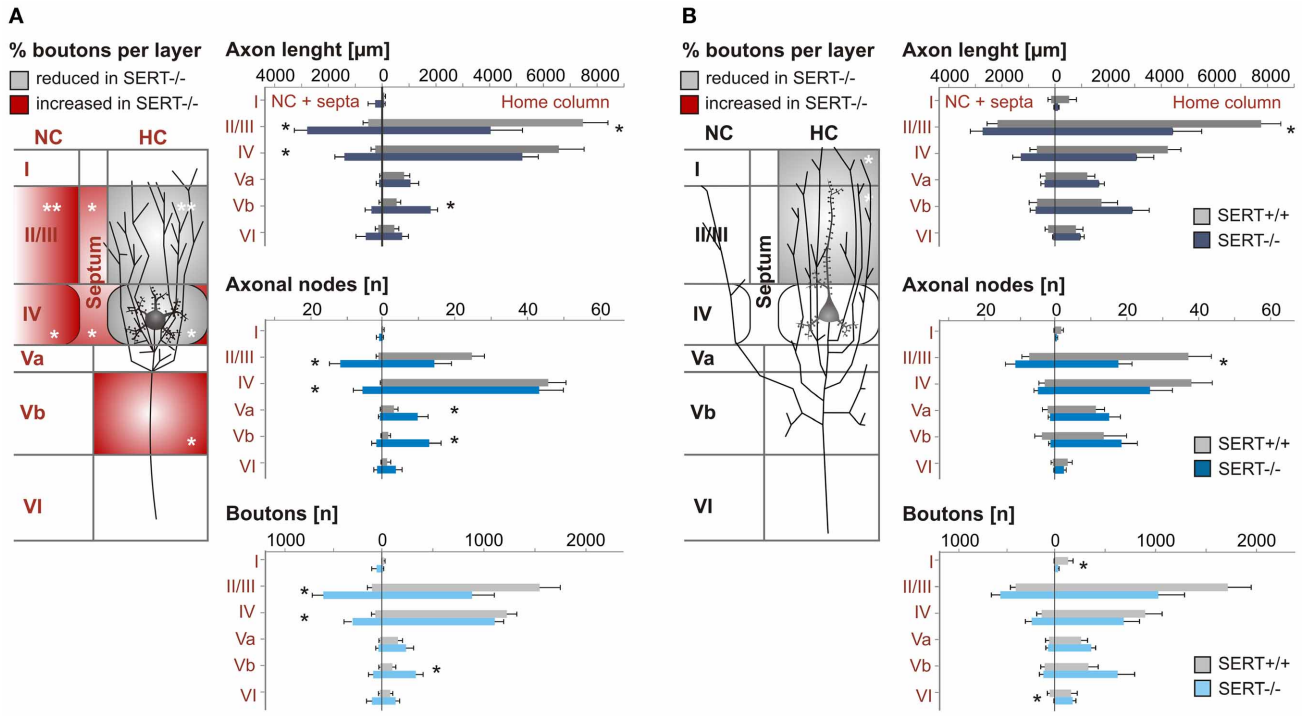

FIGURE 6 | Quantitative analysis of the axonal projection pattern of excitatory spiny stellate (A) and pyramidal cells (B) in SERT$^{+/+}$ and SERT$^{-/-}$ layer IV barrel cortex. Schematic drawings (*left panels*) illustrate the significant changes in relative bouton distribution within the individual layers (I–VI), highlighting layers specific shifts from home column (HC) to septal or neighboring column (NC) and *vice versa*. Histograms (*right panels*) represent the most relevant layer specific axonal properties (length, nodes, and boutons number) per genotype. Data are mean ± SEM, asterisks mark significant differences between genotypes. $^{*}p < 0.05$; $^{**}p < 0.01$.

has lost its predominant one-to-one association to the individual HB by projecting extensively into the layer IV septa as well as into the neighboring barrels. Together with our finding that TCAs are less arborized and possess fewer boutons within their respective HB, this loss in topographic precision could result in a reduced transmission efficiency of tactile sensory signals between individual barreloids of the VPM and the cortical layer IV. *In vitro* studies have shown that the VPM to layer IV synapse forms and refines into its topographical organization during the first post-natal week, after which time the ability to induce plasticity at the layer IV synapse decreases (Feldman et al., 1999; Fox, 2002). The SERT$^{-/-}$ phenotype observed at P21 could represent an immature system where the critical developmental time window is delayed. Recent *in vivo* studies have shown that sensory deprivation could reorganize TCA receptive fields in adult (3-month-old) animals (Oberlaender et al., 2012a,b). It remains to be investigated whether the SERT$^{-/-}$ TCA topography can be rescued over time, through sensory experience.

ALTERED BUT NOT ABOLISHED BARREL FIELD ORGANIZATION IN SERT$^{-/-}$ RATS

A reduction in the synaptic transmission of peripheral input toward the cortex either through a reduced TCA structure or through reduced synaptic activity at the TC synapse results in an altered development of the barrel field, which could also be consequent to elevated 5-HT levels during development. Whereas some studies have reported that increases of 5-HT during critical periods of barrel cortex development, prevent the formation of the barrel field (Cases et al., 1996; Persico et al., 2001; Salichon et al., 2001; Rebsam et al., 2002), our observations point to a weaker phenotype in the SERT$^{-/-}$ rat. Although the barrel formation is clearly visible, the delimitation between barrel and septa is more diffuse. The individual barrels of SERT$^{-/-}$ are smaller in comparison to those of the SERT$^{+/+}$ rats and the septal areas are significantly enlarged. Although, the CO pattern shows that the neuronal clustering of granular cells within the layer IV seems to be less defined, we found that, similar to SERT$^{+/+}$ rats, the dendritic organization of excitatory SpSt cells of the SERT$^{-/-}$ rats remains aligned along the edges of the barrel borders. Also, the cause and consequences of a widened septum in SERT$^{-/-}$ rats is unknown. Interestingly, the neurons within the septa of the layer IV mainly project to the whisker related area of the primary motor cortex (M1) (Alloway et al., 2004). A modification in the circuits involved in sensorimotor control of whisking behavior could hypothetically result in changes in active sensory discrimination. Indeed, SERT$^{-/-}$ mice have been reported to show impairments in the spontaneous gap crossing task (Pang et al., 2011).

INTRINSIC, ACTIVE, AND PASSIVE PROPERTIES OF EXCITATORY NEURONS IN LAYER IV OF SERT$^{-/-}$ RAT

In the rodent somatosensory system, the main excitatory target neurons of the TCAs originating from the VPM are the SpSt

and the pyramidal neurons within the barrels of S1 in layer IV (for review see Petersen, 2003; Brecht, 2007). Previous studies have shown that the layer IV excitatory neurons establish a highly interconnected local excitatory network which is capable of amplifying the sparse thalamic input before it is relayed toward the supragranular layers (Stratford et al., 1996; Feldmeyer et al., 1999, 2002; Schubert et al., 2003). Our results show that in SERT$^{-/-}$ rats, these excitatory networks undergo cell type specific structural changes. These changes will be discussed in detail below. However, the intrinsic electrophysiological properties of layer IV excitatory neurons were not affected by excessive exposure to 5-HT during development. This key neuronal feature regulates and defines neuronal excitability as well as the temporal characteristics of the information output (Connors and Gutnick, 1990). Neither passive, nor active electrophysiological properties of both SpSt and pyramidal cells showed detectable differences across genotypes. Although *in vitro* studies have shown that acute 5-HT exposure influences neuronal excitability and synaptic plasticity (Waterhouse et al., 1986; Schmitz et al., 1995; Foehring et al., 2002) our findings imply that constant increased 5-HT levels do not lead to lasting changes in the intrinsic neuronal excitability which could play a role in modulating the reception of incoming thalamic input. Interestingly, many studies have also shown a role for 5-HT in GluR1 insertion and AMPA receptor trafficking (Derkach et al., 2007; Makino and Malinow, 2009; Jitsuki et al., 2011; Lesch and Waider, 2012). Future studies will be necessary in order to identify possible changes in plasticity and synaptic transmission within the barrel cortex of SERT$^{-/-}$ rats.

CELL TYPE SPECIFIC CHANGES IN GENERAL DENDRITIC ORGANIZATION

The dendritic organization of neurons is regulated by presynaptic glutamatergic release as well as by surrounding monoaminergic levels (Gonzales-Burgos et al., 1996; Levitt et al., 1997; Hayashi et al., 2010). As mentioned above, SpSt cells within the barrels of layer IV typically have spatially confined dendritic trees that are restricted to the inside of a barrel and clusters around the thalamic afferent terminals (Staiger et al., 2004; Callaway and Borrell, 2011). Also, the layer IV pyramidal and star pyramidal cells, pooled in our study, give rise to an apical dendritic tree that extends the neuronal receptive surface toward the supragranular layers (Schubert et al., 2003; Staiger et al., 2004). We found no changes in the general principles of barrel-associated dendritic organization of layer IV excitatory cells of SERT$^{-/-}$ rats. However, the SpSt and pyramidal cells both showed an increased number of elongated dendrites, that were altogether less complex in the SpSt when compared to those of SERT$^{+/+}$ SpSt cells. In contrast, layer IV pyramidal cell dendrites were indistinguishable between genotypes. Although the loss in dendritic complexity, number of primary dendrites or elongated dendrites is in line with previous studies performed in various regions of the rodent brain (Bou-Flores et al., 2000; Norrholm and Ouimet, 2000; Lee and Lee, 2012), it contradicts others (Lee, 2009). These different results could be based on (i) the cell-type and region specific differences in 5-HT receptor expression (for review see Gaspar et al., 2003; Daubert and Condron, 2011), (ii) the age of the animals at which the study is carried out, and (iii) the timing as well as the duration of the increased exposure to the high 5-HT levels. Indeed, the effect of 5-HT receptor activation on basic dendritic (re)organization can be rapid. The reduced dendritic complexity of SpSt cells in SERT$^{-/-}$ could lead to an altered local signal processing of tactile information [see Varga et al. (2011)]. The cell type specific nature of these dendritic alterations could be explained by the fact that the two classes of excitatory cells receive different sources of intracortical inputs, i.e., SpSt cells are almost exclusively involved in local intra-barrel signal processing, whereas pyramidal cells are wired across layers and columns (Schubert et al., 2003).

LOSS OF INTRACOLUMNAR DOMINANCE OF EXCITATORY LAYER IV OUTPUT CONNECTIVITY

Even though many studies have broadly investigated the link between 5-HT and the resulting disorganized topography of TC axonal projections toward S1 (for review see van Kleef et al., 2012), the resulting effect of this disorganization on intracortical connectivity has not been elucidated. In layer IV, excitatory neurons typically show two main projection domains: one within the HB in layer IV, the other in the supragranular layers II/III (Lübke et al., 2000; Petersen and Sakmann, 2000; Feldmeyer et al., 2002; Staiger et al., 2004; Lübke and Feldmeyer, 2007). There is a clear distinction between the projection patterns of layer IV SpSt and pyramidal cells. SpSt cells project almost exclusively within their home column whereas pyramidal cells, in addition to their prominent intracolumnar projections, also project into NCs (Staiger et al., 2004). Interestingly, our data demonstrate that in SERT$^{-/-}$ rats, the excitatory cells within the layer IV have lost their prominent intracolumnar associated projection pattern, most importantly of the SpSt type. SpSt cells show a significant expansion of their projections into the septa and transcolumnar layer IV and II/III, at the expense of weakened intracolumnar projections. Also, the ascending axons of excitatory layer IV neurons preferentially form contacts with the basal dendrites of pyramidal cells within the supragranular layers II/III (Feldmeyer et al., 2002). The loss of such columnar organization within these important modules of the canonical microcircuitry of S1 (for review see Douglas and Martin, 2004; Lübke and Feldmeyer, 2007; Schubert et al., 2007) could imply important changes in the feed forward of sensory information toward the associative layers. However, the nature of the cellular population contacted by these increased transcolumnar projections still needs to be identified and could involve both excitatory and/or inhibitory neuronal populations.

Another interesting finding of this study was the presence of significantly increased downward projections of layer IV excitatory cells toward the layer Vb. The latter projections of layer IV cells toward the infragranular layers are known to be typically sparse (Gilbert and Wiesel, 1983; Callaway and Wiser, 1996; Lübke et al., 2000; Schubert et al., 2001, 2003; Staiger et al., 2004) and the consequences of these alterations may be complex since neurons in the layer Vb of S1 are a secondary target of TCAs of the VPM as well as major cortical output neurons [for review see Brecht (2007)]. Consequently, this connection may potentially modulate intracortical signal processing at the input as well as at the cortical output level. Furthermore, it has been observed that during development, layer IV excitatory cells initially project

toward infra-granular layers and later on, preferentially project to supra-granular layers (Callaway and Katz, 1992; Bender et al., 2003). The extensive infra-granular projections of the SERT$^{-/-}$ could therefore reflect an immature system.

It will be of interest to investigate whether the reorganization of intracortical wiring found in SERT$^{-/-}$ rats is long lasting and persists throughout the life-span. Especially the intracortical connections such as the layer IV to II–III synapse which remains plastic and can be modulated throughout adulthood (Feldman, 2000; Feldmeyer et al., 2002; Fox, 2002; Bender et al., 2003; Shepherd et al., 2005) where the refinement could be compensated for at older ages.

FUNCTIONAL CONSEQUENCES

The typical topographic columnar organization of the barrel cortex is characterized by the segregated thalamic afferents projecting to the layer IV barrels and the subsequent cell type specific intracolumnar projections toward the layers II–III. It is hypothesized that the intracortical sensory networks can correctly interpret complex spatial and temporal aspects of incoming sensory information, through the coexistence of neurons that can keep the spatial specificity of information within one column (signal segregators) and neurons that integrate information (signal integrators) across cortical columns (Schubert et al., 2003, 2007). In the barrel cortex, layer IV SpSt cells have small suprathreshold receptive fields (Brecht et al., 2004) and are thought to be the archetype of a signal segregator since they (i) receive spatially precise information via TCAs of the VPM, (ii) receive intracortical information almost exclusively from neurons in their HB, and (iii) transmit information within their column (for review, see Brecht, 2007). Our findings show that in SERT$^{-/-}$, both TCAs as well as the intracortical projections of the excitatory layer IV cells do not possess this spatial specificity. It is of interest to evaluate the behavioral consequences of these structural deficits. Indeed, *in vivo* physiological and behavioral studies on rodents having been exposed to high 5-HT levels during brain development show changes in stimulus evoked cortical activity (Esaki et al., 2005; Pang et al., 2011) as well as a reduced tactile performance (Lee, 2009; Pang et al., 2011).

Also, it would be of great interest to clarify how the cumulated effects of the TC disorganization, the altered excitatory layer IV projections and the constant elevated 5-HT levels in SERT$^{-/-}$ rats, could affect other crucial parts of the intracortical networks. Two of these candidate networks are (i) the associative layers II/III, central for the precise integration and interpretation of tactile sensory information arising from layer IV (Douglas and Martin, 2004; Lübke and Feldmeyer, 2007; Schubert et al., 2007), and (ii) the inhibitory interneuronal population, critical in dynamically shaping the receptive field properties and ensuring coincidence detection of excitatory signals by allowing a window of opportunity for integrating incoming thalamic information within the layer IV barrels (Gabernet et al., 2005; Sun et al., 2006; Cruikshank et al., 2007; Kimura et al., 2010).

ACKNOWLEDGMENTS

This study was funded by a Brain and Cognition grant of the Dutch Organization for Scientific Research awarded to Judith Homberg and Dirk Schubert (#433-09-311). We thank Professor Jochen Staiger as well as Ingo Helmich for their helpful comments on the manuscript. We also thank Jos Dederen, Judith van Westen as well as Esther van Leijsen for technical assistance.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 February 2013; paper pending published: 27 March 2013; accepted: 22 May 2013; published online: 07 June 2013.

Citation: Miceli S, Negwer M, van Eijs F, Kalkhoven C, van Lierop I, Homberg J and Schubert D (2013) High serotonin levels during brain development alter the structural input-output connectivity of neural networks in the rat somatosensory layer IV. Front. Cell. Neurosci. 7:88. doi: 10.3389/fncel.2013.00088

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 10 May 2013
doi: 10.3389/fncel.2013.00067

Neonatal citalopram exposure decreases serotonergic fiber density in the olfactory bulb of male but not female adult rats

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Manipulation of serotonin (5HT) during early development has been shown to induce long-lasting morphological changes within the raphe nuclear complex and serotonergic circuitry throughout the brain. Recent studies have demonstrated altered raphe-derived 5HT transporter (SERT) immunoreactive axonal expression in several cortical target sites after brief perinatal exposure to selective 5HT reuptake inhibitors such as citalopram (CTM). Since the serotonergic raphe nuclear complex projects to the olfactory bulb (OB) and perinatal 5HT disruption has been shown to disrupt olfactory behaviors, the goal of this study was to further investigate such developmental effects in the OB of CTM exposed animals. Male and female rat pups were exposed to CTM from postnatal day 8–21. After animals reach adulthood (>90 days), OB tissue sections were processed immunohistochemically for SERT antiserum. Our data revealed that the density of the SERT immunoreactive fibers decreased ∼40% in the OB of CTM exposed male rats, but not female rats. Our findings support a broad and long-lasting change throughout most of the 5HT system, including the OB, after early manipulation of 5HT. Because dysfunction of the early 5HT system has been implicated in the etiology of neurodevelopmental disorders such as autism spectrum disorders (ASDs), these new findings may offer insight into the abnormal olfactory perception often noted in patients with ASD.

Keywords: serotonin transporter, olfactory bulb, selective serotonin reuptake inhibitors, sexual dimorphism, autism spectrum disorders

INTRODUCTION

Serotonin (5HT) is a widely distributed neuromodulator that plays an important role in regulating brain development (Gaspar et al., 2003). 5HT neurons in the dorsal raphe nucleus midline group and median raphe nucleus have axons distributed throughout the brain including the cortex (Waterhouse et al., 1986) and the olfactory bulb (OB) (McLean and Shipley, 1987). Manipulation of this system during early stages of neurodevelopment has been shown to produce sex-specific neurobehavioral modifications that persist well into adulthood (Csaba et al., 2003; Hohmann et al., 2007; Uçeyler et al., 2010). For example, serotonergic neurotoxin injections with 5,7-dihydroxytrypatmine into the bilateral medial forebrain bundles of neonatal mice induced long-term changes in social and sensory behaviors, as well as a depletion of the 5HT-immunoreactive (5HT-ir) fibers in the cerebral cortex (Boylan et al., 2007). Similarly, brief neonatal exposure to the selective serotonin reuptake inhibitor (SSRI) citalopram (CTM) lead to a sex-specific reduction of SERT-ir fibers in the neocortex (∼40%) and hippocampus (∼55%) of adult male rats (Maciag et al., 2006; Weaver et al., 2010). These male rats displayed reductions in novel object exploration, reduced social juvenile play (Rodriguez-Porcel et al., 2011), altered auditory sensory information processing, and reduced interhemispheric callosal connections (Simpson et al., 2011). In accordance with these findings seen in rats exposed neonatally to SSRIs, the inbred BTBR mice also display abnormalities in social juvenile play and have decreased (∼20–30%) 5HT transporter (SERT) binding capacity throughout the brain (Gould et al., 2011). Depletion of 5HT in the OB affected olfactory recognition in neonate rats (Dulcy et al., 2010), and caused glomerular atrophy (Moriizumi et al., 1994). In addition, olfactory sensitivity has been shown to decrease after 3 weeks of CTM administration in adult mice (Lombion et al., 2008).

These lines of converging information demonstrate the importance of 5HT in the proper development of 5HT circuitry, and when manipulated via environmental and/or genetic means, lead to abnormal behaviors and altered innervation patterns within 5HT efferents. Therefore, our hypothesis was that brief neonatal CTM exposure will have a sex-specific effect on SERT-ir fibers within the OB, with male abnormalities being more robust.

MATERIALS AND METHODS

All procedures were approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee

and complied with the Association for Assessment and Accreditation of Laboratory Animal Care International and National Institutes of Health guidelines.

ANIMALS AND TISSUE PREPARATION

After the delivery of timed-pregnant Long Evans rats, the offspring were cross fostered to produce litters of 4∼5 pups. The pups were tattooed for identification on postnatal day (PN) 6, weaned at PN28, and housed in groups of 2∼3/cage under standard laboratory conditions with *ad-libitum* access to food and water. Beginning on PN8, the pups were injected subcutaneously with CTM (10 mg/kg, Tocris, Ellisville, MO) or saline in a volume of 0.1 ml twice daily (total CTM dose of 20 mg/kg/day or saline volume of 0.2 ml/day) for 14 days (PN8–21). This time window corresponds roughly from late stages of gestation to first 3 years of postnatal life in humans (Maciag et al., 2006). In addition, a non-treatment (NT) group (handled but without injection) was also included as a control for the effects of injection. The rationale behind the CTM dose was based on similar blood serum levels detected in rodents (Kugelberg et al., 2001) and humans (Bjerkenstedt et al., 1985), and similar dosages have been used and reported in previous rodent studies (Maciag et al., 2006; Weaver et al., 2010; Simpson et al., 2011).

After reaching adulthood (PN > 60), animals were deeply anesthetized with pentobarbital (75 mg/kg, i.p.) and perfused through the ascending aorta with 0.9% saline, followed by 3.5% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). The brains were extracted and stored in 3.5% paraformaldehyde solution and 25% sucrose overnight at 4°C until slicing.

IMMUNOHISTOCHEMISTRY

Rats from each treatment group were randomly divided into subsets and then processed and analyzed in these subsets to minimize immunostaining variability. For example, three rat brains (one non-treatment, one saline exposed, and one CTM exposed) were sliced and processed on the same day. Comparison of the SERT-ir fibers in rat OB was based initially on three male experimental subsets and three female experimental subsets. Two additional female sets were added to ensure no effect of treatment since no obvious alterations were noted in the female subsets.

In general, we followed the three-step indirect immunohistochemical procedures and quantification methods described previously (Maciag et al., 2006; Weaver et al., 2010). Specifically, 60 μm coronal sections were taken throughout the OB using a freezing microtome and placed in individual wells. One out of every six sections were incubated in rabbit anti-SERT antibody (1:1000, Immunostar, Hudson, WI) for 24 h at room temperature followed by another 24 h at 4°C. After rinse, sections were incubated in biotinylated anti-rabbit IgG (1:100, BA1000, ABC kit, Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Axon profiles were then visualized by incubating the sections in Cy3-conjugated streptavidin (1:200, Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 1 h at room temperature in the dark. For each step of staining within a subset, total volumes of solution were created and distributed equally across animals to eliminate variability in concentrations within subsets. Finally, sections were mounted on gelatin-coated slides, allowed to air dry, and were then covered with DPX (mixture of distyrene, plasticizer, and xylene).

To control for non-specific labeling, two basic procedures were utilized. First, we conducted experiments where sections were processed according to the protocol, except that the primary SERT antiserum was omitted. Following this procedure, no SERT immunoreactivity was detected. Another control study also yielded negative immunostaining when an inappropriate secondary antibody such as biotinylated anti-mouse IgG (1:100, BA9200, ABC kit, Vector Laboratories, Burlingame, CA) was used for linkage.

IMAGE ACQUISITION AND ANALYSIS

Digital photomicrographs of sections containing the area of the main OB (Paxinos and Watson, 1986, bregma at 6.7 mm) were taken with a consistent exposure time at 20× magnification using a Nikon E800 epifluorescent microscope equipped with a SenSys cool camera (Roper Scientific). The 20× magnification yielded 344 × 437 μm terminal field area. For each animal, three different sub-regions of the OB were chosen: the glomerular layer, the external plexiform layer, and the granule cell layer.

A previous study suggested that the glomerular layer in the dorsal and medial region of the OB has a higher number of SERT-ir fibers than those located in the lateral region (Gomez et al., 2005). Therefore, in order to reduce the sampling bias for the glomerular layer, we routinely took four images of the SERT-ir fibers from the middle portion of both medial and lateral regions of the OB. The same strategies of acquiring pictures were used for the external plexiform and the granule cell layers.

MetaMorph imaging software (Universal Imaging Systems) was used as described previously (Maciag et al., 2006; Weaver et al., 2010) to quantify the density of the SERT-ir fibers in the OB. Briefly, the signals on the photo were first adjusted to the same brightness. After thresholding the photo, the background was excluded and the SERT fibers were selected. A rectangular region (125 × 100 μm for the glomerular layer, 437 × 295 μm for the external plexiform layer, and 437 × 280 μm for the granule cell layer) was created and placed on the photograph to include the specified layer. The area of this region also served as the reference point. The percentage of accumulated area of SERT fibers divided by the area of the rectangular region was defined as the SERT fiber density within this specific layer. The fiber density for each layer of an individual animal was determined by averaging the densities obtained from the four images. Data were analyzed statistically using MANOVAs with *post-hoc* Tukey's HSD tests (SPSS 19, IBM).

RESULTS

DISTRIBUTION AND MORPHOLOGY OF SERT-ir FIBERS

In both male and female control rats, the majority of SERT-ir fibers appear rather smooth and fine in caliber with very few varicosities (**Figure 1A**). Most of SERT-ir fibers in the granule cell layer tended to run parallel to the pia surface, and many of the SERT-ir fibers in the external plexiform layer were either perpendicular or parallel to the pia surface. In contrast, SERT-ir fibers in the glomerular layer were mostly randomly distributed. This

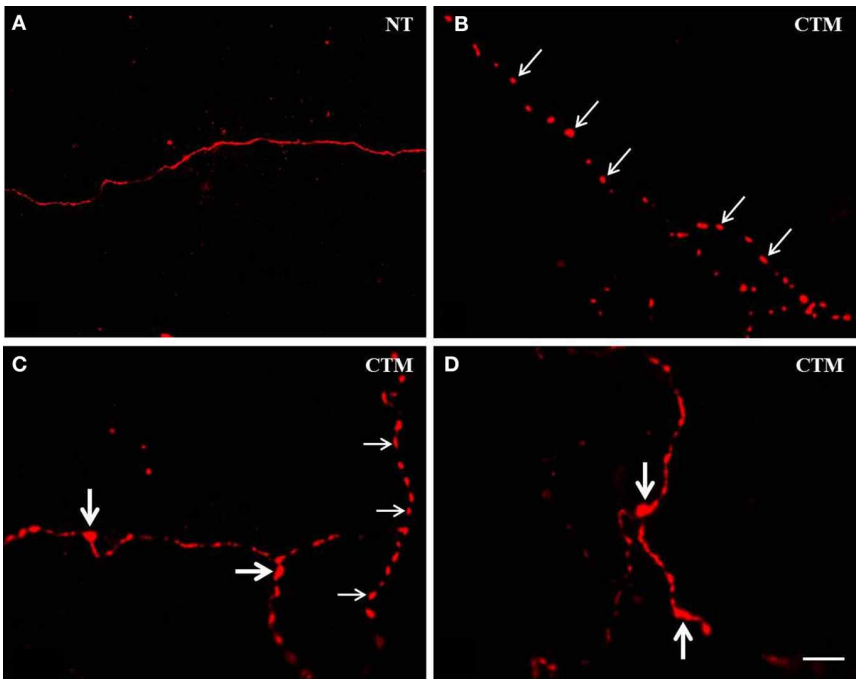

FIGURE 1 | Representative photomicrographs showing the altered morphology of SERT-ir fibers in the OB of male rats. (A) Normal SERT-ir fibers exhibit a smooth and fine morphology with very few varicosities. **(B,C)** Beaded-like SERT-ir fibers (thin arrows) were noted in CTM exposed male rats. **(C,D)** The SERT-ir fibers with large bouton-like appearance (thick arrows) were noted in CTM exposed male rats. Scale bar = 2 μm. NT, non-treatment; CTM, citalopram; OB, olfactory bulb; SERT, serotonin transporter.

general pattern of SERT-ir fiber distribution did not change in CTM exposed animals.

In contrast to the smooth and fine morphology of the SERT-ir fibers commonly seen in control animals, sex-specific varicosities were frequently noted in the glomerular layer of CTM exposed male animals (**Figures 1B,C**). These varicosities were found less frequently in the external plexiform and the granule cell layers. The distance between these varicosities along the SERT-ir fibers was usually very close and they were mainly in the range of ∼1–3 μm apart. In addition, enlarged bouton-like swellings (**Figures 1C,D**) were occasionally noted in the granule cell layer of CTM exposed male rats. In contrast to these layer-specific morphological changes in male rats neonatally exposed to CTM, such changes were not seen in the female populations.

DENSITY OF THE SERT-ir FIBERS IN MALE RATS

Representative photos demonstrating the alteration of SERT-ir fiber density in the OB of male rats were shown in **Figure 2**. A one way MANOVA revealed a significant effect of treatment in the density of SERT-ir fibers within the glomerular layer of male rats, $F_{(2,\ 6)} = 10.82$, $p = 0.010$, within the external plexiform layer, $F_{(2,\ 6)} = 6.47$, $p = 0.032$, and within the granule layer, $F_{(2,\ 6)} = 38.81$, $p < 0.000$. Compared to SAL exposed rats, Tukey's HSD *post-hoc* tests revealed that SERT-ir fiber density within the OB of CTM exposed male rats was significantly reduced ∼39% in the glomerular layer ($p = 0.015$), ∼38% in the external plexiform layer ($p = 0.034$), and ∼38% in the granule cell layer ($p = 0.001$) (**Figure 3A**). Importantly, there were no significant differences in SERT-ir fiber density between NT and SAL exposed rats within any of the three OB layers ($p \geq 0.79$) (**Figure 3A**), demonstrating that the effect was not due to the injections. The data from each experimental subset of male rats are listed in **Table 1**.

DENSITY OF THE SERT-ir FIBERS IN FEMALE RATS

Interestingly, SERT-ir fiber density within the OB of female rats showed a different pattern compared to that discovered in male rats. Representative photos were shown in **Figure 4**. Specifically, a one way MANOVA revealed a non-significant effect of treatment within the glomerular layer $F_{(2,\ 12)} = 0.173$, $p = 0.843$, within the external plexiform layer $F_{(2,\ 12)} = 0.300$, $p = 0.746$, and within the granule layer $F_{(2,\ 12)} = 0.464$, $p = 0.640$ (**Figure 3B**). The data from each experimental subset of female rats are listed in **Table 2**.

DISCUSSION

We examined the neurodevelopmental effects of neonatal CTM exposure on the expression of SERT-ir fibers in the OB of adult rats. Our data revealed that disruption of the 5HT system during early life lead to a sex-specific and long-lasting change in the morphology and density of the SERT-ir fibers within the OB of adult male rats. This suggests a sexually dimorphic response to altered levels of neonatal 5HT, which is consistent with previous rodent studies (Csaba et al., 2003; Hohmann et al., 2007; Uçeyler et al., 2010), and further supports this model in the etiology of neurodevelopmental disorders (Rodriguez-Porcel et al., 2011; Simpson et al., 2011).

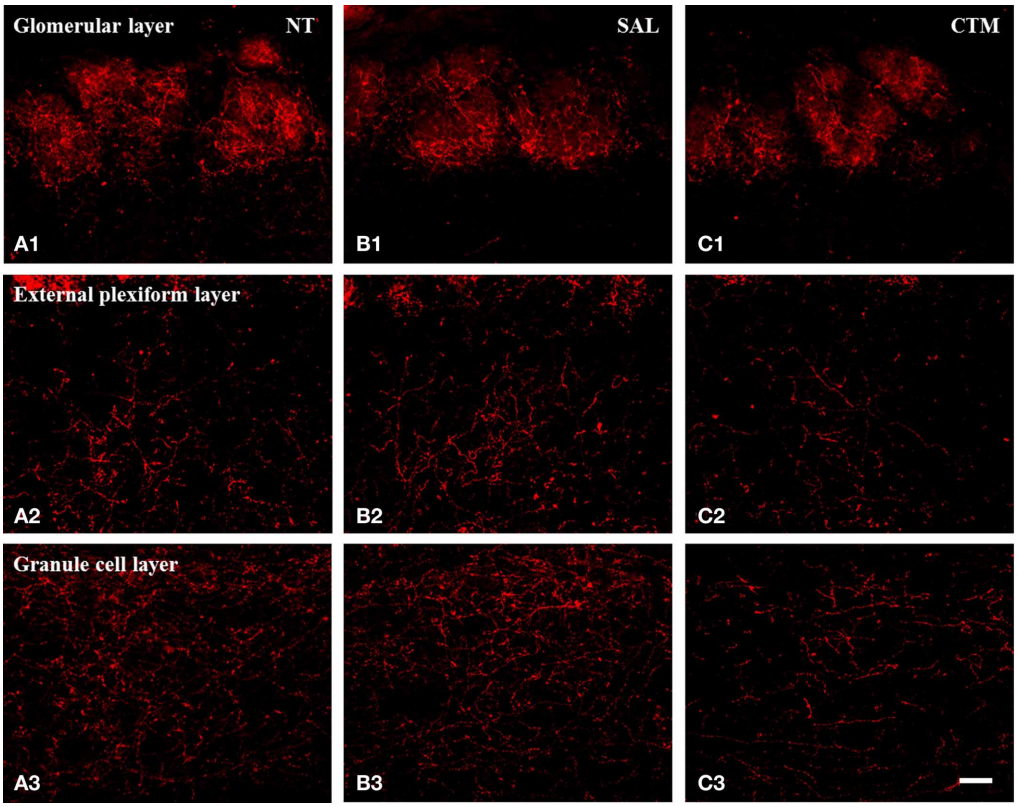

FIGURE 2 | Representative photomicrographs illustrating the distribution pattern of SERT-ir fibers in the OB of male rats. **(A1–C1)** The glomerular layer. **(A2–C2)** The external plexiform layer. **(A3–C3)** The granule cell layer. Note that SERT-ir fiber density in CTM **(C1–C3)** exposed rats was reduced in all three layers compared to NT **(A1–A3)** and SAL **(B1–B3)** exposed male rats. Scale bar = 50 μm. NT, non-treatment; SAL, saline; CTM, citalopram; OB, olfactory bulb; SERT, serotonin transporter.

TECHNICAL CONSIDERATIONS

It has been well-documented that the 5HT immunostaining patterns within the OB are reliably represented by using SERT immunostaining, but minor differences between the two biomarkers have been noted. For example, a greater number of SERT-ir fibers have been observed in the infraglomerular layer of the OB compared to 5HT-ir fibers, and the authors suggested that 5HT immunostaining may not effectively reveal neuronal processes in cells with a low concentration of intracellular 5HT (Gomez et al., 2005). Furthermore, the SERT protein was found within serotonergic axon bundles in addition to axon terminals, which makes it a useful biomarker for examining serotonergic axons (Zhou et al., 1998).

The layer-specific density of 5HT-ir fibers in the OB of normal adult male rats has been reported to be ∼8.8% in the glomerular layer, ∼3.5% in the granule cell layer, and ∼1.8% in the external plexiform layer (McLean and Shipley, 1987). In the current study, a similar pattern of the SERT-ir fiber density was observed in control animals with ∼13.3% in the glomerular layer, ∼8.4% in the granule cell layer, and ∼2.8% in the external plexiform layer, supporting the relative reliability of these two immunomarkers.

Appropriate measures were employed to ensure that our analysis was not biased by our staining and sampling procedures. For example, image quantification was done independently by two investigators blind to experimental conditions in order to verify interrater reliability. In addition, the staining procedures were processed in sets to minimize staining variability across groups. In fact, the Levene's test of equality of error variances was not significant within any of the three OB layers of male rats ($p \geq 0.604$) or female rats ($p \geq 0.113$), demonstrating equal error variances across groups.

COMPARISON OF SERT-ir FIBER MORPHOLOGICAL CHANGES WITH OTHER STUDIES

In the present study, varicosities (∼1–3 μm apart) on SERT-ir fibers were frequently noted in the glomerular layer of CTM exposed male rats, while these varicosities were less often found in the external plexiform and the granule cell layers. This is consistent with previous studies that found more 5HT-ir fibers with varicosities (∼2–20 μm apart) in the glomerular layer compared to the infraglomerular layer (McLean and Shipley, 1987), and SERT-ir fibers with varicosities within the glomerular layer (Gomez et al., 2005) of normally developing rodents. The increased prevalence of dystrophic fibers seen in CTM exposed animals suggests altered function and may impair serotonergic signaling throughout the brain.

An increased number of dystrophic thick SERT-ir fibers has been noted in the amygdala, the hippocampus, and the cortex

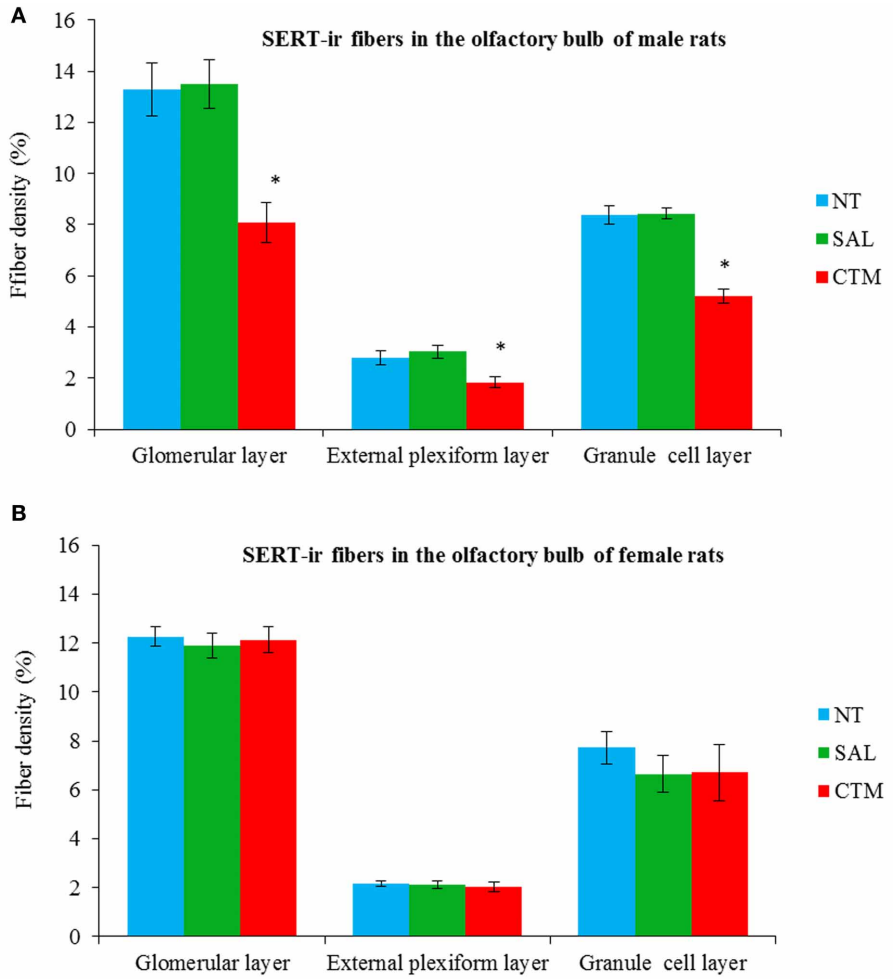

FIGURE 3 | Quantitative analysis of SERT-ir fiber density in the OB of both male and female rats. (A) The density of the SERT-ir fibers was significantly decreased in all three layers of the OB in CTM exposed male rats compared to SAL exposed male rats. **(B)** However, such changes were not significantly different in female rats. Error bars = SEM. *Represents $p < 0.05$ compared to SAL group. NT, non-treatment; SAL, saline; CTM, citalopram; OB, olfactory bulb; SERT, serotonin transporter.

Table 1 | SERT-ir fiber density in the olfactory bulb of male rats.

Subset	Glomerular layer (%)			External plexiform layer (%)			Granule cell layer (%)		
	NT	SAL	CTM	NT	SAL	CTM	NT	SAL	CTM
1	11.8	13.0	6.6	3.0	2.5	1.9	7.7	8.7	5.4
2	15.3	15.3	8.4	3.1	3.3	2.1	8.5	8.0	5.6
3	12.7	12.1	9.2	2.2	3.2	1.4	8.9	8.5	4.6
Mean	13.3	13.5	8.1	2.8	3.0	1.8	8.4	8.4	5.2
SEM	1.0	0.9	0.8	0.3	0.2	0.2	0.4	0.2	0.3

NT, non-treatment; SAL, saline; CTM, citalopram; SEM, standard error of the mean.

of autistic patients (Azmitia et al., 2011). Interestingly, rodents exposed neonatally to CTM showed an increased number of thick and rod-like SERT-ir fibers and/or fine and beaded SERT-ir fibers in the cortex and hippocampus (Maciag et al., 2006; Weaver et al., 2010). However, in the present study, very few SERT-ir fibers in the OB showed this pattern after neonatal exposure to CTM. One possible explanation for this discrepancy could be that the neurochemical composition of the OB raphe projecting system may be different from cortical raphe projecting system. For example, the majority of cortical projecting 5HT neurons are in the midline

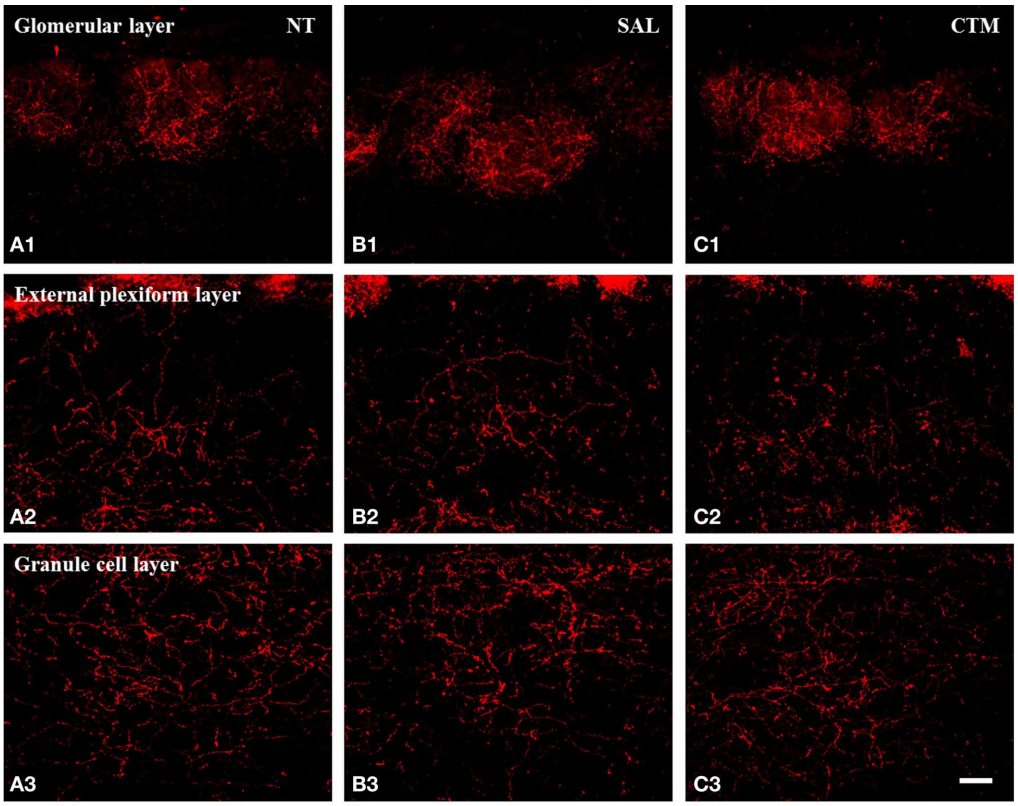

FIGURE 4 | Representative photomicrographs illustrating the distribution pattern of SERT-ir fibers in the OB of female rats. **(A1–C1)** The glomerular layer. **(A2–C2)** The external plexiform layer. **(A3–C3)** The granule cell layer. Note that SERT-ir fiber density remained unchanged in all three layers of NT **(A1–A3)**, SAL **(B1–B3)**, and CTM **(C1–C3)** exposed female rats. Scale bar = 50 μm. NT, non-treatment; SAL, saline; CTM, citalopram; OB, olfactory bulb; SERT, serotonin transporter.

Table 2 | SERT-ir fiber density in the olfactory bulb of female rats.

Subset	Glomerular layer (%)			External plexiform layer (%)			Granule cell layer (%)		
	NT	SAL	CTM	NT	SAL	CTM	NT	SAL	CTM
1	11.9	11.8	13.6	1.9	2.6	2.6	8.8	5.3	10.4
2	12.2	12.1	11.1	2.0	2.2	1.6	8.1	7.5	7.3
3	12.1	10.0	11.2	2.2	1.8	1.6	6.1	7.3	6.2
4	11.4	13.2	11.6	2.3	2.2	2.4	9.3	8.6	6.5
5	13.8	12.3	13.2	2.5	1.7	1.8	6.3	4.6	3.1
Mean	12.3	11.9	12.1	2.2	2.1	2.0	7.7	6.7	6.7
SEM	0.4	0.5	0.5	0.1	0.2	0.2	0.7	0.7	1.2

NT, non-treatment; SAL, saline; CTM, citalopram; SEM, standard error of the mean.

subgroup of the raphe complex co-express nitric oxide and 5HT (Simpson et al., 2003; Lu et al., 2010), and it is currently unknown whether this is the case for the OB projecting raphe neurons.

SEXUAL DIMORPHISM OF THE 5HT SYSTEM

At present, a limited number of studies have examined sexual differences within the developing 5HT system of human or rodent brains. It was reported that cortical 5HT content in normal male mice was twice that of female mice at PN3 (Connell et al., 2004). In healthy young humans, 5HT synthesis capacity was higher in boys compared to girls (Chugani et al., 1999), and similarly higher in healthy adult men compared to women (Sakai et al., 2006). In contrast, 5HT levels were reported to be higher in the brainstem and limbic forebrain of female rats compared to male rats (Carlsson and Carlsson, 1988).

The major support for the sexually dimorphic development of the 5HT system is from rodent studies that have demonstrated sex-specific effects after early life manipulation of brain

5HT levels. For example, it was reported that 5HT depleted male animals showed reduced exploration in response to spatial rearrangement and object novelty, however, this effect was not found in females (Hohmann et al., 2007). Similarly, physical activity was reduced in adult male SERT knock-out mice compared to females (Uçeyler et al., 2010). Furthermore, after a single injection of 5HT into newborn rats, 5HT levels were reduced in the striatum of adult male rats and displayed increased sexual activity, while no obvious differences were detected in adult female rats (Csaba et al., 2003). Interestingly, perinatal exposure to CTM affected male locus coeruleus circuit function but was not seen in females (Darling et al., 2011). Because a variety of early-life 5HT-system manipulations produce a constellation of long-term and sex-specific effects, it is not surprising that we observed a sex-specific reduction of SERT-ir fiber density in the OB of only male rats after neonatal CTM exposure.

Taken together, the current investigation and previous studies suggest that a sexual dimorphism does exist in both the normally developing 5HT system and after manipulations of brain 5HT levels. At present, the precise biological mechanism(s) of such sexual differences during early development are not known. Nonetheless, the neurodevelopmental consequences of these sexual differences need to be explored as it pertains to neurodevelopmental and psychiatric disorders that exhibit sex specificity.

FUNCTIONAL SIGNIFICANCE AND CLINICAL IMPLICATIONS

Recently, our laboratory and others have suggested that manipulation of perinatal 5HT levels induce numerous neurological and behavioral abnormalities, similar to what have been observed in autistic patients. For example, patients with autism spectrum disorder (ASDs) have been known to show impaired social interactions (Wing and Gould, 1979), a disrupted 5HT system in the brain (Chugani et al., 1999; Makkonen et al., 2008; Nakamura et al., 2010), as well as under-connectivity between two cortical hemispheres (Just et al., 2004; Vidal et al., 2006; Courchesne et al., 2007; Damarla et al., 2010). In addition, a recent study reported that exposure to SSRIs during pregnancy increased the risk of ASD diagnosis by a factor of more than two (Croen et al., 2011). These new lines of evidence suggest that dysregulation of 5HT levels may be one of the contributing factors for ASDs.

In addition to the core symptoms commonly seen in ASD, autistic patients are also known to display high incidence of sensory abnormalities (Rogers and Ozonoff, 2005). For example, it was reported that over 90% of children with ASDs had multiple sensory abnormalities (Leekam et al., 2007). Abnormal responses to multisensory information such as touch, oral, and olfactory stimuli have been proposed as predictors of the social severity in children with ASDs (Hilton et al., 2010). Other studies reported either disrupted olfactory identification (Suzuki et al., 2003; Bennetto et al., 2007; May et al., 2011) or altered odor sensitivity (Tomchek and Dunn, 2007; Dudova et al., 2011) in patients with ASDs. At present, the neurochemical and neuroanatomical foundations of the altered olfactory information processing in patients with ASDs remain unclear, but this model of early-life 5HT manipulation may help elucidate the cause of these widespread sensory abnormalities.

Lastly, 5HT has been shown to play an important role in the rodent olfactory system, and disruption of 5HT levels lead to abnormal olfactory odor transmission. For example, 5HT was reported to be necessary in conditioned olfactory learning in rats (McLean et al., 1993; Moriizumi et al., 1994; Dulcy et al., 2010), mediated through $5HT_{2A/2C}$ receptors (McLean et al., 1996; Hardy et al., 2005). These previous findings along with our present data suggest a potential link between an altered 5HT system in the OB and the abnormal olfactory function noted in rats and patients with ASDs. However, it may be premature to suggest that an altered serotonergic system in the OB offers a possible clinical explanation for abnormal odor information process frequently noted in patients with ASDs. Therefore, additional behavioral tests of olfactory function in our animal model warrant further investigation.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health under grant number: EUREKA MH084194.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 23 January 2013; accepted: 22 April 2013; published online: 10 May 2013.

Citation: Zhang J, Dennis KA, Darling RD, Alzghoul L, Paul IA, Simpson KL and Lin RCS (2013) Neonatal citalopram exposure decreases serotonergic fiber density in the olfactory bulb of male but not female adult rats. Front. Cell. Neurosci. 7:67. doi: 10.3389/fncel.2013.00067

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 19 June 2013
doi: 10.3389/fncel.2013.00093

Serotonin homeostasis and serotonin receptors as actors of cortical construction: special attention to the 5-HT_{3A} and 5-HT_6 receptor subtypes

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Cortical circuits control higher-order cognitive processes and their function is highly dependent on their structure that emerges during development. The construction of cortical circuits involves the coordinated interplay between different types of cellular processes such as proliferation, migration, and differentiation of neural and glial cell subtypes. Among the multiple factors that regulate the assembly of cortical circuits, 5-HT is an important developmental signal that impacts on a broad diversity of cellular processes. 5-HT is detected at the onset of embryonic telencephalic formation and a variety of serotonergic receptors are dynamically expressed in the embryonic developing cortex in a region and cell-type specific manner. Among these receptors, the ionotropic 5-HT_{3A} receptor and the metabotropic 5-HT_6 receptor have recently been identified as novel serotonergic targets regulating different aspects of cortical construction including neuronal migration and dendritic differentiation. In this review, we focus on the developmental impact of serotonergic systems on the construction of cortical circuits and discuss their potential role in programming risk for human psychiatric disorders.

Keywords: 5-HT, somatosensory cortex, cerebral cortex, development, plasticity, 5-HT3 receptor, 5-HT6 receptor, circuit assembly

INTRODUCTION

The mammalian cerebral cortex is critical for sensory-motor integration, higher-order cognitive functions, and emotional regulation. It processes information through the activation of neural networks composed of excitatory glutamatergic pyramidal neurons and local modulatory interneurons that release γ-aminobutyric acid (GABA), neuropeptides, and vasoactive substances (Peters and Jones, 1984; Peters and Kara, 1985a,b; Baraban and Tallent, 2004; Karagiannis et al., 2009; Tricoire and Vitalis, 2012). Developmental perturbations impacting the maturation of cortical circuits can confer risk for neuropsychiatric disorders (Insel, 2010; Thompson and Levitt, 2010; Marin, 2012). Our labs have contributed to a model, in which such developmental vulnerability is often restricted to sensitive periods. The concept of sensitive developmental periods for the indelible modulation of complex behaviors is similar to that described for sensory systems (i.e., visual cortex, ocular dominance plasticity), but modulating factors, and underlying mechanisms are much less well-understood.

Building cortical circuits relies on a series of precisely timed events that take place mainly during embryonic and early postnatal development (reviewed in Marin and Rubenstein, 2001; Bystron et al., 2008; Corbin et al., 2008; Batista-Brito and Fishell, 2009; Rakic, 2009; Vitalis and Rossier, 2011). Critical components include the proliferation, migration, and differentiation of neurons and glial cells, with differentiation including the appropriate growth and guidance of axons toward their targets. These steps are genetically programmed and phylogenetically conserved, yet they are malleable and plastic. As cell-autonomous signaling unfolds over time, the various cortical cell-types are continuously in contact with and responding to their environment. Cell extrinsic signals are very diverse in nature and include monoamines, guidance cues, growth factors, cell adhesion molecules, and various components of the extracellular matrix. In particular, the monoamine 5-HT has emerged as an important regulator of neural circuit formation (previously reviewed in Gaspar et al., 2003; Vitalis and Parnavelas, 2003).

In developing rodent embryos, cortical 5-HT mainly arises from placental sources at the onset of cortical development and from serotonergic afferents by E16–E17 (Bonnin et al., 2011). This dual source of 5-HT is conserved in humans and permits 5-HT signaling during development, even before embryonic serotonergic neurons have differentiated and are able to release 5-HT. Not surprisingly, 5-HT modulates neuronal proliferation, migration, and differentiation, and is implicated in the etiology of many neuropsychiatric disorders, including mental retardation, autism, depression, and anxiety (for reviews, see Berger-Sweeney and Hohmann, 1997; Levitt et al., 1997; Whitaker-Azmitia, 2001;

Gu, 2002; Gaspar et al., 2003; Homberg et al., 2009; Oberlander et al., 2009; Daubert and Condron, 2010; Lesch and Waider, 2012). In the context of developmental plasticity under normal conditions as well as in disease, it is important to appreciate that 5-HT signaling is influenced by many factors, including nutrition (Serfaty et al., 2008), perinatal stress (Peters, 1990; Papaioannou et al., 2002a,b), infection (Winter et al., 2008, 2009), 5-HT metabolism and storage (Cases et al., 1996; Vitalis et al., 1998, 2007; Noorlander et al., 2008; Popa et al., 2008), genetic alterations (Lira et al., 2003; Murphy and Lesch, 2008; Pluess et al., 2010; Karg et al., 2011; Bonnin et al., 2011), and pharmacological compounds such as selective 5-HT reuptake inhibitors (Ansorge et al., 2004, 2008).

Here we review findings demonstrating that early-life 5-HT signaling regulates cellular events implicated in the assembly of cortical circuits. We highlight recent studies that have revealed the role of specific 5-HT receptors in the construction of such circuits: the ionotropic 5-HT type 3A receptor (5-HT_{3A}) and the metabotropic 5-HT type 6 receptor (5-HT_{6}). Finally, we review clinical studies suggesting that altered 5-HT homeostasis or signaling could increase risk for human stress-related psychopathologies such as mood and anxiety disorders.

STRUCTURE AND DEVELOPMENT OF THE RODENT CEREBRAL CORTEX

NEURONAL COMPONENTS

The cerebral cortex of adult mammals is a laminated structure comprised of six layers that each contain a complement of pyramidal (glutamatergic) and non-pyramidal (GABAergic) neurons (Peters and Jones, 1984). Pyramidal neurons make up ∼80% of all adult cortical neurons, sending excitatory output axons to other cortical areas and to distant parts of the brain (Peters and Kara, 1985a; Thomson and Lamy, 2007; Spruston, 2008). The vast majority of cortical GABAergic cells are interneurons that only make local connections. GABAergic interneurons are extremely diverse, differing in shape, electrophysiological properties, and the combination of neuropeptides and calcium-binding proteins that they express (Peters and Kara, 1985b; Cavanagh and Parnavelas, 1988; DeFelipe, 1993; Kawaguchi and Kondo, 2002; Blatow et al., 2005; Tomson and Lamy, 2007; PING et al., 2008; Karagiannis et al., 2009; Xu et al., 2010; Vitalis and Rossier, 2011; Tricoire and Vitalis, 2012; DeFelipe et al., 2013). Using these differentiating characteristics, one can at a first approximation distinguish four main classes of interneurons populating the somatosensory cortex (PING et al., 2008; DeFelipe et al., 2013). First, fast-spiking interneurons that express parvalbumin (Parv), and act as an inhibitory gate for incoming sensory information (Inoue and Imoto, 2006; Sun et al., 2006). Second, adapting martinotti cells that express somatostatin (SOM), and are thought to control dendritic information through local feedback inhibition (Karube et al., 2004). Third, adapting bipolar interneurons that express vasoactive intestinal peptide (VIP) and calretinin (CR), and preferentially target other interneurons and receive direct input from the thalamus (Férézou et al., 2007; Vitalis and Rossier, 2011). Fourth, adapting neurogliaform interneurons that express neuropeptide Y (NPY) and/or nitric oxide (NO), and that are responsible for the slow GABAergic inhibition of pyramidal cells and interneurons (Karagiannis et al., 2009; Oláh et al., 2009; Perrenoud et al., 2012a,b; Tricoire and Vitalis, 2012).

DEVELOPMENT OF THE CEREBRAL CORTEX

Origins and migration of pyramidal neurons and the formation of cortical layers

The cerebral cortex develops from neuroepithelial germinal cells of the telencephalic pallium and subpallium that massively proliferate (from E11 to E12 in mice), to form the cerebral vesicles. The first neurons generated, Cajal-Retzius (C-R) cells and subplate (SP) cells, form transient and heterogeneous populations of cells that originate from both pallial and subpallial territories and establish the preplate (PP; Boulder Committee, 1970; Uylings et al., 1990; Bystron et al., 2008). SP and reelin secreting C-R cells provide positioning cues and instructions to developing cortical neurons and afferents (Supèr et al., 2000; Soriano and del Rio, 2005; Herz and Chen, 2006; Lakatosova and Ostatnikova, 2012). The first pyramidal neurons generated arise sequentially from the cortical ventricular zone (VZ), from which they translocate or migrate radially to form a layer within the PP, the so-called cortical plate (CP), thus splitting the PP into a superficial marginal zone (MZ; presumptive layer I containing the C-R cells) and a deep SP. At the beginning of CP formation (E13–E14 in mice), pyramidal cells are generated from radial glial cells (RGCs), whereas later (E15–E17 in mice), they mainly originate from intermediate progenitor cells (IPC; or basal progenitors) deriving from RGC cells (see Kriegstein and Noctor, 2004; Noctor et al., 2004; Corbin et al., 2008 for reviews). The neurons of the CP assemble into layers II–VI in an "inside-out" sequence: the deepest cellular layers are assembled first and those closest to the surface last.

Origins and migration of GABAergic neurons

In rodents, most GABAergic neurons are generated outside the cortical VZ, mainly in the medial (E11–E14 in mice) and the caudal (E14–E17 in mice) parts of the ganglionic eminence (MGE and CGE, respectively) in the basal telencephalon (for reviews, see Marin and Rubenstein, 2001; Wonders and Anderson, 2006; Batista-Brito and Fishell, 2009; Rudy et al., 2011; Vitalis and Rossier, 2011), and more ventrally in the entopeduncular region (AEP) and the preoptic region (POA; Gelman et al., 2009). These areas are specified through a combination of distinct transcription factors and morphogenes, and produce different classes of interneurons. The ventral and dorsal parts of the MGE express the homeobox transcription factor Lhx6 and generate two large classes of interneurons: fast-spiking/Parv+ interneurons and adapting/SOM+ interneurons (Xu et al., 2004; Butt et al., 2005, 2007; Miyoshi et al., 2007; Wonders et al., 2008). Later, the CGE—a region that expresses the transcription factor Gsh2 (Fogarty et al., 2007) but lacks the transcription factors Nkx2.1, Nkx6.2, and Lhx6 (Flames et al., 2007)—generates an average of 30% of the total population of GABAergic interneurons, which mainly express VIP, CR, and NPY (Lee et al., 2010; Vucurovic et al., 2010; Rudy et al., 2011; Vitalis and Rossier, 2011). Once produced, interneurons migrate toward the CP. They initially follow parallel migratory streams, first in the intermediate zone and MZ, and later along the subventricular zone (SVZ), before they switch their

migratory mode and incorporate into the developing CP through radial migration. Interestingly, some of the later generated mainly CGE-derived interneurons pause longer (until around P1–P2) in the SVZ before entering CP. In mice, cortical migration is almost completed by P4, and followed by cortical expansion. However, during the first postnatal days and decreasing with age the SVZ retains the capacity to produce CR-expressing interneurons that incorporate into the cerebral cortex at postnatal stages (Inta et al., 2008; Riccio et al., 2012). These key events are recapitulated in **Figure 2**.

SOURCES OF 5-HT TO THE RODENT CORTEX

5-HT SYNTHESIS

5-HT is synthesized from the essential amino-acid tryptophan. In the blood stream, 90% of tryptophan is linked to serum-albumin. A proportion reaching ∼10% when the blood-brain barrier becomes fully functional (postnatal day 12) and decreasing with age is free to cross the developing blood-brain barrier (Ribatti et al., 2006). Tryptophan is accumulated in 5-HT producing cells by a non-specific transporter with high affinity to several uncharged aromatic amino-acids. Tryptophan is then hydroxylated in these cells into 5-hydroxytryptophan by the tryptophan hydroxylase. Tryptophan hydroxylase type 2 (Tph2) is expressed in serotonergic neurons of the raphe nuclei (Côté et al., 2003; Walther et al., 2003), while peripheral tissues mostly express tryptophan hydroxylase type 1 (Tph1). 5-hydroxytryptophan is then further decarboxylated into 5-HT by the aromatic amino-acid decarboxylase (AADC). 5-HT is catabolized in the cytoplasm of 5-HT transporter (SERT) expressing cells by monoamine oxidase A or B (MAOA or MAOB). MAOA has higher affinity to 5-HT than MAOB, but both enzymes are co-expressed in rodent serotonergic neurons between E12 and P7 (Vitalis et al., 2002a). After P7, the expression of MAOB becomes predominant, and MAOA deficiency could be partially compensated for by the increased expression of MAOB in serotonergic neurons (Cases et al., 1996; Vitalis et al., 2002a; Cheng et al., 2010).

5-HT of the embryonic telencephalon is not only produced locally by serotonergic neurons of the raphe nuclei, but also originates from extra-CNS (embryonic periphery, placental) as well as extra-embryonic (maternal) sources. In the two following sections, we briefly recapitulate the development of the serotonergic system and review the various sources of telencephalic 5-HT during embryonic and early postnatal life.

DEVELOPMENT OF THE SEROTONERGIC SYSTEM IN RELATION TO TELENCEPHALIC DEVELOPMENT

Serotonergic neurons of the brainstem are subdivided into 9 groups forming two clusters: the caudal division (B1–B4; including the raphe pallidus, obscurus, magnus, and pontis) projecting to the spinal cord and the cerebellum, and the rostral division [B5–B9; including the dorsal (B6, B7) and median raphe nuclei (B5, B8)] projecting to the forebrain (Lidov and Molliver, 1982; Steinbusch and Nieuwenhuys, 1983; Törk, 1990; **Figure 1A**). Recent genetic and developmental approaches revealed differential rhombomeric identities of raphe 5-HT neurons, which introduce a new layer of functional classification (Jensen et al., 2008; Kiyasova and Gaspar, 2011). Together with genetic tracing and topographic projection mapping, we will soon have a much better understanding of the anatomical organization of the 5-HT systems.

In mice, dorsal raphe neurons differentiate in the brainstem by E10–E11 (E12–E15 in rats). This period coincides with the beginning of telencephalic vesicle formation (Wallace and Lauder, 1983; Aitken and Törk, 1988). Serotonergic neurons generated rostral to the isthmus (B6–B9 groups; dorsal and median raphe) send axons only one day after their genesis. These axons reach the cortico-striatal junction by E14 in mice (by E16 in rats; Wallace and Lauder, 1983; **Figure 1B**), during the peak of migration of cortical GABAergic interneurons generated in the MGE. 5-HT-containing axons enter the cortical anlage as two tangential streams, one above and the other below the CP (Wallace and Lauder, 1983; Aitken and Törk, 1988). The former is distributed in the MZ where pioneering C-R cells are located and with which they are in close appositions, making transient synaptic contacts (Radnikow et al., 2002; Janusonis et al., 2004).

Below the CP, 5-HT afferents are mainly restricted to the IZ and the SP (Wallace and Lauder, 1983). At E14, the developing cerebral cortex (Bayer and Altman, 1991) and the ganglionic eminences produce deeper-layer neurons (glutamatergic and GABAergic, respectively) that are in the process of migration to their final positions. By E16–E17 in mice, thalamocortical axons (TCAs) penetrate the cortical anlage and are in close apposition with 5-HT axons running in the IZ. In parallel, cortical neurons begin to establish their polarity, sending their axons toward their respective targets and developing numerous dendritic processes. At the end of corticogenesis, 5-HT axons gradually arborize sending numerous branches into the CP (Wallace and Lauder, 1983). During this period a large proportion of GABAergic interneurons enter the CP where they radially migrate to reach their final positions (see above). Progressively, serotonergic axons become evenly distributed in the different cortical territories and show their mature pattern of innervation by P21 (Steinbusch, 1981). However, dorsal raphe and median raphe projections differ anatomically. The dorsal raphe projections have been described as generally thin, displaying numerous branches with pleiotropic varicosities and preferentially arborize in cortical layers IV and V that receive thalamic inputs. By contrast, median raphe projections are characterized by large spherical varicosities that can form true chemical synapses (Törk, 1990). They preferentially arborize in layer I and lower white matter, give collaterals that could surround neuronal cell bodies and proximal dendrites, and preferentially contact interneurons containing VIP- and cholecystokinin (CCK) in various species (Törk, 1990; Hornung and Celio, 1992; Férézou et al., 2007). Interestingly, in the mature brain, a dense plexus of 5-HT-positive fibers is present in the SVZ, in close apposition with progenitor cells of this region (Jahanshahi et al., 2011). At all stages 5-HT could be released along the entire axonal network thus diffusing into the entire extracellular fluid. It is still not clear whether subsets of serotonergic axons preferentially release 5-HT in synaptic clefts vs. volume transmission.

OTHER SOURCES OF 5-HT

Although 5-HT is likely to act as a trophic or instructive factor during early periods of cortical development, its sources have

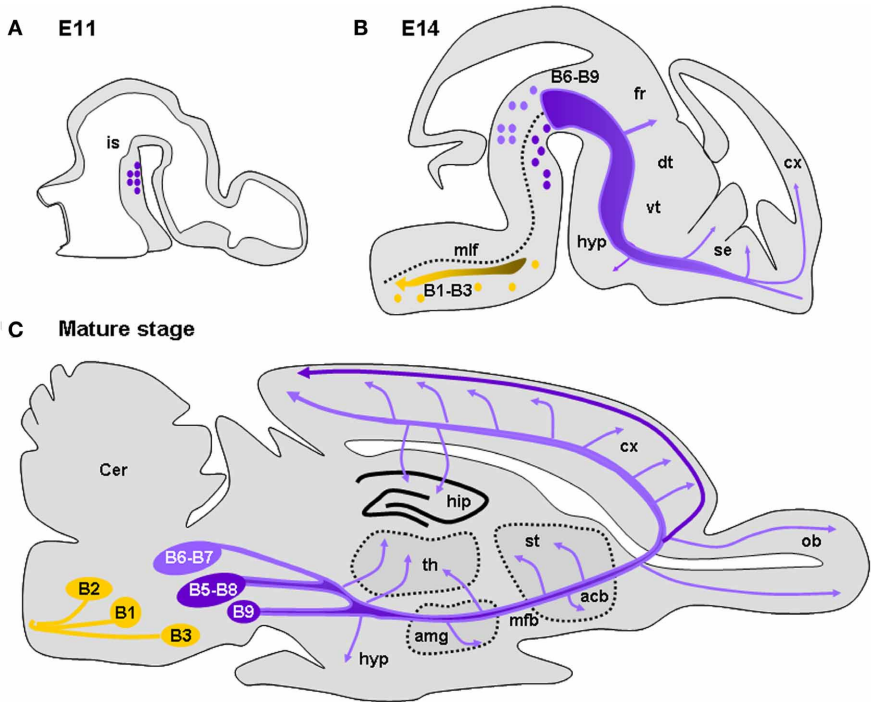

FIGURE 1 | Development and location of serotonergic neurons and projections. (A) Drawing of a sagittal section of an E11 mouse brain illustrating the location of 5-HT neurons caudal to the isthmus (is). **(B)** Drawing of a sagittal section of an E15 mouse brain showing the 5-HT-containing cell groups and their main projections. 5-HT cell groups are classically distributed in nine groups (B1–B9). The posterior groups (B1–B3; yellow dots) mainly consist in raphe magnus sending projections to the medulla, the spinal cord, and the periphery (yellow dots and arrow). The raphe dorsal (B6, B7) located dorsal to the medial longitudinal fasciculus (mlf) and the raphe median (B5, B8) send ascending serotonergic innervations destine to the telencephalon and diencephalon (purple). Serotonergic afferents innervating the forebrain travel initially together along the median forebrain bundle (mfb). Arrows indicate regions where axons are seen to deviate from the major ascending pathway: along the fasciculus retroflexus (fr) toward the habenula, in the hypothalamus (hyp), the striatum, and the septum (se). **(C)** Drawing of a sagittal section of a mature mouse brain showing the 5-HT-containing cell groups and their main projections. Arrows indicate regions highly innervated by 5-HT afferents: the hypothalamus (hyp), the amygdala (amg), the thalamus (th), the accumbens (acc), the striatum (st), the olfactory bulb (ob), the cerebral cortex (cx), and the hippothalamus (hip). In the cerebral cortex, layers V and VI receive preferentially afferents arising from the dorsal raphe (light purple) while layer I receives afferents mainly arising from the median raphe (dark purple). Drawings are adapted from Wallace and Lauder (1983) and Steinbusch and Nieuwenhuys (1983).

remained elusive. Evidence indicates that 5-HT is supplied to the developing cerebral cortex before 5-HT axons reach their targets or even before serotonergic neurons are generated. In line with this observation, 5-HT receptors are expressed in the rostral forebrain, craniofacial region, and peripheral region days before serotonergic axons enter these regions (Buznikov et al., 2001). Furthermore, *ex vivo* application of 5-HT or alteration of 5-HT levels during early embryonic stages can alter normal development of various embryonic structures before serotonergic neurons have innervated these structures (Lauder, 1988; Shuey et al., 1992; Moiseiwitsch and Lauder, 1995; Whitaker-Azmitia et al., 1996; Buznikov et al., 2001; Witaker-Azmitia, 2001). Recently, the placenta (that is of embryonic origin) has been identified as an important source of 5-HT for the developing embryo (Bonnin et al., 2011; **Figure 2**). Syncytiotrophoblastic cells of the placenta contain Tph1, AADC, and MAO (Grimsby et al., 1990; Shih et al., 1990), and convert tryptophan of maternal origin into 5-HT as soon as E10–E11 (Bonnin et al., 2011). Genetically modified mice in which 5-HT neurons fail to fully differentiate or to produce normal amounts of 5-HT levels do not display severe cortical defects when gestating in heterozygous dams with an almost unaltered serotonergic system, suggesting that sources of 5-HT independent of embryonic serotonergic neurons could be sufficient to permit normal cortical development. Examples include mice lacking the transcription factors Lmx1b (Smidt et al., 2000) or Pet-1 (Hendricks et al., 1999), in which all or 70–80% of 5-HT raphe neurons fail to develop, respectively, and in mice lacking Tph2 Alenina et al., 2009; Gutknecht et al., 2012; Migliarini et al., 2012. Further analysis revealed that Pet-1 knockout embryos developing in heterozygous dams have normal 5-HT levels before the closure of the brain-blood barrier (before E15; Daneman et al., 2010). In addition, $SERT^{+/-}$ embryos developing in $SERT^{-/-}$ or wild type dams had similar levels of 5-HT before E15 (Bonnin et al., 2011). Together, these results revealed that the placenta is an important source of 5-HT for the embryonic CNS before E15 but questioned the contribution of maternal 5-HT that was suspected in earlier studies (Shuey et al., 1992; Yavarone et al., 1993; Côté et al., 2003, 2007).

Outside the CNS, 5-HT is also synthesized in the periphery of the developing embryo. In particular, high levels of 5-HT are

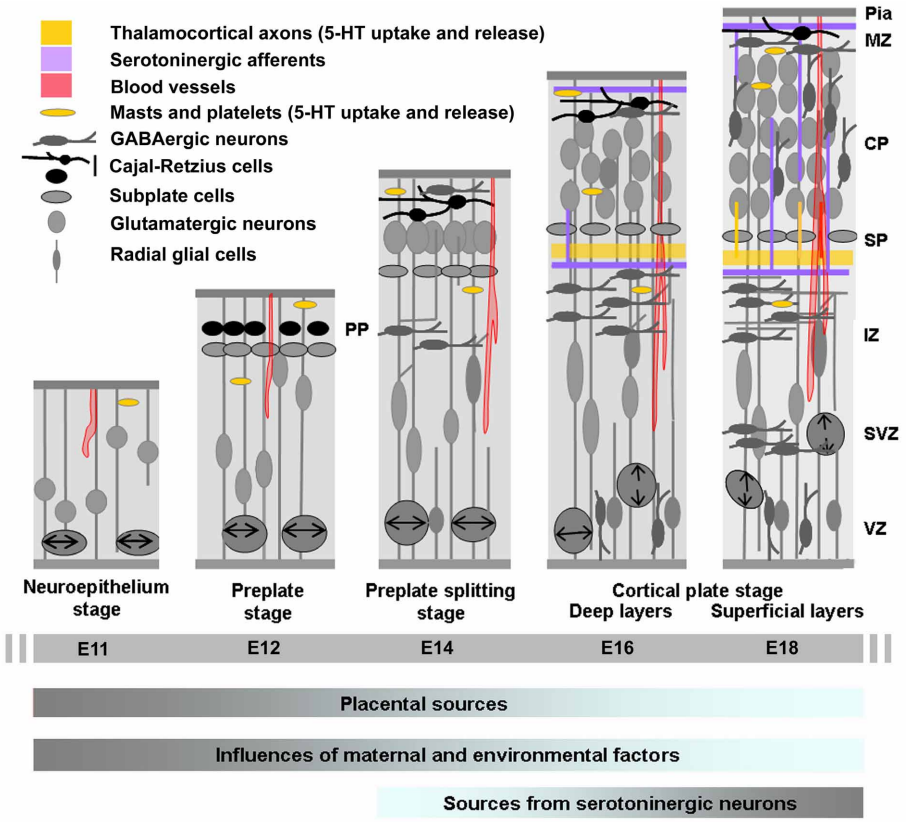

FIGURE 2 | Cortical development in relation to sources of 5-HT. Cortical neurogenesis in the mouse neocortex occurs from embryonic day E10–11 (left) to E17 (right) begins with an intense proliferation of the progenitor cells located in the ventricular zone (VZ) of the subpallium and more ventrally of the pallium (not shown in the drawing). These populations of cells give rise to most of the GABAergic neurons (subpallium) and glutamatergic neurons and glial cells (pallium) of the cerebral cortex. Once generated, neurons migrate toward the pial surface and complete their differentiation in the cortical plate (CP). Glutamatergic neurons destined to populate the deeper layers of the cortex are generated and then migrate away form the VZ earlier than the neurons destined for progressively more superficial layers. GABAergic neurons arise from more ventral structure and migrate tangentially in the developing CP. On E13, the cerebral wall is bilaminar consisting of the VZ and overlying primitive plexiform layer. By E17–E20 the thickness of the overlying intermediate zone/with matter and developing cortical plate are at their maximum widths, with all neuronal cells having exited the cell cycle and migrated to their final laminar distribution within the developing cortex. At this stage GABAergic neurons enter the CP by radial migration. The cortical anlage is vascularized very early and carries platelets and mast cells that could provide 5-HT to the developing embryo. During the initial phase of cortical development 5-HT is mainly synthesized in the placenta while later on it is produced by serotonergic neurons of the embryo (gray is high and blue is low). IZ, intermediate zone; PPL, primordial plexiform layer; SP, subplate; SVZ, subventricular zone [Adapted from Uylings et al. (1990) and Corbin et al. (2008)].

produced in the myenteric plexus (from E15 to E16), by enterochromaffin cells of the lining lumen of the digestive tract (from E18), by neuroepithelial cells of the respiratory tracts, by pinealocytes (from E11 to E12) and by parafollicular cells of the thyroid. After being released from 5-HT producing cells, 5-HT could be taken up by SERT expressing cells including platelets and mast cells (Jankovic, 1989; Zhuang et al., 1996) that become numerous around E12 in mice. These cells could cross the blood-brain barrier and transit across blood vessels that start to invade the developing cortex by E10–E11 in mice (Daneman et al., 2010). However, overall peripheral structures are thought to contribute only to a small proportion of cortical 5-HT during development.

In addition, sensory thalamic neurons projecting to primary sensory cortices (i.e., somatosensory, visual, auditory) transiently express SERT (E15–P15) and the vesicular monoamine transporter type 2 (VMAT2) that are respectively responsible for the uptake and packaging of 5-HT into synaptic vesicles (Cases et al., 1996, 1998; Vitalis et al., 1998; Lebrand et al., 1996, 1998; Gaspar et al., 2003; Vitalis and Parnavelas, 2003; **Figure 2**). While equipped with these transporters, thalamic neurons may release 5-HT in an activity-dependent fashion by transiently adopting a serotonergic phenotype even without expressing TPH or MAOs (Vitalis et al., 2002a). Interestingly, it has been suggested that TCAs could be implicated in the proliferation and migration of glutamatergic neurons, and it is thus possible that release of 5-HT by TCAs could contribute to the regulation of these processes (Kennedy and Dehay, 1997; Edgar and Price, 2001). Fate mapping of SERT-expressing cells in mice revealed that in addition to the thalamus, also the cortex, hippocampus, hypothalamus, and brainstem harbor neurons that transiently adopt a serotonergic phenotype (Narboux-Nême et al., 2008). Within the cortex, transient SERT expression starts between E15 and P0 and is confined

to layers V and VI (infralimbic, prelimbic, and anterior cingulate cortex) or layers II, V, and VI (posterior cingulate and retrosplenial cortex). The role of 5-HT signaling by these neurons remains to be elucidated. However, because of the spatial and temporal aspects of this phenomenon, it is tempting to speculate that transient serotonergic neurons might influence cortical maturation and circuit formation.

5-HT RECEPTORS WITH SPECIFIC ATTENTION TO THE 5-HT$_{3A}$ AND 5-HT$_6$ SUBTYPES

TRANSDUCTION PATHWAYS

At least 14 genes that encode for 5-HT receptors have been identified and cloned in the mammalian brain (Hoyer et al., 1994, 2002; Raymond et al., 2001; Hannon and Hoyer, 2008; Millan et al., 2008). In addition, alternative splicing and RNA editing add to the diversity of 5-HT receptors. With the exception of the 5-HT$_3$ receptors, all 5-HT receptors are coupled to G-proteins, leading to a categorization into four groups according to their second messenger coupling pathways. The 5-HT$_1$ and 5-HT$_5$ receptors are coupled to Gi/Go proteins and exert their inhibitory effects on adenylate cyclase inhibiting cAMP formation. The 5-HT$_2$ receptors are coupled to Gq proteins and stimulate phospholipase C to increase the hydrolysis of inositol phosphates and elevate intracellular Ca^{2+}. The 5-HT$_{4,6,7}$ receptors are coupled to Gs proteins and are positively linked to adenylate cyclase and increase cAMP formation. 5-HT$_3$ (5-HT$_{3A}$ and 5-HT$_{3B}$) receptors belong to a family of ligand-gated ion channel receptors that include nicotinic acetylcholine receptors, GABA$_A$ receptors, and glycine receptors and that are modulated by intracellular cyclic AMP (Hoyer et al., 1994). The 5-HT$_3$ receptors respond to neurotransmitter release via direct (through the 5-HT$_3$ receptor itself) or indirect (via the activation of the voltage-gated Ca^{2+} channels) increase of Ca^{2+} entry into the cell (reviewed in Chameau and van Hooft, 2006). 5-HT$_3$ receptors are composed of five subunits, with the majority being homomers of 5-HT$_{3A}$ receptors. Heteromeric 5-HT$_{3AB}$ receptors have been observed in specific brain regions and display lower Ca^{2+} permeability than the homomeric 5-HT$_{3A}$ receptors (Tecott et al., 1993; Morales and Bloom, 1997; Davies et al., 1999; Morales and Wang, 2002). Furthermore, the co-assembly of the 5-HT$_3$ with the alpha4 subunit of the nicotinic acetylcholine has been reported to confer increased permeability to Ca^{2+} (Kriegler et al., 1999; Chameau and van Hooft, 2006).

EXPRESSION PATTERNS

The expression of 5-HT receptors during cortical development is not yet fully characterized. However, the recent use of transgenic animals (i.e., carrying the GFP/YFP under the control of a specific 5-HTR promoter) and open *in situ* hybridization databases (i.e., Allen Brain Atlas) have started to provide valuable insights. For example, 5-HT$_{1A,F}$ are expressed in neocortical proliferative zones in E14.5 rodent brain (Hillion et al., 1994; Bonnin et al., 2006) and the 5-HT$_{2B}$ are expressed in the proliferative zones of the human occipital cortex (Lidov and Rakic, 1995). The 5-HT$_{1A,B,D}$, 5-HT$_{2A}$, 5-HT$_{2C}$, and 5-HT$_{3A}$, are expressed in specific subpopulations of postmitotic neurons (Hillion et al., 1994; Johnson and Heinemann, 1995; Tecott et al., 1993; Morales and Bloom, 1997; Bonnin et al., 2006; Chameau et al., 2009; Vucurovic et al., 2010; Tanaka et al., 2012), whereas the 5-HT$_6$ is expressed in both migrating interneurons and pyramidal neurons (Riccio et al., 2011; **Figure 3**). Although a complete developmental time-course of 5-HT$_6$ expression in the dorsal pallium is not available, 5-HT$_6$ expression is detected in the developing rat brain as early as E12 and is maintained stable until adult age (Grimaldi et al., 1998). In adulthood, 5-HT$_6$ receptors are expressed in layers II–VI of the rodent postnatal and mature cerebral cortex (Ward et al., 1995; Hamon et al., 1999; Gerard et al., 1997), and pyramidal neurons and glial cells of the human prefrontal cortex (Marazziti et al., 2013). Interestingly, human prefrontal cortex expression of the 5-HT$_6$ receptor peaks in toddlers (Lambe et al., 2011).

The dynamic expression pattern of the 5-HT$_{3A}$ receptor is recapitulated in **Figure 4**. In the mouse cortical anlage, 5-HT$_{3A}$ is expressed as early as E12 in PP neurons expressing reelin (C-R cells) and/or GABA (Chameau et al., 2009; Vucurovic et al., 2010). During the period of intense production of GABAergic neurons, the 5-HT$_{3A}$ is expressed by newly postmitotic (Tuj-1+) neurons located in the CGE and AEP/PO, where about 30% of cortical GABAergic neurons are generated (Lee et al., 2010; Vucurovic et al., 2010). Using homochronic *in utero* grafting in combination with a transgenic mouse line expressing GFP under the control of the 5-HT$_{3A}$ promoter (5-HT$_{3A}$:GFP animals) we have shown that this expression was protracted

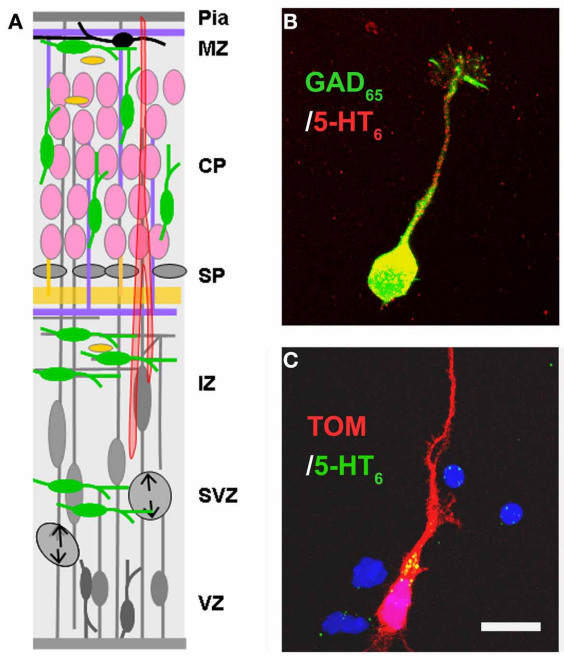

FIGURE 3 | Expression of the 5-HT$_6$ receptor. (A) Drawing shown in **Figure 2** has been modified in order to depict the expression of 5-HT$_6$ at E17–E18. At this stage 5-HT$_6$ is expressed in developing interneurons (green), in differentiating pyramidal neurons (pink). **(B,C)** Expression of 5-HT$_6$ in cell cultures (E17 + 1DIV) in GABAergic neurons expressing GAD$_{65}$ (green) and in pyramidal neurons labeled with TOM after *in utero* electroporation performed at E14.5. Scale bar: 10 μm.

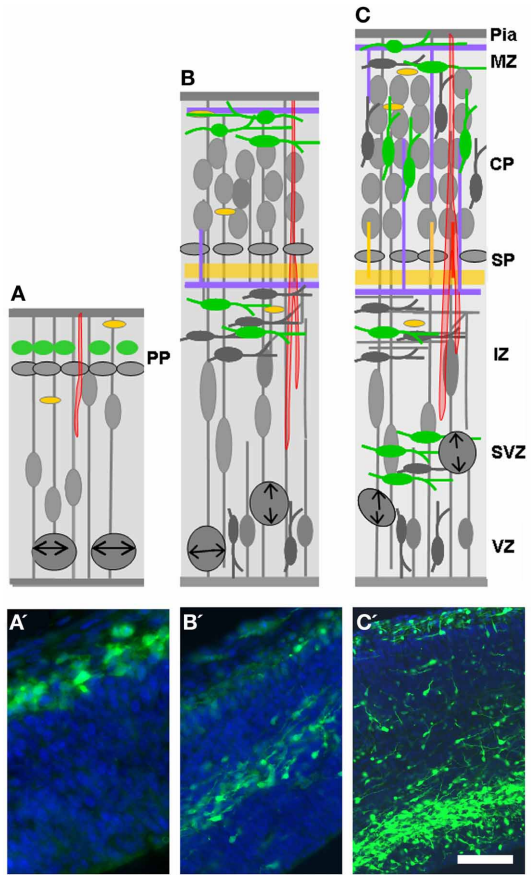

FIGURE 4 | Expression of the 5-HT$_{3A}$ receptor. (A–C) Drawings shown in **Figure 2** have been modified in order to depict the expression of 5-HT$_{3A}$ (green) during development. Note that 5-HT$_{3A}$ is expressed by pioneer Cajal-Retzius cells of the marginal zone (MZ; **A**, E12) and by a subpopulation of late generated GABAergic neurons arising from the CGE (E14 and E16–E17). **(A′–C′)** Corresponding photomicrographs of the drawings presented in (**A–C**). Scale bar: **(A)** 20 μm; **(B)** 100 μm; **(C)** 120 μm.

in two large subpopulations of cortical GABAergic neurons that could be distinguished based on their electrophysiological properties, molecular contents, and morphologies. The first one corresponded to multipolar interneurons expressing NPY and displaying late spiking and accommodating properties while the second one corresponded to small bipolar and doublet bouquet interneurons expressing VIP and displaying adapting and bursting properties (Vucurovic et al., 2010; Lee et al., 2010; Rudy et al., 2011; Vitalis and Rossier, 2011). During postnatal stages and decreasing with age 5-HT$_{3A}$ receptors are also expressed in young neurons (doublecortin+ and/or CR+), which are generated in the SVZ and migrate toward the olfactory bulb and various cortical and subcortical regions (Inta et al., 2008; Riccio et al., 2012). In addition, we recently found that 5-HT$_{3A}$ receptors are expressed during postnatal development (P0–21) in a pool of migrating interneurons, which are probably generated from local transient amplifying precursors within the white matter, ventral to the anterior cingulate cortex (Riccio et al., 2012).

IMPACT OF 5-HT IMBALANCE ON CORTICAL CIRCUIT ASSEMBLY

5-HT AND CELL PROLIFERATION

It has been postulated for some time that 5-HT regulates the proliferation of a wide variety of cell types including cortical neurons. Indeed, studies that pharmacologically or genetically deplete maternal and embryonic brain 5-HT levels or restrict tryptophan availability have found reduced embryonic brain size as a major consequence. Chronic pCholophenylalanin (PCPA)-treatment, which inhibits 5-HT synthesis, alters the proliferation of serotonergic target cells (i.e., the hippocampal field and cerebral cortex) when administrated daily to pregnant dams from E8 to E12 (Lauder and Krebs, 1978) or from E12 to E17 (Vitalis et al., 2007). Similar observations were made after reserpine-treatments that deplete 5-HT (Holson et al., 1994), or after lesions of serotonergic fibers such as those observed after high cocaine administration (Clarke et al., 1996). However, there are several drawbacks in these initial studies. For example, chronic treatments are likely to induce secondary alterations, which might be ultimately responsible for the effects observed. Another major problem is the selectivity of the neurotransmitter system affected. This is particularly problematic for reserpine-treatments that deplete all monoamines. Recently, the generation of transgenic models selectively targeting specific 5-HT-related genes in different neuronal populations have started to provide more specific insights. For instance mice deficient for *tph1* or *tph2* showed body weight reduction and delayed maturation of upper cortical layers (Côté et al., 2007; Alenina et al., 2009; Narboux-Neme et al., 2013). A 2 h pulse labeling experiment revealed that heterozygous embryos growing in null mutant tph1$^{-/-}$ mice showed an ∼30% reduction of BrdU-positive cells in the VZ when compared to tph1$^{-/-}$ embryos growing in heterozygous mice (Côté et al., 2007). Together these studies suggest that 5-HT regulates the proliferation of neuronal precursors, but additional studies are needed to refine these initial observations and confirm this conclusion.

Initial *in vitro* studies have failed to show that 5-HT could modulate the proliferation of cortical progenitors (Dooley et al., 1997; Lavdas et al., 1997), as the proportion of cells that integrated BrdU was similar in untreated and treated cultures. However, since 5-HT had an anti-apoptotic effect the dilution of BrdU+ cells may have masked this proliferative effect. Furthermore, it was demonstrated that stimulation of the 5-HT$_2$ and 5-HT$_3$ had no effect on cortical neurogenesis (Dooley et al., 1997; Vitalis and Parnavelas, 2003). This is consistent with the fact that 5-HT$_{3A}$ is not expressed in pallial and subpallial proliferative zones (Vucurovic et al., 2010). In contrast, the 5-HT$_{1A}$ appears to mediate such a role. *In vivo*, PCPA-induced microcephaly is reversed after treatment with a 5-HT$_{1A}$ agonist. Furthermore, in the adult rodent brain, 5-HT$_{1A}$ promotes neurogenesis in the subgranular zone of the dentate gyrus (Brezum and Daszuta, 1999, 2000; Gould, 1999; Haring and Yan, 1999) and such a role has been postulated to be a key feature of antidepressant therapies (Guthrie and Gilula, 1989; Santarelli et al., 2003). Recently, the analysis of mice lacking MAOA and B, which display high 5-HT levels but normal dopamine and norepinephrine levels during embryonic and early postnatal development, revealed a specific reduction

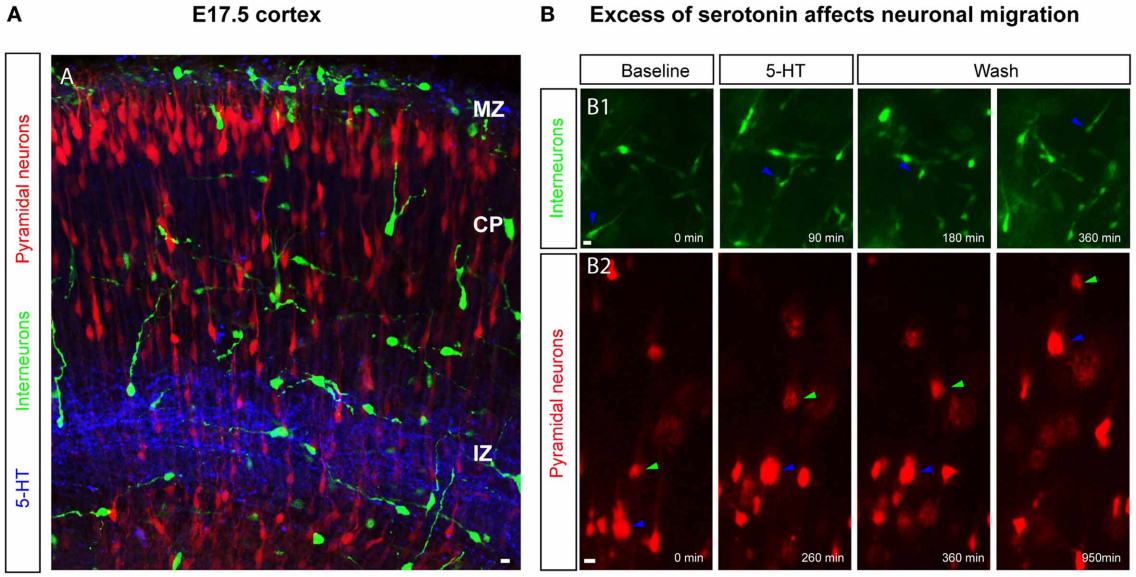

FIGURE 5 | 5-HT levels impact the migration of GABAergic and glutamatergic neurons. (A) 5-HT immunoreactivity is present in the marginal zone (MZ) and in the intermediate zone (IZ) at embryonic day E17.5, a time point when pyramidal neurons and cortical interneurons are migrating to constitute upper cortical layers. Upper-layer pyramidal neurons (in red) are labeled through electroporation of progenitor cells in the dorsal pallium at E14.5 or E16.5 with a Tomato-expressing plasmid and interneurons are labeled using GAD65-GFP or 5-HT_{3A}-GFP transgenic mice. **(B)** Time-lapse imaging on cortical slices revealed that an excess of 5-HT and 5-HT_6 manipulation affects the migration of cortical interneurons (B1) and pyramidal neurons (B2) in a reversible manner. Scale bar: 10 μm.

of symmetric divisions of intermediate precursor cells (Corbin et al., 2008) in the SVZ during late corticogenesis (E17.5; Cheng et al., 2010). This unexpected alteration was reverted after E14.5–E19.5 PCPA-treatment. In addition, neurosphere formation was modulated by 5-HT in a dose-dependent manner *in vitro*, with proliferative effects observed for concentration ranging from 10 to 100 ng/ml and inhibitory effects observed for higher concentration (1000 ng/ml). Interestingly, these inhibitory effects were associated with decreased 5-HT_{1A} labeling of neuronal precursors (Cheng et al., 2010). Together, these studies identified 5-HT_{1A} as a largely positive regulator of neuronal proliferation in embryonic and postnatal life. Hence, 5-HT might modulate cortical density through its proliferation-inducing action on progenitors.

Additional mechanisms exist through which 5-HT could potentially modulate proliferation and cortical density. 5-HT could be involved in modulating the length of the cell cycle or participate in progenitor cell death regulation. Interestingly, E12–E17 PCPA-treatment reduces the number of cells expressing Ki67 (a proliferation marker), promotes early GFAP expression, and impairs the normal development/organization of radial glial processes (Vitalis et al., 2007). In turn, early differentiation of RGCs could reduce cortical neurogenesis. Alternatively, hypo-5-HT induced microcephaly could be due to increased death of postmitotic neurons or neuronal progenitors. Indeed, 5-HT_2 stimulation promotes the survival of glutamatergic neurons *in vitro* with a maximal effect observed for stages E16 and E18 in rats (Dooley et al., 1997), and 5-HT_{1A} stimulation increases neuroprotection in models of ischemia and protects neuronal cultures against serum withdrawal (Bielenberg and Burkhardt, 1990; Azmitia et al., 1995; Ahlemeyer et al., 2000). Furthermore, activation of 5-HT_2 reverts increased apoptosis observed in VMAT2:KO mice, in which dopamine, norepinephrine, and serotonin are depleted (Stankovski et al., 2007). Such a role was also observed in mice lacking TrkB, the high affinity receptor for the brain-derived neurotrophins factor (BDNF) and neurotrophin 4, and in both cases 5-HT_2 activation was able to normalize the caspase 3–9 cascades (Vitalis et al., 2002b; Stankovski et al., 2007).

During early development, 5-HT could also influence cortical proliferation through the modulation of gap junctions that coordinate cell-cell assembly (Guthrie and Gilula, 1989; Lo Turco and Kriegstein, 1995; Bittman et al., 1997). Interestingly, monoaminergic receptor activation modulates postnatal gap junction coupling in various brain regions including the developing neocortex, where regulation appears to occur at the level of connexin subunit phosphorylation (Roerig and Feller, 2000). Pharmacologic evidence suggests that 5-HT promotes uncoupling of gap junctions through 5-HT_{2R} stimulation (Roerig and Feller, 2000). However, to our knowledge, no study has investigated the action of 5-HT receptor modulation on gap junction coupling in the embryonic cortex.

5-HT AND NEURONAL MIGRATION

5-HT modulates the migration of various cell types and this effect is maintained across most phyla. For example, 5-HT acts as a permissive signal that triggers cell motility of mature lymphocytes in the vertebrate immune system (chick, fish, rodent; Khan and Deschaux, 1997; Boehme et al., 2004) and of microglial cells toward the central nervous system (Krabbe et al., 2012). In the non-vertebrate developing CNS a role for 5-HT in promoting-directed neuronal migration has been reported for

Caenorhabditis elegans (Kindt et al., 2002). In the mammalian cortex, a role for 5-HT in regulating the migration of cortical neurons has emerged recently with studies focused on the late phase of corticogenesis. Using a pharmacological approach and cortical slices, high 5-HT levels have been shown to decrease the migratory speed of non-GABAergic and GABAergic neurons (Riccio et al., 2009, 2011; **Figure 5**). In cortical explants of E17.5 or P0 mouse brain, in which pyramidal neurons were labeled by *in utero* electroporation at E14.5 or E16.5 respectively, neuronal migration was analysed using video-microscopy in control condition or after acute bath application of 5-HT. This study revealed that acute application of high 5-HT concentration leads to a reversible decrease in the migration speed of glutamatergic neurons running in the IZ. Interestingly, $SERT^{-/-}$ mice exhibit an abnormal distribution of pyramidal neurons in the most superficial regions of the CP at E19 (presumptive layers II–III) suggesting that 5-HT excess could lead to a delay in the migration of cortical pyramidal neurons *in vivo*. Furthermore, activation of the 5-HT_6 receptor recapitulates these events: application of a specific 5-HT_6 agonist to E17.5 or P0.5 cortical explants reduced the migratory speed of pyramidal neurons labeled at E14.5 or E16.5 respectively, suggesting that the 5-HT_6 receptor is involved in regulating neuronal migration (Riccio et al., 2011). Similarly to non-GABAergic neurons, GABAergic neurons expressing GAD_{65} reversibly and in a dose-dependent manner decrease their migratory speed following acute high levels of 5-HT application *ex vivo* (Riccio et al., 2009). 5-HT also induced a retraction of the leading processes of GABAergic neurons migrating into the IZ and CP. RT-PCR performed on cells sorted by flow cytometry and obtained from E18.5 cortical slices of GAD_{65}:GFP mice, revealed that these cells expressed the 5-HT_{3A} and the 5-HT_6 receptors. Again, 5-HT_6 agonist application mimicked 5-HT-induced effects on GABAergic neurons. Furthermore pharmacological manipulation of the cAMP-signaling pathway partially modulates the 5-HT_6 mediated effects on cortical interneuron migration (Riccio et al., 2009). Interestingly, recent large-scale proteomic strategies have revealed that the 5-HT_6 receptor binds to a large variety of signaling molecules that play a critical role during brain development including the mTOR pathway (Meffre et al., 2012). It is thus likely that the effects on migration elicited by the pharmacological manipulation of 5-HT_6 receptors also involve these signaling pathways. Studies are currently underway to test this hypothesis.

It must be noted that the impact of 5-HT on the migration of cortical neurons was revealed using high doses of 5-HT. As in other cell types (Moiseiwitsch and Lauder, 1995), it is possible that 5-HT produces opposite effects on neuronal migration depending on the levels of extracellular 5-HT. In cortical explants maintained in a serum-free medium containing lower concentration of 5-HT than those used in experiments described above (5 uM), glutamatergic neurons reach their laminar location faster than in explants maintained in serum-free medium alone, suggesting that 5-HT may enhance the radial migration of these neurons (Lepore et al., 2001). Furthermore, decreasing 5-HT levels during development delayed or disrupted cortical migration suggesting 5-HT could also act as a positive drive on cortical migration (Stankovski et al., 2007; Vitalis et al., 2007). In animals treated with PCPA during the peak of migration (E12/E13 to E17 in rats), GABAergic neurons accumulated at the level of the SP and showed a marked deficit to integrate in the developing CP (Vitalis et al., 2007). Long-lasting consequences of E12–E17 PCPA-treatment lead to a marked reduction of CR- and CCK/VIP-positive GABAergic neurons, two neuronal populations that express the 5-HT_{3A} receptor (Férézou et al., 2007). Interestingly, mice lacking Tph2 also display reductions of selective GABAergic populations in limbic structures (Waider et al., 2013). 5-HT_{3A} is protractedly expressed by 30% of GABAergic neurons and it could be that this population is particularly sensitive to 5-HT depletion during corticogenesis. 5-HT_{3A} is associated with F-actin that decorates the tips of the of dendritic and axonal growth cones. Interestingly, pharmacological alteration of F-actin induced a modification in the distribution of 5-HT_{3A} (Emerit et al., 2002). In addition, 5-HT_{3A} mediates calcium entry into the cell (see above). Together these results suggest that 5-HT_{3A} activation could play a role in promoting the migration of cortical interneurons. Such a role is under investigation.

5-HT AND DIFFERENTIATION

Lauder and Krebs were the first to report that 5-HT depletion delays neuronal maturation in areas normally receiving 5-HT afferents (Lauder and Krebs, 1978; Lauder, 1993). These investigators defined differentiation as the cessation of cell division measured by incorporation of ^{3}H-thymidine. After these pioneering studies, numerous groups have shown that 5-HT can influence neuritic outgrowth in various phyla (such a role was intensively investigated in Aplysia) and in various regions of the CNS (Haydon et al., 1984, 1987; Whitaker-Azmitia et al., 1996; Lieske et al., 1999; Lotto et al., 1999; Kondoh et al., 2004; Fricker et al., 2005 and see below). Here we review the role for 5-HT on dendritic and axonal morphogenesis during cortical development.

5-HT and dendritic maturation of cortical neurons

After termination of neuronal migration, cortical neuron subtypes differentiate at their specific laminar position and assemble into precise cortical circuits. During this process, projection neurons extend an elaborated dendritic arbor, which is contacted by the axons of different subtypes of excitatory neurons and inhibitory interneurons in a subdomain-specific manner. The molecular rules that govern the precise connectivity between different subtypes of inhibitory interneurons and excitatory projection neurons are largely unknown. In this context, reelin-secreting C-R cells have been identified as key regulators of cortical development, including neural migration, neural positioning, and dendritic arborization (Supèr et al., 2000; Soriano and del Rio, 2005; Lakatosova and Ostatnikova, 2012). C-R cells receive serotonergic projections with which they make transient synaptic contacts (Janusonis et al., 2004). Reelin secretion is regulated in part by the amount of brain 5-HT during late embryogenesis since 5-methoxytryptamine, a broad 5-HT receptor agonist, reduces reelin levels circulating in the blood at P0 (Janusonis et al., 2004). Reduced reelin levels in turn lead to malformation of microcolumns in the presubicular cortex of the P7 rat pups.

Microcollumns are the basic microcircuit-units of the cortex (Jones, 2000; Mountcastle, 2003), and intriguingly are structurally abnormal in autism spectrum disorder (ASD). The 5-HT_{3A} is expressed by ∼80% of C-R cells at P0 and its synaptic activation is sufficient to induce action-potential firing of C-R cells, suggesting that 5-HT_{3A} could play a role in regulating reelin release and dendritic development (Chameau et al., 2009). Indeed, developmental 5-HT_{3A} blockade induces a hypercomplexity of apical dendrites of layers II–III pyramidal neurons sparing the basal dendrites (Janusonis et al., 2004). In line with this finding, application of the N-terminal region of reelin rescued the dendritic phenotype of cortical pyramidal neurons in 5-HT_{3A}:KO cortical slices, whereas reelin blockade leads to increased growth of apical dendrites (Chameau et al., 2009). These data suggest that, increased reelin secretion due to over-activation of the 5-HT_{3A} receptor would decrease growth of apical dendrites. This hypothesis was recently investigated *in vivo* using selective 5-HT reuptake inhibitors (SSRI). Interestingly, fluoxetine administration from E8 to E18 decreases the dendritic basal and apical arbor complexity of layer II/III pyramidal neurons in the somatosensory cortex. This effect is specific to the developmental period as SSRI have opposite consequences at mature stages (Table 1 in Homberg et al., 2009). Furthermore, the effects of SSRIs on developing dendrites were abolished when administered to 5-HT_{3A}:KO mice or after pharmacological blockade of the 5-HT_{3A} receptor (Chameau et al., 2009; Smit-Rigter et al., 2012). Moreover, 5-HT_{3A} signaling is responsible for the anxiety-like behaviors that are induced by prenatal fluoxetine treatment in wild type mice (Smit-Rigter et al., 2010). These results suggest that developmental excess of 5-HT increases reelin secretion by over-activating 5-HT_{3A} receptors expressed on C-R cells, consequently inhibiting dendritic growth of pyramidal neurons.

However, other 5-HT receptors may contribute to modulating the morphology of cortical neurons. The 5-HT_{1A} receptor for example is also known to modulate dendritic development (Sikich et al., 1990; Ferreira et al., 2010). Although its role has not been investigated in the cerebral cortex, several studies have clearly shown and dissected its role in the hippocampus. Indeed, mice lacking 5-HT_{1A} display increased dendritic arborization of CA1 pyramidal cells associated with cognitive impairments (Klemenhagen et al., 2006; Tsetsenis et al., 2007). Furthermore, the use of conditional expression of 5-HT_{1A} in mice otherwise lacking this receptor revealed that it is playing a critical role during the postnatal window corresponding to dendritic maturation of CA1 pyramidal neurons (Gross et al., 2002). During this time 5-HT_{1A} appears to limit dendritic growth cone retraction and extension by possibly remodeling actin filaments (Ferreira et al., 2010). As 5-HT_{1A} is strongly expressed in the developing CP (Figure 1 in Bonnin et al., 2006) such a role could also be expected for cortical neurons. Together these studies suggest that a fine tuning of 5-HT_{1A} activation may be required for appropriate dendritic maturation of cortical neurons. Finally, one should keep in mind that 5-HT also act as a trophic factor during development and 5-HT deficiency induces a reduction of dendritic arborization and complexity. Indeed, animals fed with low tryptophan diet (González-Burgos et al., 1996; Feria-Velasco et al., 2002) or depleted of 5-HT during the embryonic period (Vitalis et al., 2007) display cortical pyramidal neurons with decreased dendritic complexity and spine density. It is thus probable that 5-HT regulates dendritic maturation and spine density through different types of 5-HT receptors that remain to be identified.

5-HT and axonal development within the cerebral cortex

The first clear demonstration that 5-HT acts on cellular processes involved in the formation of cortical circuits comes from the work performed on the rodent somatosensory cortex (**Figure 6**). The serendipitous generation of a mouse displaying deficiency in the gene encoding for MAOA was at the starting point of these discoveries. These studies showed that excessive 5-HT amounts (nine-fold increase at P0) in the developing cortex induced an abnormal organization of TCAs growing in the layer IV of the primary somatosensory cortex (Cases et al., 1995, 1996; **Figure 7**). These alterations, that were later interpreted as an abnormal refining of TC axons, are due to a specific rise of 5-HT occurring during early postnatal development (P0–P4). Indeed, such alterations could be induced in wild type rodents by pharmacological inactivation of MAOA during this sensitive period (Vitalis et al., 1998).

In addition, pharmacological normalization of 5-HT levels in MAOA:KO mice by P0–P4 PCPA-treatment was sufficient to normalize the organization of S1 in MAOA:KO mice (Cases et al., 1996; **Figure 7**). Therefore, the first few days after birth represent the sensitive time-period for 5-HT effects on axonal segregation in the rodent barrel cortex. Later, it was shown that genetic SERT deficiency affected S1 organization similarly. The 5-HT 5-HT_{1B} and 5-HT_{1D} receptors, that are transiently expressed on TC axons during development, play a key role in this process, since the barrel cortex phenotype is rescued in SERT:KO and MAOA:KO mice that are also deficient for 5-HT_{1B} receptors (Persico et al., 2001; Salichon et al., 2001; Rebsam et al., 2002; van Kleef et al., 2012; **Figure 7**). The general model thus supports the view that increased extracellular levels of 5-HT lead to an over-activation of 5-HT_{1B} receptors expressed on TCAs. This increased 5-HT_{1B} signaling may inhibit glutamate release by TCAs and impair barrel cortex formation directly at presynaptic and indirectly at postsynaptic levels. Interestingly, 5-HT excess does not only impair S1 organization, since abnormal axonal patterning of TCAs was also observed in the primary visual cortex (Upton et al., 1999; Salichon et al., 2001). This intriguing role of 5-HT signaling during circuit formation may apply to all primary sensory cortices that are innervated by neurons transiently capable of 5-HT uptake (Hansson et al., 1998; Lebrand et al., 1998). Surprisingly, perinatal 5-HT deficiency induces only little changes on the organization of TCAs. Lowering brain 5-HT levels prenatally using PCPA or PCA only leads to a reduction of barrel field size (20% average) without altering its general organization (Bennett-Clarke et al., 1994; Osterheld-Haas et al., 1994; Narboux-Neme et al., 2013).

Although no evidence to date indicates that developmental excess of 5-HT during stages of embryonic developmental directly affects the patterning of TCAs, it has been shown that TCAs express 5-HT_{1B} and 5-HT_{1D} receptors at a time when TCAs are navigating from the subpallium toward the pallium (Bonnin et al., 2007). *In vivo* embryonic down-regulation of 5-$HT_{1B/C}$ receptors in TCAs using *in utero* electroporation leads to abnormal TCA

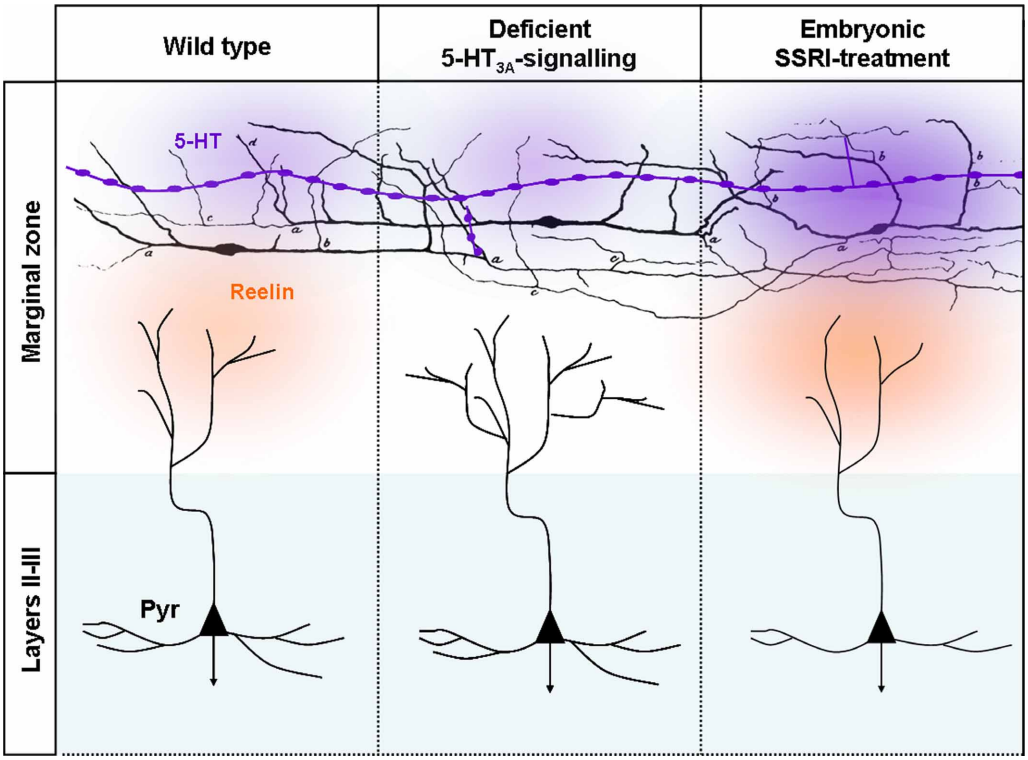

FIGURE 6 | Deficient 5-HT$_{3A}$-signaling or embryonic SSRI-treatment alters the morphologies layers II–III pyramidal dendrites. Serotonergic afferents (purple) containing large vesicles rich in 5-HT are located in the marginal zone and below the cortical plate that send branches toward MZ. 5-HT could be released from the entirety of the axonal length. In MZ, 5-HT fibers make synaptic contacts with C-R neurons that were initially drawn by Cajal (1891). In control conditions 5-HT activates 5-TH$_{3A}$ receptors located on C-R neurons. 5-HT and the activation of the 5-HT$_{3A}$ has been shown to induce reelin secretion that in turn modifies the morphology of apical dendrites by controlling the growth and sprouting of their arborization. In absence of 5-HT$_{3A}$ receptor that could be observed in 5-HT$_{3A}$-knockout mice or *ex vivo* following pharmacological blockade of the 5-HT$_{3A}$ the morphology of apical dendrites become exuberant while basal dendrites that are far from the source of reelin are preserved. Following SSRI-treatment during embryogenesis excess extracellular 5-HT leads to an increased reelin secretion and to a reduction in the complexity of apical and basal dendrites.

pathfinding indicating that 5-HT receptors are functional before birth and can regulate TCAs guidance at early stages of cortical development (Bonnin et al., 2007). Furthermore, it has been shown that 5-HT modifies the attractive vs. repulsive responsiveness of TCAs to netrin-1 (Bonnin et al., 2007), an important guidance molecule for TCAs. Given these findings, it is thus likely that developmental excess of 5-HT could also affect these earlier stages of thalamocortical pathfinding and lead to abnormal thalamocortical long-range wiring (Bonnin and Levitt, 2011; Bonnin et al., 2012).

FROM RODENT MODELS TO HUMAN PATHOLOGY–TRANSLATIONAL CONSIDERATIONS

The work reviewed reveals that developmental imbalance of 5-HT homeostasis or 5-HT receptor signaling has an impact on various processes involved in the formation of cortical circuits in rodents. Whether these developmental changes can also occur in humans remains largely unknown. The nature and severity of neocortical circuit alterations induced by 5-HT-related perturbations are likely to depend on a broad variety of factors including the timing of the insult. For instance, altered neuronal migration was observed during the late phase of rodent gestation, a developmental phase which corresponds to the second trimester in humans. In contrast, altered dendritic growth was observed largely during the first two postnatal weeks in rodents, a phase corresponding to the third trimester in humans. In this respect, we provide a rodent to human correspondence of cortical development in **Figure 8** to ease comprehension.

One of the most clinically relevant situations leading to a developmental excess of 5-HT in humans is the exposure of the human fetus to SSRIs during pregnancy. SSRIs cross the placenta, reach the developing brain and are poorly metabolized by the fetus. Given the high incidence of mood disorders in pregnant women, prescription of SSRIs is frequent during this period. These drugs are considered relatively safe and beneficial during pregnancy, largely because they buffer the negative effects of maternal stress on the fetal-developing brain without causing major teratogenic effects. However, multiple negative effects of SSRI treatment during pregnancy have recently been identified, with the limitation that it is often difficult to control for confounding effects of maternal psychopathology. Ultrasonic investigation of human fetuses provides evidence that SSRIs taken during pregnancy alter the brain physiology starting as early as the beginning of second trimester (Mulder et al.,

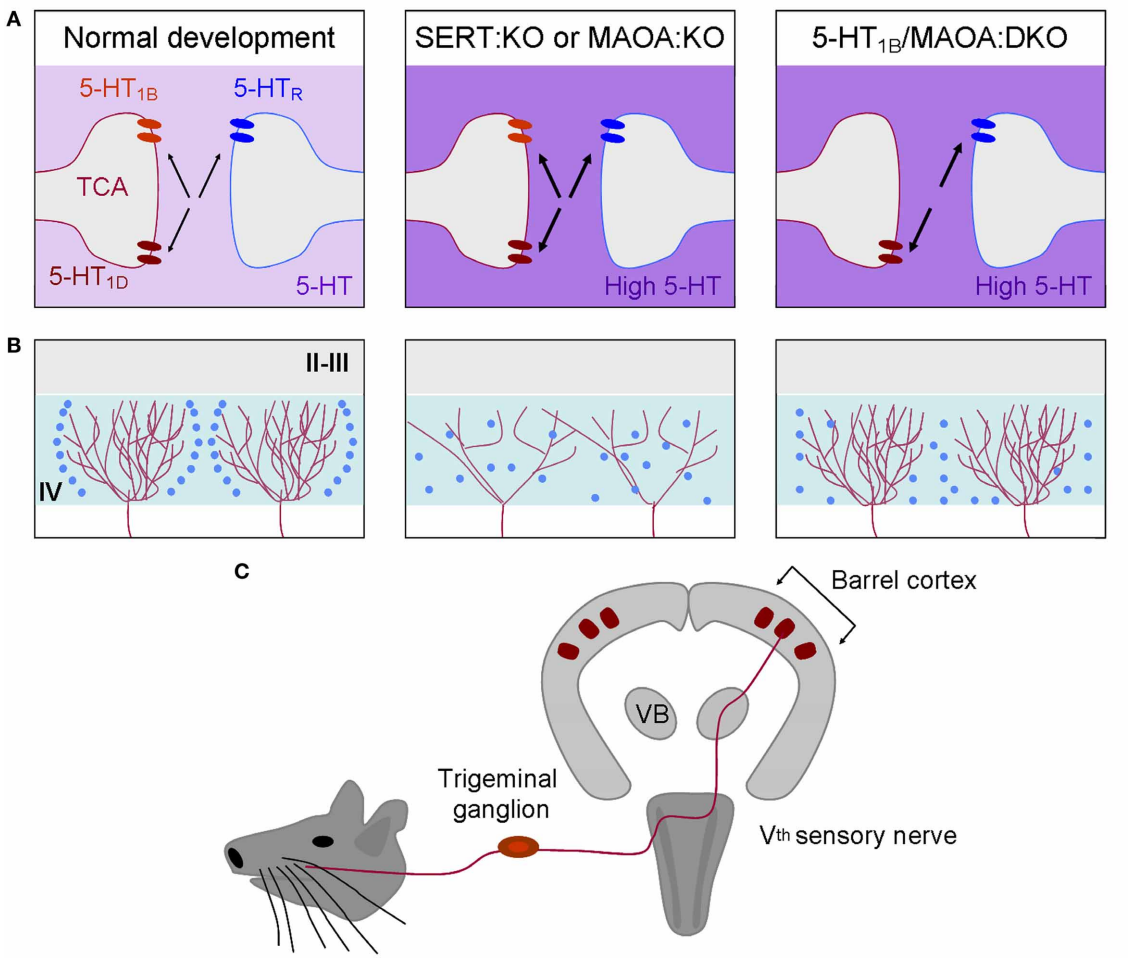

FIGURE 7 | The developmental organization of the somatosensory cortex depends on brain 5-HT levels. The rodent barrel cortex is characterized by a one-to-one correspondence between the sensory system and its cortical projection area **(C)**. Each whisker on the rodent snout is somatotopically represented in the trigeminal nucleus, the ventrobasal thalamic nucleus (VB), and the primary somatosensory cortex (barrel). In the layer IV (IV) of the primary somatosensory cortex (barrel cortex), cortical barrels are constituted by regions of dense arborization of thalamocortical afferents (red) and by granular neurons (blue) that segregate around them. During normal development, SERT, 5-HT$_{1B}$, and 5-HT$_{1D}$ are expressed transiently by thalamocortical axons (left column). In SERT:KO or MAOA:KO mice displaying excessive extracellular 5-HT levels (deep purple) during development the organization of thalamocortical axons and the segregation of granular neurons are altered (middle column). 5-HT$_{1B,1D}$ receptors are the direct targets of 5-HT excess. 5-HT$_{1B}$ receptors act as regulators of thalamocortical development through the inhibition of glutamate release. Removing the 5-HT$_{1B}$ in MAOA:KO mice is sufficient to restore a normal thalamocortical organization (right column). 5-HT$_{1B}$:KO mice display normal barrel organization suggesting that 5-HT$_{1B}$- and 5-HT$_{1D}$-signaling are redundant and that 5-HT$_{1D}$ is sufficient to maintain a normal organization of thalamocortical axons. However, granular neurons do not completely segregate suggesting that other 5-HT receptors, such as the 5-HT$_{2A}$, could act on granular neurons and participate in the organization of cortical barrels. **(A)** Depicts what is occurring at the thalamocortical and granular interface. **(B)** Represent the organization of two adjacent barrels.

2011). Combined recordings of general motor activity, rapid eye movements, and fetal heart rate variability indicate that fetuses exposed to SSRIs during gestation have abnormal increases in motor movements during phases of non-REM sleep compared to fetuses from drug-free mothers with comparable levels of anxiety and depressive symptoms. Furthermore blood flow recordings at 36 weeks gestation in the middle cerebral artery were significantly decreased in fetuses exposed to SSRIs during gestation (Rurak et al., 2011). At birth, babies prenatally exposed to SSRIs display a wide range of neurobehavioral alterations, including lower APGAR scores, increased irritability, and blunted pain reactivity (Casper et al., 2003; Oberlander et al., 2005), as well as reduced fetal head growth (El Marroun et al., 2012). More recently prenatal antidepressants were shown to shift developmental milestones on infant speech perception tasks *in utero* and at 6 and 10 month of age (Weikum et al., 2012), suggesting a role for 5-HT in modulating critical time period maturation in humans. At later time-points, children exposed to SSRIs during pregnancy display increased internalizing behaviors (Oberlander et al., 2010) and decreased scores on psychomotor developmental scales (Casper et al., 2011). The most worrisome finding comes from a recent study reporting a two-fold increase in the risk for

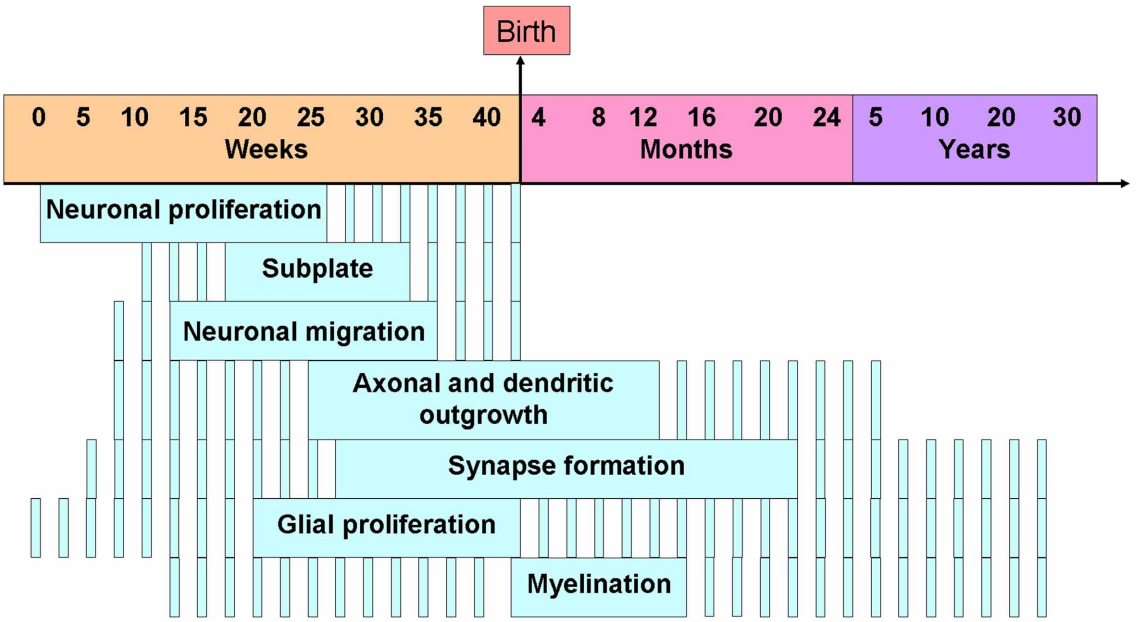

FIGURE 8 | Timing of the human developmental processes in the cerebral cortex. Note that the dashed lines means that the process is active and plain lines means that the process is at its peak. Adapted from de Graaf-Peters and Hadders-Algra (2006).

autism-spectrum disorders in children exposed to SSRIs during pregnancy (Croen et al., 2011). The risk appeared higher when exposure to SSRIs occurred during the second trimester and with higher dosage of SSRIs, suggesting deleterious effects on early neural circuit formation.

A second cause of excessive 5-HT-signaling in humans can be of genetic origin. The common 5-HT transporter-linked polymorphic region (SERTLPR) short (s) allele variant leads to decreased levels of SERT expression *in vitro* compared to the long (l) allele, and to a state of SERT hypofunction (Murphy and Lesch, 2008). This s-allele variant has been extensively investigated in the field of psychiatry and a large body of work in non-human primates and humans reveals that the hypofunctional s-allele interacts with early-life adversity to increase risk for a wide range of psychopathological traits. When exposed to high levels of maternal anxiety during pregnancy, 6 months old infants and children carrying the s-allele showed respectively higher levels of negative emotionality compared to l-allele carriers (Pluess et al., 2010) and increased scores of anxiety and depression (Oberlander et al., 2010). Finally an interaction between the s-allele and severe forms of adversity occurring later during childhood have been observed in many independent studies and lead to an increased risk for depressive symptoms in early adulthood (Karg et al., 2011). These findings indicate that the common hypofunctional s-allele is associated to an increased risk to broad spectrum of psychopathology in the presence of developmental adversity. The effect size of the s-allele is small and it is thus likely that the abnormal cortical circuit alterations observed in SERT deficient rodent models will only occur in humans in more severe forms of genetic or environmental SERT deficiency. In a clinical perspective, it is possible that only an accumulation of risk factors will lead to the cortical circuit alterations detected in rodents. For example, it is possible that these early life circuit alterations could emerge in fetuses carrying hypofunctional SERT variants and being exposed to SSRIs. Furthermore other risk alleles could interact with SERT deficiency to further increase the risk for neural circuit alterations. For instance, PTEN, a gene associated to ASDs (Levitt and Campbell, 2009) interacts with SERT haploinsufficiency to modify brain size and social behaviors in rodents (Page et al., 2009). Overall, these findings point to the general conclusion that various different clinical dimensions including autism, depression, and anxiety-related phenotypes are associated to conditions of SERT deficiency during development. Knowledge derived from animal studies is beginning to provide important insight into the developmental and cellular mechanisms that underlie these complex phenotypes. They support the general hypothesis that developmental excess of 5-HT can lead to early neural circuit alterations, which will act as an important vulnerability factor for a spectrum of psychiatric symptoms.

Rodent studies have revealed that the 5-HT_{3A} and the 5-HT_6 receptors regulate cellular events involved in cortical circuit formation. However, their implication in determining vulnerability to human psychiatric disorders remains to be elucidated. Interestingly it has been reported that a 5-HT_{3A} genetic variant interacts with early-life adversity to increase risk for depressive symptoms and decrease fronto-limbic gray matter (Gatt et al., 2010). In addition this variant also interacts with polymorphisms in the brain-derived neurotrophic factor gene to predict emotion-elicted heart-rate, electroencephalogram asymmetry, and self-reported negativity bias (Gatt et al., 2010). These studies point to a potential developmental interaction between the 5-HT_{3A} receptor and early-life stress in mediating risk for

mood disorders, confirming the intricate connection between early-life stress and the serotonergic systems. A role for the 5-HT_6 receptor in determining risk for human psychiatric disorders still remains elusive. Human variants in the 5-HT_6 receptor have initially been associated to an increased risk for schizophrenia but a recent meta-analyis reported negative findings (Kishi et al., 2012). Interestingly and in a developmental perspective, 5-HT_6 antagonists have recently been shown to reverse cognitive deficits induced by early-life social isolation (Marsden et al., 2011). In two different developmental rat models of schizophrenia specifically neonatal phencyclidine and postweaning isolation, the mammalian target of rapamycin (mTOR) pathway was found to be persistently upregulated in the prefrontal cortex (Meffre et al., 2012). Interestingly it has been shown that 5-HT_6 signaling acts on the mTOR pathway and that 5-HT_6 antagonists injected in adulthood could reverse the cognitive defects induced by early-life insults and normalize mTOR signaling pathway modifications (Meffre et al., 2012). In a broader perspective procognitive behavioral effects of 5-HT_6 receptor antagonists have been observed in different types of animal models including socially isolated reared rats (Marsden et al., 2011). More specifically it has been shown that 5-HT_6 receptor antagonists could reverse deficits in novel object discrimination induced by isolation rearing and that these procognitive effects could be linked to increased hippocampal-prefrontal cortex glutamatergic neurotransmission, further suggesting the relevance of the 5-HT_6 receptor as a potential therapeutical target in cognitive deficits (Marsden et al., 2011).

PERSPECTIVES

Data obtained in rodents and humans lead to the general hypothesis that genetic and environmental factors that influence 5-HT signaling during specific sensitive periods of development critically impact cellular events involved in the formation and maturation of cortical circuits. These various factors act in concert in predisposing to or protecting against cortical dysfunction. The central aspect of this conceptual framework is that type and timing of altered 5-HT signaling determine cortical circuit alterations and behavioral/cognitive consequences. Future studies will aim to focus on cell-type specific targets of 5-HT during development in order to gain a more precise understanding of the diversity of cellular events and receptors that are involved in cortical circuit formation. These studies should help us to better understand how 5-HT signaling during development can impinge on specific sets of neural circuits and how these circuit specific alterations are linked to the broad range of behavioral dimensions resulting from early-life 5-HT dysregulation.

ACKNOWLEDGMENTS

Tania Vitalis thanks people of the "sleep neuronal networks" team and Hervé Langzam for support and fruitful discussions. Founding was provided by the CNRS, ESPCI ParisTech and INSERM (for Tania Vitalis), by the Swiss National Foundation and NCCR Synapsy grant (for Alexandre G. Dayer) and the National Institute of Mental Health, the Brain and Behavior Research Foundation, and the Sackler family (for Mark S. Ansorge).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 20 March 2013; accepted: 27 May 2013; published online: 19 June 2013.

Citation: Vitalis T, Ansorge MS and Dayer AG (2013) Serotonin homeostasis and serotonin receptors as actors of cortical construction: special attention to the 5-HT_{3A} and 5-HT_6 receptor subtypes. Front. Cell. Neurosci. 7:93. doi: 10.3389/fncel.2013.00093

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 27 May 2013
doi: 10.3389/fncel.2013.00076

The serotonin 5-HT$_3$ receptor: a novel neurodevelopmental target

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Serotonin (5-hydroxytryptamine, 5-HT), next to being an important neurotransmitter, recently gained attention as a key-regulator of pre- and postnatal development in the mammalian central nervous system (CNS). Several receptors for 5-HT are expressed in the developing brain including a ligand-gated ion channel, the 5-HT$_3$ receptor. Over the past years, evidence has been accumulating that 5-HT$_3$ receptors are involved in the regulation of neurodevelopment by serotonin. Here, we review the spatial and temporal expression patterns of 5-HT$_3$ receptors in the pre- and early postnatal rodent brain and its functional implications. First, 5-HT$_3$ receptors are expressed on GABAergic interneurons in neocortex and limbic structures derived from the caudal ganglionic eminence. Mature inhibitory GABAergic interneurons fine-tune neuronal excitability and thus are crucial for the physiological function of the brain. Second, 5-HT$_3$ receptors are expressed on specific glutamatergic neurons, Cajal–Retzius cells in the cortex and granule cells in the cerebellum, where they regulate morphology, positioning, and connectivity of the local microcircuitry. Taken together, the 5-HT$_3$ receptor emerges as a potential key-regulator of network formation and function in the CNS, which could have a major impact on our understanding of neurodevelopmental disorders in which 5-HT plays a role.

Keywords: serotonin, 5-HT$_3$ receptor, development, interneurons, neuroblasts

INTRODUCTION

In addition to its role as a classical neurotransmitter, it is now well established that serotonin (5-hydroxytryptamine, 5-HT) plays a pivotal role in the development of the mammalian central nervous system (CNS). 5-HT is one of the first neurotransmitters to appear during development (E13 in the rat, Lauder, 1990; and E11 in the mouse, Pfaar et al., 2002) and acts a neurotrophic factor in early embryonic CNS development and thus even before synapse formation of cortical neurons is completed. Therefore, it aids to establish CNS organization, supporting as well serotonergic (autoregulation) as also non-serotonergic circuit formation during pre- and early postnatal periods (Sodhi and Sanders-Bush, 2004; Vitalis et al., 2007; Daubert and Condron, 2010). 5-HT signaling is involved in cell division, differentiation, survival, and neuronal migration (Dooley et al., 1997; Lavdas et al., 1997; Azmitia, 2001; Vitalis et al., 2007). It further regulates dendrite formation (Vitalis et al., 2007) and synaptogenesis of cortical neurons (Chubakov et al., 1986; Matsukawa et al., 2003) and is released from sprouting axons even before initial synapse formation (Vitalis and Parnavelas, 2003). Genetic or pharmacological disruption of 5-HT signaling leads to disruption of circuit formation as well as alteration of cell morphology, for example in the somatosensory cortex (Gaspar et al., 2003) and interneuronal circuits (Vitalis et al., 2007). Further, disruption of the 5-HT system during early development by stress or drug exposure is associated with altered cognitive ability, neurodevelopmental disorders such as autism spectrum disorders (ASD) and increased incidence of psychopathologies as schizophrenia (Whitaker-Azmitia, 2001).

The myriad of functions of 5-HT in developmental processes corresponds to the expression of a vast amount of receptors, each with its spatial and temporal expression patterns. Seven receptor families for 5-HT have been identified, including the G protein-coupled receptors 5-HT$_1$, 5-HT$_2$, and 5-HT$_{4-7}$ and the only ligand-gated ion channel 5-HT$_3$. Thus far, 5-HT$_1$ and 5-HT$_2$ receptors have received the most attention as effectors of the actions of 5-HT during CNS development (Borella et al., 1997; Azmitia, 2001; Whitaker-Azmitia, 2001; Gaspar et al., 2003; Puig et al., 2004; Bonnin et al., 2006). However, recent evidence suggests that the 5-HT$_3$ receptor is involved in several mechanisms which determine the formation of neuronal circuits from embryonic stages onward. In this review, we summarize those recent findings which suggest that 5-HT$_3$ receptors emerge as a novel target during the development of the CNS.

EXPRESSION OF 5-HT$_3$ RECEPTORS DURING DEVELOPMENT

The 5-HT$_3$ receptor belongs, together with the nicotinergic acetylcholine, the GABA$_A$, and the glycine receptor, to the Cys-loop family of ligand-gated ion channels (Barnes and Sharp, 1999; Chameau and van Hooft, 2006; Walstab et al., 2010; Lummis, 2012). To date, two subunits (5-HT$_{3A}$ and 5-HT$_{3B}$) have been identified in rodents (Maricq et al., 1991; Davies et al., 1999), and additional three subunits (3$_C$–3$_E$) have been identified in humans (Niesler et al., 2007). Functional 5-HT$_3$ receptors can be built from the same (only 5-HT$_{3A}$) or different subunits (5-HT$_{3A}$ and 5-HT$_{3B}$ receptor subunits). The receptor composition is crucial for its function (Chameau and van Hooft, 2006;

Thompson and Lummis, 2007), in such a way that incorporation of 5-HT$_{3B}$ leads to an increase in single channel conductance and decrease in Ca^{2+} permeability (Davies et al., 1999; Noam et al., 2008). Whether the 5-HT$_{3B}$ subunit is a major determinant of 5-HT$_3$ receptor function in the CNS is still a subject of debate (van Hooft and Yakel, 2003; Chameau and van Hooft, 2006; Jensen et al., 2008) and appears to, at least in part, depend on species-specific expression patterns. Yet, the putative expression of 5-HT$_{3B}$ subunits as part of a heteromeric 5-HT$_3$ receptor complex in the CNS remains of interest, especially in view of the profound effects on Ca^{2+} permeability and associated downstream effectors. Most studies of 5-HT$_3$ receptor expression and function in the CNS in rodents focus on 5-HT$_{3A}$ receptors and the terms 5-HT$_3$ and 5-HT$_{3A}$ are used as equivalent here.

5-HT$_3$ RECEPTORS ARE EXPRESSED IN CAUDAL EMINENCE-DERIVED IMMATURE AND MATURE INTERNEURONS DURING CORTICOGENESIS

In the CNS, the 5-HT$_3$ receptor is first observed in the subpallial ganglionic eminence (GE), the major source of interneurons in the basal telencephalon, at E12.5 (Johnson and Heinemann, 1995; Miquel et al., 1995; Tecott et al., 1995). The rodent GE generates later neocortical GABAergic interneurons which migrate tangentially into the cortical plate. In contrast, neocortical glutamatergic neurons originate in the pallial ventricular zone (VZ) and migrate radially into the cortex (Corbin et al., 2001; Nadarajah and Parnavelas, 2002). Different areas of the GE give rise to various subpopulations of GABAergic interneurons which can be subclassified by their morphology and neuropeptide expression (Flames and Marín, 2005; Rudy et al., 2011; Vitalis and Rossier, 2011).

5-HT$_3$ receptor-positive interneurons compromise ∼30% of the superficial GABAergic interneurons in the somatosensory cortex (Lee et al., 2010). They coexpress cholecystokinin (CCK), vasoactive intestinal peptide (VIP), and/or neuropeptide Y (NPY) and, at smaller fractions, calretinin (CR) and/or reelin, but not parvalbumin (PV) or somatostatin (SST; Morales and Bloom, 1997; Férézou et al., 2002; Inta et al., 2008; Lee et al., 2010; Vucurovic et al., 2010). Further expressing several morphological and electrophysiological properties, 5-HT$_3$ receptor-positive interneurons form a rather heterogeneous group of cells, whose potential common properties remain to be fully characterized (for a recent review, see Rudy et al., 2011). 5-HT$_3$ receptor-expressing neocortical interneurons are not only excited by 5-HT but also acetylcholine via nicotinic receptors (Lee et al., 2010). At least a subset of 5-HT$_3$ receptor-positive cells receives monosynaptic thalamocortical input leading to strong depolarization of these cells (Lee et al., 2010). Therefore, 5-HT$_3$ receptor-expressing cells might be part of potential feedforward inhibitory thalamocortical networks whose sensitivity is potentially regulated by serotonergic and/or cholinergic input (Lee et al., 2010; Rudy et al., 2011). Further discussion of potential functional significance of 5-HT$_3$ receptors on these interneurons was published recently (Rudy et al., 2011).

The major source of 5-HT$_3$ receptor-expressing neocortical interneurons is the caudal part of the GE (CGE; Lee et al., 2010; Vucurovic et al., 2010). Based on recent publications, there is no expression of 5-HT$_3$ receptor in the medial GE (MGE; Lee et al., 2010; Vucurovic et al., 2010), which is the area PV- and SST-expressing cortical interneurons are derived exclusively from (Miyoshi et al., 2007). Note that embryonic 5-HT$_3$ receptor expression was mistakenly described in the MGE in earlier publications (Tecott et al., 1995).

Recently, the generation of enhanced green fluorescent protein (EGFP)-expressing 5-HT$_{3A}$ receptor reporter mice by Inta et al. (2008) and the GENSAT (Gene Expression Nervous System Atlas) project allowed for detailed analysis and fate mapping of 5-HT$_3$ receptor-positive cells during embryonic corticogenesis (Lee et al., 2010; Vucurovic et al., 2010). 5-HT$_3$ receptor-positive superficial neocortical interneurons were found to be generated in the CGE around E13.5–14.5 (Vucurovic et al., 2010). Similar, Miyoshi et al. (2010) described the genesis of cortical interneurons in the CGE to begin at E12.5 and peak at E16.5. Therefore, CGE-derived interneurons are some of the latest cells to integrate into neocortical layers, which by this time point are already populated by other interneurons including MGE-derived interneurons (Butt et al., 2005; Miyoshi et al., 2007; peak of MGE-derived cortical interneuron genesis at E14.5: Miyoshi et al., 2010). 5-HT$_3$ receptor-positive neuroblasts thereby migrate at least partly through the neocortical subventricular zone (SVZ) and intermediate zone (IZ; Tanaka and Nakajima, 2012). Further, unlike MGE-derived interneurons, 5-HT$_3$ receptor-expressing interneurons do occupy preferentially superficial cortical layers I–III (Miyoshi et al., 2007; Lee et al., 2010; Vucurovic et al., 2010). Additionally, they migrate into the neocortical layers in an "outside-in" (Vucurovic et al., 2010) rather than the "inside-out" integration manner of PV- and SST-expressing interneurons. Such "outside-in" neurogenesis was previously described as a feature of CR interneurons (Rymar and Sadikot, 2007). Interestingly, in contrast to PV-interneurons, the birthdate of these CR-expressing interneurons does not match that of neighboring projection neurons in the corresponding layer (Yozu et al., 2004; Rymar and Sadikot, 2007). This might be true as well for the 5-HT$_3$ receptor-positive interneurons. Therefore, 5-HT$_3$ receptor-expressing CGE-derived neocortical interneurons might form a group of cells with very specific, yet unknown, characteristics and might follow different migration- and integration cues than other major groups of interneurons like PV-positive interneurons (Lee et al., 2010; Miyoshi et al., 2010).

In grafting experiments, Vucurovic et al. (2010) found that CGE-derived cells also populated several limbic structures including the bed nucleus, hippocampus, and amygdala. These were derived earlier from the CGE then the neocortical cells, which is in line with earlier genesis of interneurons in these regions (Vucurovic et al., 2010).

Furthermore, next to the CGE, embryonic 5-HT$_3$ receptor expression was also observed in cells of the entopeduncular area (AEP) and peroptic area (POA; Lee et al., 2010; Vucurovic et al., 2010). The further development of these cells has not been characterized yet. Cells from the POA might contribute to interneurons in the neocortex (Gelman et al., 2009, 2011) and thus it was proposed that the POA might also give rise to 5-HT$_3$ receptor-positive interneurons of the neocortex (Rudy et al., 2011). However, Vucurovic et al. (2010) found no evidence of POA cells migrating into neocortical regions but the cells rather contributed,

dependent on their birthdate, to cells of the dentate gyrus (DG), amygdala, endopiriform nucleus, and the claustrum.

5-HT$_3$ RECEPTORS ARE EXPRESSED IN POSTNATAL IMMATURE NEURONS

5-HT$_3$ receptors are expressed in migrating neuroblasts in several migratory streams derived from the SVZ in the early postnatal brain (Inta et al., 2008; Vucurovic et al., 2010). The SVZ, and therefore these neuroblasts, are not derived from the CGE but from the lateral GE (LGE). Migratory streams in the early postnatal rodent brain are part of the ongoing neurogenesis and migration of neurons after birth. These migratory streams include the rostral migratory stream (RMS) populating mainly the olfactory bulb (OB), the dorsal migratory pathway (DMP) above the hippocampus directed toward the occipital cortex, the ventral migratory pathway (VMP) heading toward the striatum and nucleus accumbens, and the external migratory pathway (EMP) aiming toward latero-dorsal brain regions (Inta et al., 2008). Neuroblasts of the RMS do not only migrate into and maturate within the OB but also integrate into the cortex (Le Magueresse et al., 2011). Next to cortical interneurons derived from embryonic interneuron genesis, these neuroblasts maturate into a novel, recently described subclass of CR-positive interneurons with unique firing pattern ("small axonless neurons") which are uniquely generated in the early postnatal period and mainly integrate into deeper layers of olfactory and orbital cortices (Le Magueresse et al., 2011). Additionally, 5-HT$_3$ receptor-positive postnatal SVZ-derived neuroblasts, so-called immature white matter interstitial cells, were recently described to populate the corpus callosum (von Engelhardt et al., 2011).

Of the several postnatal migratory streams harboring 5-HT$_3$ receptor-positive neuroblasts, only the RMS persists into adulthood as an area of secondary neurogenesis (Alvarez-Buylla and García-Verdugo, 2002; Abrous et al., 2005) containing 5-HT$_3$ receptor-positive neuroblasts (Inta et al., 2008; Chen et al., 2012). Similar to early postnatal RMS neuroblasts, they migrate and integrate into the OB, where they maturate to CR- and VIP-positive but calbindin- (CB) negative interneurons. Interestingly, and in contrast to cortical interneurons derived from the CGE, about one-third and one-tenth of the 5-HT$_3$ receptor-expressing interneurons in the OB are PV- and SST-positive, respectively (Chen et al., 2012). Adult SVZ neurogenesis is of particular clinical interest because SVZ-derived neuroblasts can migrate into the cortex upon traumatic events or in neurodegenerative diseases to replace cortical neurons. Indeed, upon stroke in adult mice 5-HT$_3$ receptor-positive neuroblasts integrate into the cortex and maturate to CR-positive interneurons (Kreuzberg et al., 2010). However, the majority of these cells loses 5-HT$_3$ receptor expression upon maturation (Kreuzberg et al., 2010).

To conclude, 5-HT$_3$ receptor-expressing neuroblasts are present in several locations in the early postnatal and adult brain. Nevertheless, both the regulation of migration and maturation of embryonic CGE- and adult SVZ-derived neuroblasts as well as the functional role of 5-HT$_3$ receptors during these processes are yet unresolved. Only little is known about downstream signaling upon activation of 5-HT$_3$ receptors and subsequent Ca^{2+} ionic influx. Investigating a potential function of 5-HT$_3$ receptors in regulating neuroblast migration and maturation therefore would be promising. Some recent studies proposed regulation of cytoskeletal remodeling in neurons by 5-HT$_3$ receptors. For example, 5-HT$_3$ receptor agonists were found to promote neurite elongation of GABAergic cortical interneurons (Vitalis and Parnavelas, 2003). Activation of 5-HT$_3$ receptors further promotes dendrite formation in primary thalamic neurons *in vitro* (Persico et al., 2006; note contradictory: Lotto et al., 1999). In growth cones, cohesion spots, and dendrites of hippocampal neurons and in human embryonic kidney (HEK) cells, 5-HT$_3$ receptors were found to form clusters with the light chain (LC1) of microtubule-associated protein 1B (MAP1B) and the tubulin cytoskeleton (Sun et al., 2008) and these clusters lead to the formation of F-actin-rich lamellipodia (Emerit et al., 2002). 5-HT$_3$ receptors follow the tubulin and F-actin networks for receptor routing and precise tuning at the neuronal membrane surface (Grailhe et al., 2004; Ilegems et al., 2004). Further, LC1 might regulate the receptor function in these cells (Sun et al., 2008). Therefore, 5-HT$_3$ receptors and the cytoskeleton are highly interacting, which might not only lead to the specific transport of 5-HT$_3$ receptors into synaptic sites and regulation of receptor function, but also 5-HT$_3$ receptors might evoke signaling involved in cytoskeletal remodeling. 5-HT$_3$ receptor activity in immature and mature interneurons might be crucial for their activity as well as development.

Interestingly, it was recently reported that electrophysiological activity is essential for the postnatal correct migration and axonal and dendritic integration of CGE-derived reelin- and CR-, but not VIP-positive neurons (García et al., 2011). Whereas this activity is glutamate-dependent after P3, the source of activity before P3 is yet unclear. Serotonergic input via 5-HT$_3$ receptors might be a candidate source of such perinatal activity.

CONCLUSION I: 5-HT$_3$ RECEPTORS ARE A POTENTIAL CENTRAL PART OF MATURATING INTERNEURONS DURING PRE- AND POSTNATAL CORTICAL DEVELOPMENT

5-HT$_3$ receptors are expressed on embryonic immature CGE-derived GABAergic interneurons as well as neuroblasts in early postnatal migratory streams and the adult SVZ. Therefore, they might be involved in (fine)regulation of neuronal excitability and thus migration, maturation, and network formation of inhibitory networks from early embryonic to adult stages (**Figure 1**).

EXPRESSION OF 5-HT$_3$ RECEPTORS ON CEREBELLAR GRANULE AND CORTICAL CAJAL–RETZIUS CELLS

Next to the pre- and postnatal central expression of 5-HT$_3$ receptors on mature and immature interneurons, recent evidence showed also expression on two specific types of glutamatergic cells: cerebellar granule cells and cortical Cajal–Retzius cells. First, ubiquitous post-/extra- and presynaptic expression of 5-HT$_3$ receptors was recently observed in glutamatergic granule cells of the cerebellum within the first three postnatal weeks in rodents (Oostland et al., 2011, 2013). 5-HT$_3$ receptors are important for the serotonergic regulation of short-term synaptic plasticity at parallel fiber-Purkinje cell synapses during the early postnatal sensitive period and regulate the maturation state of these synapses (Oostland et al., 2011). They further regulate the

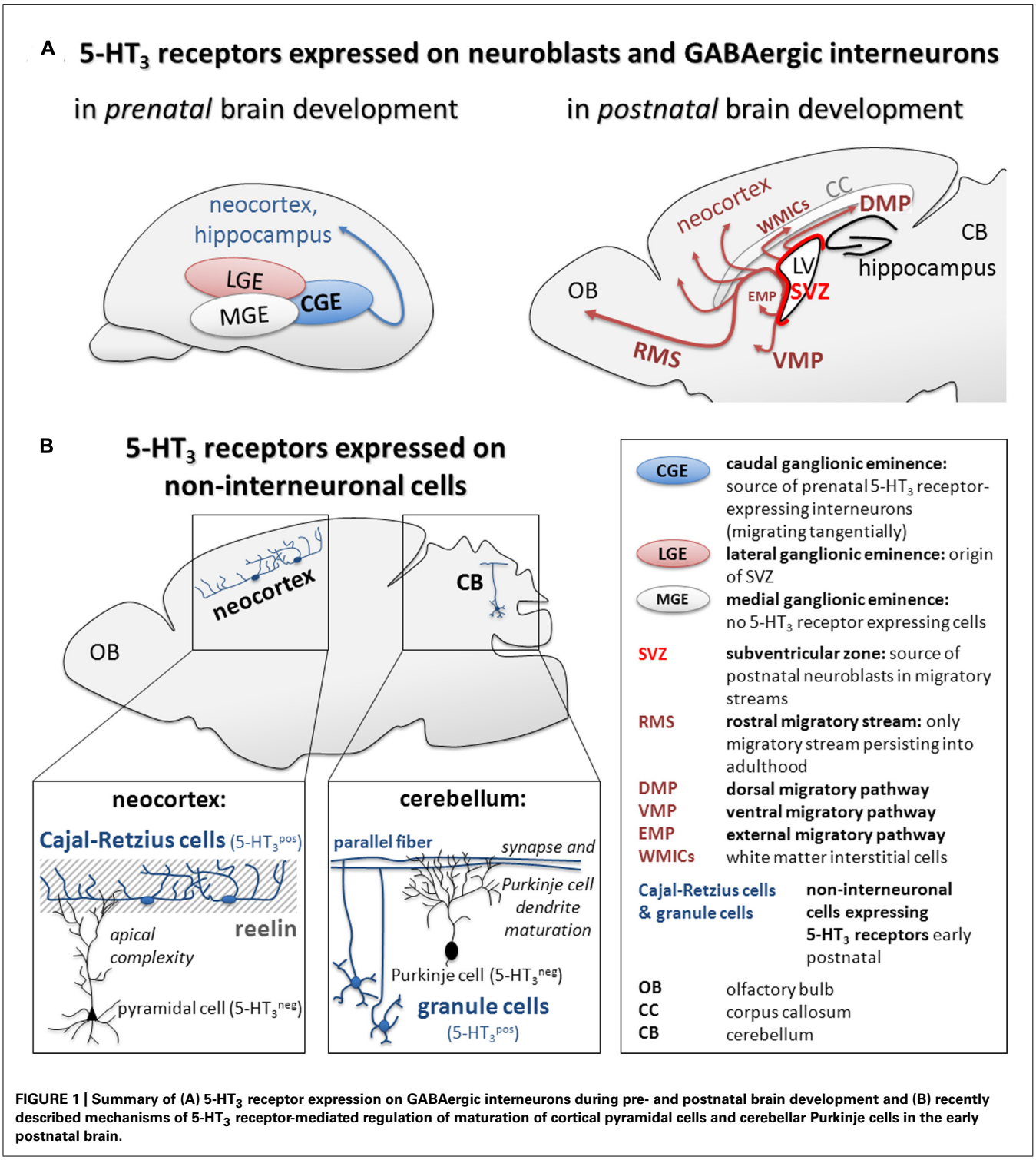

FIGURE 1 | Summary of (A) 5-HT$_3$ receptor expression on GABAergic interneurons during pre- and postnatal brain development and (B) recently described mechanisms of 5-HT$_3$ receptor-mediated regulation of maturation of cortical pyramidal cells and cerebellar Purkinje cells in the early postnatal brain.

time course of early postnatal morphological maturation of Purkinje cells as indicated by higher dendritic length and complexity in 5-HT$_3$ receptor knock-out mice and *in vitro* after treatment with a 5-HT$_3$ receptor antagonist (Oostland et al., 2013). 5-HT$_3$ receptor knock-out animals further show delayed climbing-fiber elimination (Oostland et al., 2013). However, morphology and physiology of Purkinje cells in 5-HT$_3$ receptor knock-out mice appears normal in adult mice, thus indicating a narrow postnatal time window of serotonergic, 5-HT$_3$ receptor-mediated regulation of cerebellar maturation and connectivity (Oostland et al., 2013). Further research might explore a function of 5-HT$_3$ receptors in the development of early life motor coordination and learning.

Second, glutamatergic Cajal–Retzius cells were recently described to express 5-HT$_3$ receptors upon birth (Chameau et al., 2009; Lee et al., 2010). Cajal–Retzius cells are transient neurons located in the marginal zones of the neocortex and hippocampus during CNS development (Marín-Padilla, 1998). In the cortex, they are strategically located in layer I, the area where the apical dendrites of pyramidal neurons terminate and secrete the extracellular matrix glycoprotein reelin. Reelin plays a major role as guidance factor for cell migration, cell positioning, and neuronal process outgrowth (Frotscher, 1997). Cajal–Retzius cells in mice are innervated by serotonergic fibers as early as E16. Disruption of the serotonergic system during embryonic development results in lower levels of reelin and a disturbed corticogenesis with disrupted formation of cortical columns (Janusonis et al., 2004). The regulation of corticogenesis by Cajal–Retzius cells is at least partly dependent on 5-HT$_3$ receptor signaling (Chameau et al., 2009). Chameau et al. (2009) not only reported expression of 5-HT$_3$ receptors specifically on Cajal–Retzius cells (but not on pyramidal neurons), but further established a novel role of 5-HT$_3$ receptors, Cajal–Retzius cells, and reelin in the postnatal maturation of cortical pyramidal neurons. Cajal–Retzius cells limit the apical dendritic outgrowth of cortical layer II/III pyramidal cells and thus complexity of cytoarchitecture and network formation. Blocking 5-HT$_3$ receptor activity with an antagonist or reelin signaling with an anti-reelin antibody leads to hypercomplexity of the apical dendrites of layer II/III pyramidal neurons in the somatosensory cortex. A similar phenotype is also present in 5-HT$_3$ receptor knock-out mice and can be rescued by application of recombinant reelin (Chameau et al., 2009). However, it remains to be investigated if, and how, the release of reelin from Cajal–Retzius cells is directly regulated by 5-HT$_3$ receptor activity. Similar findings of possibly indirect regulation of migration and regulation of cytoarchitecture in cortical pyramidal neurons were shown *in vitro* in mixed GABA- and non-GABAergic cortical neuron cultures, where 5-HT$_3$ receptor activation inhibited axonal and dendritic outgrowth and dendritic branching only in non-GABAergic cells (Hayashi et al., 2010).

The increased dendritic complexity of cortical layer II/III pyramidal neurons in 5-HT$_3$ receptor knock-out mice has been associated with altered cortical spatial organization and connectivity with larger dendritic bundles in layer III tangential sections, whereas spine density was not affected (Smit-Rigter et al., 2011). On a functional level, the increase in dendritic complexity of cortical layer II/III pyramidal neurons in 5-HT$_3$ receptor knock-out mice results in a different firing pattern of these cells (van der Velden et al., 2012), suggesting that 5-HT$_3$ receptor activity during maturation of neurons is not only important for the wiring of the local microcircuitry, but also consequently for the processing of information within the circuit. As a potential consequence of this disturbed cortical wiring and function, 5-HT$_3$ receptor knock-out mice display reduced anxiety-like behavior (Kelley et al., 2003; Bhatnagar et al., 2004) and impaired social behavior (Smit-Rigter et al., 2010), although a direct link between the cortical abnormalities and the behavioral phenotypes remains to be established.

CONCLUSION II: 5-HT$_3$ RECEPTORS REGULATE MATURATION AND DENDRITE COMPLEXITY OF NON-INTERNEURON CELLS

5-HT$_3$ receptors regulate the wiring of the local microcircuit in the cortex and the cerebellum by yet unknown either direct or indirect mechanisms via Cajal–Retzius cells and granule cells, respectively. Therefore, 5-HT$_3$ receptors may be crucially involved in the formation of higher-level neuronal structures (**Figure 1**).

PUTATIVE IMPLICATIONS FOR NEURODEVELOPMENTAL DISORDERS

5-HT$_3$ receptors are associated with several psychiatric disorders in humans. Single nucleotide polymorphism, especially the C178T polymorphism in the 5′UTR region of the 5-HT$_3$ receptor, were found to be associated with bipolar disorder (Niesler et al., 2001), schizophrenia (Niesler et al., 2001; Thompson et al., 2006), lowered harm avoidance in women (Melke and Westberg, 2003), alcohol and drug dependence (Enoch et al., 2010), lowered activity of amygdala and prefrontal cortex (Iidaka et al., 2005), prefrontal and hippocampal gray matter loss, and early life quality-dependent elevated depressed mood (Gatt et al., 2010a,b). These variants are associated with changes in 5-HT$_3$ receptor function and expression (Krzywkowski et al., 2007). However, it has to be noted that 5-HT$_3$ receptor genetics is fundamentally different between humans and rodents. 5-HT$_3$ receptor expression in humans is much more complicated including additional splice variants of 5-HT$_{3A}$, the possible expression of heteromeric receptors in the CNS, and three additional receptor genes (5-HT$_{3C-E}$), whose function and expression in the CNS have yet to be investigated.

The data presented in this review highlights the 5-HT$_3$ receptor as a crucial regulator of brain development. This also makes it interesting as novel candidate to be involved brain development pathologies such as ASD. Indeed, several studies present evidence that ASD might be caused by disruptions of the serotonergic system during brain development. Common ASD animal models are based on alterations of prenatal 5-HT levels (Whitaker-Azmitia, 2005; Boylan et al., 2007; Hohmann et al., 2007). Likewise, clinical data from ASD patients points toward a causal relationship of distortion of the serotonergic system and ASD pathology (Anderson et al., 1987; Naffah-Mazzacoratti et al., 1993; Chugani, 2002).

Investigating a potential role of 5-HT$_3$ receptors in the development of ASD, it is apparent that 5-HT$_3$ receptor knock-out mice display some features similar to ASD symptoms including impaired social behavior (Smit-Rigter et al., 2010) and a reduction in basal anxiety-related behavior (Kelley et al., 2003; Bhatnagar et al., 2004; Smit-Rigter et al., 2010). Further, in line with the potential role of the 5-HT$_3$ receptor outlined earlier in this review, these animals display some alterations in neocortical development as hypercomplexity of apical dendrites of cortical layer II/III pyramidal neurons (Chameau et al., 2009) and increased apical dendrite bundling (Smit-Rigter et al., 2011). Disruptions of neocortical development, especially in the balance between excitatory and inhibitory circuits, might at least partially underlie autism neurobiology (Polleux and Lauder, 2004; Levitt, 2005). For example, in parallel with 5-HT$_3$ receptor knock-out animals, ASD patients display a cortical column pathology with changes in cortical minicolumn size, number and cellular distribution,

and increased cortical volume (Bailey et al., 1998; Casanova et al., 2002; Carper and Courchesne, 2005). Further, reelin signaling was proposed to be impaired in ASD neurobiology (Fatemi et al., 2005). Indeed, 5-HT$_3$ gene polymorphisms were recently found to be associated with ASD (Anderson et al., 2009; Rehnström et al., 2009). However, there is yet no evidence of a role of 5-HT$_3$ receptors in the neurobiology of ASD.

Finally, recent literature draws attention to the potential risk of disturbing serotonergic circuits during fetal brain development via exposure of fetuses to selective serotonin reuptake inhibitors (SSRIs). The use of SSRIs by pregnant women, especially during the first trimester, may increase the risk of ASD in the offspring (Croen et al., 2011). In mice, early postnatal exposure to SSRIs leads to increased anxiety-like behavior (Ansorge et al., 2004). In addition, *in utero* exposure to fluoxetine leads to life-long abnormalities of cortical cytoarchitecture and increased anxiety-like behavior (Smit-Rigter et al., 2012). These effects were not present in 5-HT$_3$ receptor knock-out mice suggesting that the adverse effect of fluoxetine-exposure during brain development might be 5-HT$_3$ receptor-dependent (Smit-Rigter et al., 2012).

We conclude that, although current data is still limited, 5-HT$_3$ receptors are important for proper brain development. The 5-HT$_3$ receptor knock-out mouse has proven to be a valuable tool to elucidate some of the roles of 5-HT$_3$ receptors in neuronal development. However, the availability of more advanced tools to knock-out or -down 5-HT$_3$ receptors in a more spatially and temporally controlled manner is eagerly anticipated.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 February 2013; paper pending published: 12 March 2013; accepted: 06 May 2013; published online: 27 May 2013.

Citation: Engel M, Smidt MP and van Hooft JA (2013) The serotonin 5-HT$_3$ receptor: a novel neurodevelopmental target. Front. Cell. Neurosci. 7:76. doi: 10.3389/fncel.2013.00076

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 12 June 2013
doi: 10.3389/fncel.2013.00072

Genetic and pharmacological manipulations of the serotonergic system in early life: neurodevelopmental underpinnings of autism-related behavior

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Serotonin, in its function as neurotransmitter, is well-known for its role in depression, autism and other neuropsychiatric disorders, however, less known as a neurodevelopmental factor. The serotonergic system is one of the earliest to develop during embryogenesis and early changes in serotonin levels can have large consequences for the correct development of specific brain areas. The regulation and functioning of serotonin is influenced by genetic risk factors, such as the serotonin transporter polymorphism in humans. This polymorphism is associated with anxiety-related symptoms, changes in social behavior, and cortical gray and white matter changes also seen in patients suffering from autism spectrum disorders (ASD). The human polymorphism can be mimicked by the knockout of the serotonin transporter in rodents, which are as a model system therefore vital to explore the precise neurobiological mechanisms. Moreover, there are pharmacological challenges influencing serotonin in early life, like prenatal/neonatal exposure to selective serotonin reuptake inhibitors (SSRI) in depressed pregnant women. There is accumulating evidence that this dysregulation of serotonin during critical phases of brain development can lead to ASD-related symptoms in children, and reduced social behavior and increased anxiety in rodents. Furthermore, prenatal valproic acid (VPA) exposure, a mood stabilizing drug which is also thought to interfere with serotonin levels, has the potency to induce ASD-like symptoms and to affect the development of the serotonergic system. Here, we review and compare the neurodevelopmental and behavioral consequences of serotonin transporter gene variation, and prenatal SSRI and VPA exposure in the context of ASD.

Keywords: serotonin, neurodevelopment, ASD, prenatal, social behavior, connectivity, valproic acid, SSRI

INTRODUCTION

Accumulating evidence suggests an important role for the serotonergic system in the onset of mental illnesses in general and autism spectrum disorders in particular (ASD; **Box 1**). Because of serotonin's (5-HT) ability to modulate developmental processes (Gaspar et al., 2003; Homberg et al., 2010), a modification of the serotonergic system is seen as a crucial factor in the occurrence of dysfunctional developmental programming leading to abnormal behavior in adult life. Therefore, studying the behavioral consequences of early life alterations in the serotonergic system is of major importance to increase our knowledge and understanding of these mental illnesses. There are several possibilities for genetic as well as pharmacological manipulation of the serotonergic system which are of great use to unravel the complex function of 5-HT. Regulation of 5-HT levels can be influenced by genetic factors such as genetic variance in the 5-HT transporter (5-HTT) gene. The most widely studied 5-HTT polymorphism in humans is the 5-HTT Length Polymorphic Region (5-HTTLPR), which involves genetic variance in the promoter region of the 5-HTT gene (Lesch et al., 1996; section The Human 5-HTT Polymorphism) In rodents, this genetic variance is modeled by a mutation of the 5-HTT gene (5-HTT$^{-/-}$) (Kalueff et al., 2010). Although the latter mutation is not promoter specific, the behavioral consequences are very similar compared to those associated with the 5-HTTLPR in humans, including increased anxiety, depression-related behavior in the context of stress, prosocial behavior, and increased behavioral flexibility (Kalueff et al., 2010). There are also pharmacological factors influencing early developmental 5-HT levels, such as selective serotonin reuptake inhibitors (SSRIs). These antidepressant drugs are commonly prescribed to depressed pregnant women and are able to cross the placenta (Homberg et al., 2010; Olivier et al., 2011). SSRIs block the 5-HTT, and thereby give rise to high 5-HT levels not only in the mother but also in the developing fetus. Another agent that may affect 5-HT levels during development is valproic acid (VPA). This drug is used as mood stabilizer and when taken during pregnancy, affects the 5-HT system of the developing brain (Markram et al., 2007). What is particularly interesting is that

Box 1 | Autism Spectrum Disorder (ASD) endophenotypes.

ASD is a neurodevelopmental disorder manifesting within the first 3 years after birth and progressively worsening in the course of life. Core symptoms of ASD are impairments in sociability (no interest in interaction with others, dysfunction in managing complex social interactions), communicative skills and imagination (absence of spoken language or mild language impairments), and repetitive behavioral patterns (stereotype, preference for sameness, complex rituals (American Psychiatric Association, 1994). Additionally, ASD patients show abnormalities in perception, attention and memory (Ben Shalom, 2003; Dakin and Frith, 2005), as well as increased anxiety (potentially as a result of the repetitive behaviors). These symptoms may well arise from hyper-functioning of microcircuits (see section Prenatal Valproic Acid Exposure in Rats), and hypo-functioning of macrocircuits (as reflected by decreased white matter and connectivity in brains of ASD patients (Kana et al., 2011). The hyper-function of microcircuits may contribute to hyper-perception, hyper-attention, hyper-memory and hyper-emotionality. These symptoms may on their turn contribute to the progression of the disease, as overly strong reactions to experiences may become more and more extreme with each new experience especially when these experiences are emotionally charged. This may lead to obsessively detailed information processing. Due to hypo-functioning of macrocircuits this information is fragmented, leading to an inability to place the information in a broader context. Hence, the autistic patient is trapped into a limited but highly secure internal world with minimal extremes and surprises (Markram and Markram, 2010), as expressed by the DSM IV ASD core symptoms.

these genetic and pharmacological factors are all associated with common structural phenotypes in the brain and behavioral manifestations (**Figure 1**). Hence, comparing the different conditions associated with high 5-HT levels during development (genetic 5-HTT down-regulation, prenatal SSRI and prenatal VPA exposure) may lead to insights relevant for prevention, diagnosis, and/or treatment of ASD, as well as our fundamental understanding of the role of 5-HT in brain development. It is our aim to discuss these three conditions associated with increased 5-HT levels during development in human subjects as well as rodents, and discuss the possible mechanisms underlying the similarities.

THE DEVELOPMENT OF THE SEROTONERGIC SYSTEM

THE PLACENTA AS EXOGENOUS SOURCE OF SEROTONIN

Serotonergic neurons appear early during brain development, already releasing 5-HT before the establishment of conventional synapses as most of the axonal network maturation is achieved after birth in rodents. The function of this 5-HT release is to amplify its own synthesis and increase axon outgrowth (De Vitry et al., 1986; Witteveen et al., submitted). However, the influence of 5-HT is effective even before its neurons are born in the raphe nucleus. This suggests the need of an exogenous source of 5-HT at least during the early developmental stages. Synthesis of 5-HT requires two tryptophan hydroxylase (TPH) enzymes; TPH1 which is located in the pineal gland and gut enterochromaffin cells, and TPH2 which is restricted to the raphe nuclei and enteric nervous system. During development, expression of the transcripts starts at embryonic day (E) **10.5** for TPH2 and at **E12.5** for TPH1 (Cote et al., 2007). Before this stage, serotonergic signaling molecules, like the 5-HT2B receptor and plasmalemmal 5-HTT (E8–9) are already present (Buznikov et al., 2001). So the influence of 5-HT precedes that of its production. Since sites of earlier serotonin synthesis have not been found, the main source during that period has been shown to be maternal as the placenta is a source of serotonin (Cote et al., 2007; Bonnin et al., 2011; see Velasquez et al., 2013). Indeed, the essential amino acid tryptophan, which is the precursor of 5-HT, is present in placental tissue during E10.5–E14.5, which gives the placenta the necessary machinery to synthesize 5-HT. The capacity for placental 5-HT synthesis peaks at E14.5, suggesting that the placental source of 5-HT is of most importance in the period of early development (Bonnin et al., 2011), especially the forebrain. The mid/hindbrain, on the other hand, solely receives 5-HT input from the serotonergic neurons that arise at E10.5 in the dorsal raphe nuclei (for review and figures see van't Hooft and Smidt, submitted), which suggests a smaller importance of placental 5-HT for the development of the mid/hindbrain (Bonnin and Levitt, 2011). Hence, alterations in placental 5-HT likely will affect the early development of the forebrain, whereas genetic alterations in serotonergic genes are expected to affect the mid/hindbrain, as well as the forebrain in later developmental stages.

THE SEROTONIN TRANSPORTER (5-HTT)

A central position in the functioning of the serotonergic system is the 5-HTT. The 5-HTT is located in the plasma membrane of presynaptic nerve terminals from which 5-HT is released. It clears 5-HT from the extracellular space by reuptake mechanisms and thereby regulates serotonergic neurotransmission (Haenisch and Bonisch, 2011). There is only one gene encoding the 5-HTT, which is found in the central nervous system as well as peripheral tissue (Homberg et al., 2010). Expression of the 5-HTT gene starts in the serotonergic neurons of the mouse dorsal raphe nucleus at E11. By E16–E20 5-HTT is expressed in a multitude of brain regions including non-serotonergic ones such as the ganglionic eminence, thalamus, olfactory bulb, and cortex (Zhou et al., 2000). Around the second postnatal week in rodents, when neural circuits are pruned, 5-HTT expression declines in these non-serotonergic areas (Homberg et al., 2010), while it is maintained in the dorsal raphe nucleus throughout lifetime. The transient expression in various brain areas exclusively during their critical phase of development suggests that the 5-HTT plays an essential role in the establishment of brain circuits.

5-HTT GENE VARIANCE IN HUMANS AND RODENTS

THE HUMAN 5-HTT POLYMORPHISM

Abnormalities in 5-HTT function are implicated in ASD (**BOX 1**) by studies reporting reduced 5-HTT density in the frontal cortex of ASD patients (Makkonen et al., 2008; Nakamura et al., 2010) but see (Azmitia et al., 2011). The risk of abnormal functioning of 5-HTT can be increased by the genetic background of an individual. The most commonly studied genetic aberration of the 5-HTT is the 44-base pair insertion/deletion polymorphism in the promoter region of the gene (5-HTTLPR) (Champoux et al., 2002).

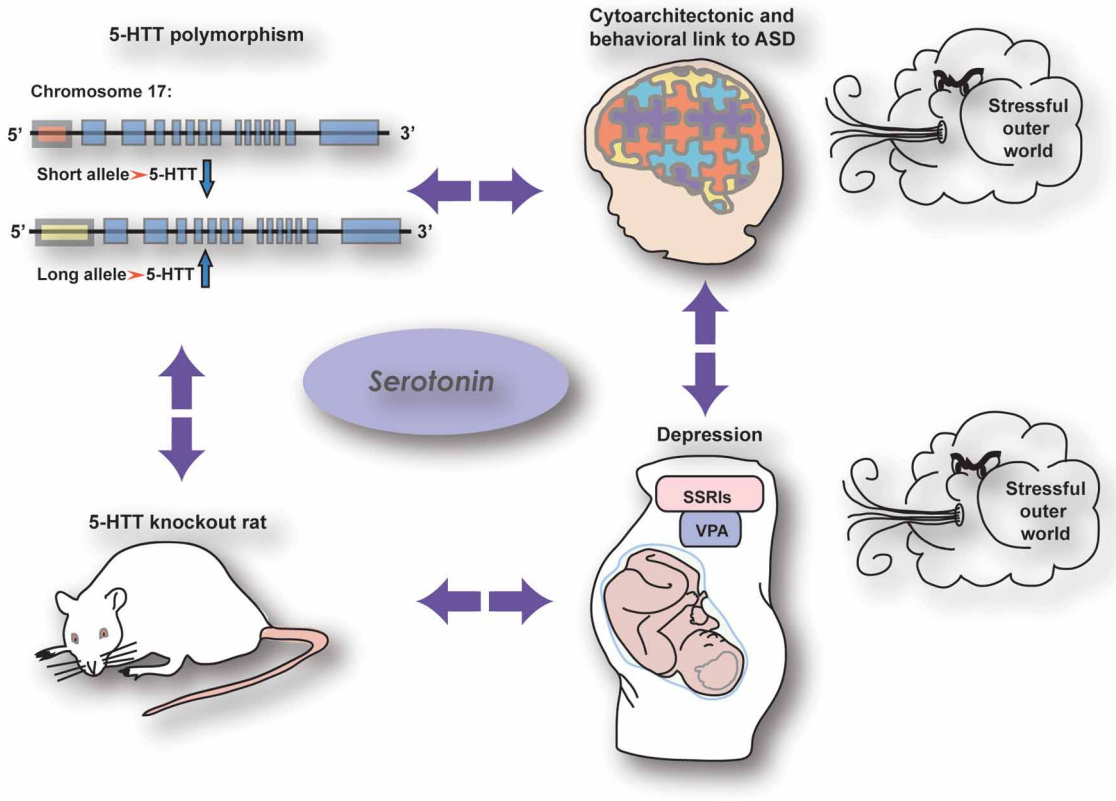

FIGURE 1 | The relationship between serotonergic genetic and pharmacological manipulations with common effects on brain wiring and behavior.

The short (s) allelic variant of 5-HTTLPR is associated with a decrease in 5-HTT transcription (Heils et al., 1997), which presumably leads to decreased expression and function of 5-HTT. Since the 5-HTT is responsible for the reuptake of 5-HT, reduced availability of 5-HTT may lead to increased extracellular 5-HT levels. This, however, cannot be directly investigated in the human brain with the currently available methodologies (Holmes et al., 2003a; Homberg and Lesch, 2011). Regardless of whether or not the 5-HTTLPR s-allele is associated with increased 5-HT levels in the brain, the 5-HTTLPR s-allele has been associated with anxiety-related traits like neuroticism (Munafo et al., 2009). A recent meta-analysis revealed that the 5-HTTLPR s-allele is associated with a bias toward negative environmental stimuli (Kwang et al., 2010; Thomason et al., 2010; Fox et al., 2011; Pergamin-Hight et al., 2012), which may explain the association between the s-allele and anxiety-related traits. There are several indications that the 5-HTTLPR s-allele is indeed a strong genetic risk factor for ASD. For instance, the s/s genotype was found to be highly significantly associated with ASD (Devlin et al., 2005), albeit this association was dependent on the nature of ethnic populations (Huang and Santangelo, 2008; Arieff et al., 2010). It has also been demonstrated that the 5-HTTLPR s-allele is specifically associated with the failure to use non-verbal communication to regulate social interactions in ASD patients (Brune et al., 2006). Furthermore, the increased platelet serotonin level as has been consistently found in a fraction of autistic patients is linked to 5-HTTLPR genotype (Coutinho et al., 2004) but see (Betancur et al., 2002). Yet, recently it was reported that according to mothers' ratings children with the 5-HTTLPR l/l genotype had more severe ASD social deficits than 5-HTTLPR s-allele carriers (Gadow et al., 2013). It is possible that factors like ethnic background, scoring method, read-outs and age, contribute to such inconsistent findings.

Presumably, the presence of the 5-HTTLPR s-allele affects the structure and function of the early brain in such a way that it is more sensitive to adverse environmental stimuli like stress and/or that connectivity between brain regions is altered. It has been well-established that the 5-HTTLPR s-allele is associated with heightened reactivity of the amygdala in response to emotional stimuli (Hariri et al., 2002; Thomason et al., 2010). The amygdala plays a central role in emotional vigilance, particularly toward stimuli with a negative valence. Yet, the amygdala is also essential in social interactions and indeed, it plays a critical role in orienting gaze and attention to socially salient stimuli (Birmingham et al., 2011). Furthermore, ASD patients show increased amygdala activity during face processing (Monk et al., 2010; Kliemann et al., 2012). Hence, amygdala hyper-reactivity in association with the 5-HTTLPR s-allele may relate to both heightened emotional processing and social impairments, although it remains to be investigated whether these processes are based on similar mechanisms. Another neuronal phenotype associated with the 5-HTTLPR s-allele is a structural (Pacheco et al., 2009) and functional (Pezawas

et al., 2005) uncoupling between the prefrontal cortex (PFC) and amygdala. Given that the PFC exerts an inhibitory control over the amygdala, a reduction in this inhibitory control is hypothesized to contribute to impaired emotion regulation in 5-HTTLPR s-allele carriers (Pezawas et al., 2005; Hariri and Holmes, 2006; Canli and Lesch, 2007; Homberg and Lesch, 2011), and thereby anxiety-related traits. Interestingly, independent from the 5-HTTLPR s-allele genotype, studies also revealed an association between altered prefrontal-amygdala connectivity and ASD. More specifically, in the so-called salience network there was a reduced connectivity between the insula and amygdala, which are considered as social brain regions (von dem Hagen et al., 2012). Furthermore, a social network involving the middle temporal gyrus, fusiform gyrus, amygdala, mPFC, and inferior frontal gyrus displayed reduced effective connectivity in ASD patients when exposed to facial expression (Sato et al., 2012). The 5-HTTLPR s-allele is also associated with increased cerebral cortical gray matter volumes in young male children with ASD (Wassink et al., 2007), for which the functional implications are unfortunately unclear. Finally, a core structural phenotype associated with ASD is decreased cortico-cortical connectivity, due to corpus callosum abnormalities. Indeed, ASD has consistently been linked with significantly less white matter density in the (anterior part of the) corpus callosum (Frazier and Hardan, 2009; Shukla et al., 2010; Hong et al., 2011; Schipul et al., 2012), suggesting aberrant long-range corticocortical connectivity. As to whether also the 5-HTTLPR s-allele is directly associated with corpus callosum changes remains to be determined. Given that there is active 5-HT uptake in the corpus callosum (Reyes-Haro et al., 2003), it is well-conceivable that the 5-HTTLPR affects corpus callosum connectivity.

5-HTT KNOCKOUT MICE AND RATS

Human studies have significantly advanced our understanding of the neural and behavioral phenotypes associated with the 5-HTTLPR, and thereby the possible role of 5-HT in neurodevelopment. However, detailed understanding of the neural correlates of the behavioral manifestations is limited due to inaccessibility of the human brain. As mentioned before, the 5-HTTLPR s-allele can be mimicked by a targeted reduction of the serotonin transporter gene in rodents (5-HTT$^{-/-}$) (Holmes et al., 2003a). These animals exhibit high extracellular 5-HT levels due to impaired 5-HT clearance in the presynaptic nerve terminal (Mathews et al., 2004), and due to reduced 5-HT reuptake and limited 5-HT recycling in the presynaptic nerve terminal, serotonin synthesis is increased (Kim et al., 2005; Haenisch and Bonisch, 2011). Dorsal raphe neurons in 5-HTT$^{-/-}$ mice show a reduced firing rate as well as desensitization and down-regulation of somatodendritic 5-HT$_{1A}$ receptors, which exert an inhibitory control over raphe action potential firing activity (Lira et al., 2003). Postsynaptic 5-HT$_{1A}$ receptors expressed in target regions of the dorsal raphe neurons, such as the frontal cortex, amygdala, septum, and hypothalamus, are decreased as well (Holmes et al., 2003a,b). These changes are likely compensatory adaptations in response to high extracellular 5-HT levels. Finally, there is convincing evidence that BDNF (brain-derived-neurotrophic factor) mRNA and protein levels are decreased in the PFC and hippocampus of 5-HTT$^{-/-}$ rats (Molteni et al., 2010), which may correspond to the lower serum BDNF levels as observed in children with ASD (Correia et al., 2010; Al-Ayadhi, 2012). Given the role of BDNF in neuroplasticity, the lower availability of BDNF may contribute to the structural and functional changes in corticolimbic structures and white matter tracks in 5-HTTLPR s-allele carriers (section The Human 5-HTT Polymorphism) and 5-HTT$^{-/-}$rodents (this section).

At the behavioral level, 5-HTT$^{-/-}$ rodents show striking similarities with phenotypes observed in 5-HTTLPR s-allele carriers. For instance, 5-HTT$^{-/-}$ mice show a reduction in exploratory locomotion in a light/dark exploration and in the homecage emergence test, as well as reduced open arm exploration in the elevated plus maze test (Haenisch and Bonisch, 2011). Since the reduction in activity is not due to impaired motor function, these results suggest an increase in anxiety-like behavior in 5-HTT$^{-/-}$ mice (Holmes et al., 2003b). Also 5-HTT$^{-/-}$ rats show anxiety-related symptoms in these behavioral tests, but without hypoactivity (Olivier et al., 2008). Whereas these behavioral tests are species specific, the finding that 5-HTT$^{-/-}$ mice and rats, as well as human 5-HTTLPR s-allele carriers show impaired fear extinction (recall) (Garpenstrand et al., 2001; Wellman et al., 2007; Narayanan et al., 2011; Nonkes et al., 2012) implies that the role of 5-HTT in emotional control is highly conserved across species. Also striking is the finding that 5-HTT$^{-/-}$ rodents consistently show a reduction in social interactions, which fit well with the pro-social behaviors reported for 5-HTTLPR s-allele carriers (Kiser et al., 2012). Furthermore, in line with the repetitive behaviors displayed by ASD patients (Pierce and Courchesne, 2001), 5-HTT knockout mice displayed higher frequencies of self-grooming than their wild-type littermates (Kalueff et al., 2010; Lewejohann et al., 2010). In the domain of communication, which is affected in ASD, it has been reported that wild-type mice show more ultrasonic vocalizations (USVs) within the 20–40 kHz range than prenatally stressed animals of both 5-HTT$^{+/+}$ and 5-HTT$^{+/-}$ genotypes, as well as non-stressed 5-HTT$^{+/-}$ animals (Jones et al., 2010). Furthermore, 5-HTT$^{-/-}$ rats show reduced prepulse inhibition (Page et al., 2009), implying the sensorimotor integration is impaired in these animals, such that they are unable to efficiently select sensory information from the external world. 5-HTT$^{-/-}$ mice also show a reduced performance in the gap test measuring the functioning of the whiskers (Pang et al., 2011). These mice reach a smaller gap distance in this task, suggesting that their vibrissa related tactile perception is less sensitive compared to those of control animals. Finally, 5-HTT knockout mice display reduced inflammatory (Palm et al., 2008) and thermal (Vogel et al., 2003) pain. Given that ASD is characterized by impairments in social interaction, perceptual changes, as well as anxiety, one may argue that 5-HTT$^{-/-}$ rodents very well-model a variety of phenotypes relevant for ASD. Notably, the interpretation of the (endo)phenotypes of 5-HTT knockout rats in the context of ASD is mainly based on face validity (Homberg, 2013). Furthermore, there are many factors like gender, age, gene × environment that influence behavior and have not and currently cannot be taken into account because of limited available information (Kas et al., 2011). Nonetheless, because the similarities between 5-HTT knockout

(endo)phenotypes and those of the VPA ASD rat model (see section Prenatal Valproic Acid Exposure in Humans) is striking (Markram et al., 2007).

Although fMRI studies in rodents are hampered by the need for anesthetics in the MRI scanner, *ex vivo* immunostaining experiments have revealed morphological alterations in prefrontal regions and the amygdala of 5-HTT$^{-/-}$ animals. For instance, excitatory pyramidal neurons in the amygdala and PFC of 5-HTT$^{-/-}$ mice showed increased dendritic branching and an increased number of spines (Wellman et al., 2007). The early guidance and innervation of the mPFC pyramidal neurons by 5-HT projections from the raphe seem to be affected as well in 5-HTT$^{-/-}$ rats as was shown by Witteveen et al. (submitted). It has also been reported that 5-HTT$^{-/-}$ mice display increased cell density in the neocortex (Altamura et al., 2007), which may correspond to the increased gray matter found in s-allele ASD patients (Wassink et al., 2007). Furthermore, corpus callosum connectivity is reduced in 5-HTT$^{-/-}$ rats, as measured by Diffusion Tensor Imaging (DTI) (Van der Marel et al., 2013) (**Figure 2**). This was noted at the level of the genu of the corpus callosum, which connects the prefrontal cortices, as has also been observed in ASD patients (Hardan et al., 2000; Vidal et al., 2006). Perhaps the most distinct morphological and functional alterations that have been reported in 5-HTT$^{-/-}$ rodents involve the barrel cortex, which is part of the primary somatosensory cortex representing the whiskers. 5-HTT$^{-/-}$ rats and mice show a distorted or nearly absent barrel pattern in cortical layer IV (Persico et al., 2000) (Miceli et al., submitted). Furthermore, Esaki et al. (2004) demonstrated that glucose uptake in the barrel cortex is significantly reduced in these mice, implying that the barrel cortex is also functionally impaired (Esaki et al., 2004). These changes may be related to altered (netrin-1-dependent) guidance of thalamocortical afferents (TCAs), which project to the barrels [see section The Serotonin Transporter (5-HTT)]. These TCAs appear less mature and less topologically organized in 5-HTT$^{-/-}$ mice and rats (Cases et al., 1998). Given that ASD (Marco et al., 2012) and potentially depression (Kundermann et al., 2009) are associated with blunted (somato)sensory responses (section Perinatal SSRI Exposure in Humans) these 5-HTT$^{-/-}$ findings are of great value

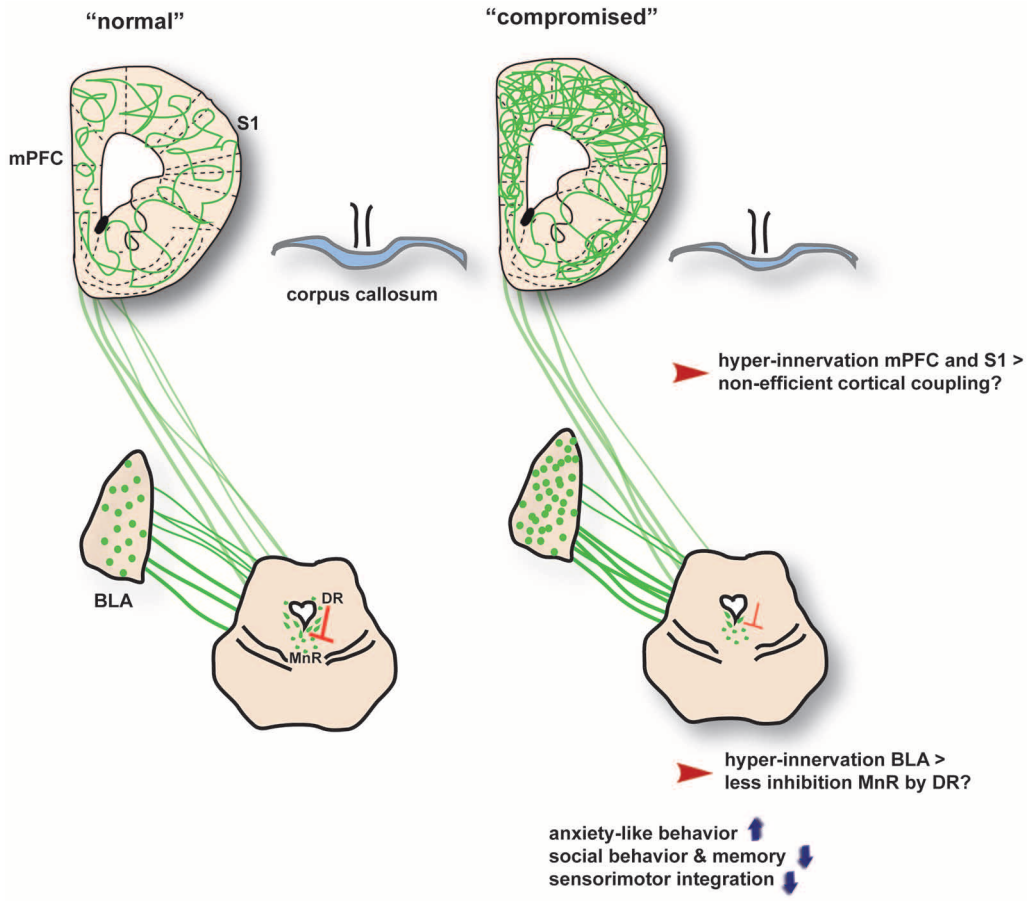

FIGURE 2 | A gross overview of the neural changes associated with increased neurodevelopmental serotonin levels across the 5-HTT, SSRI and VPA conditions (termed as "compromised"). BLA, basolateral amygdala; DR, dorsal raphe nucleus; MnR, median raphe nucleus; S1, somatosensory cortex; mPFC, medial prefrontal cortex. The interaction between the DR and MnR is based on findings in 5-HTT knockout rats by Witteveen et al., submitted.

to increase our understanding of the pathophysiology of these psychiatric conditions.

ANTIDEPRESSANT (SSRI) EXPOSURE

PERINATAL SSRI EXPOSURE IN HUMANS

SSRIs are the most frequently prescribed antidepressants to help overcome depression and anxiety-related disorders. Their main target is the 5-HTT, which is inhibited by SSRI, leading to a pharmacologically induced increase in 5-HT levels in the extracellular space. During pregnancy women have an increased risk to develop depression-like disorders, with reports of depressed pregnant women ranging between 9 and 16% (Nonacs et al., 2005; Ververs et al., 2006; Field, 2010; Gentile and Galbally, 2011). Given that depression is associated with an increased risk of preterm delivery, low birth weight, operative delivery, and admission of the newborn to the neonatal intensive care unit (Chung, 2001; Bonari et al., 2004; Field, 2010), antidepressant treatment is mandatory. With only few side effects reported in adults, and therefore regarded safe, SSRIs are the drug of choice for the treatment of depression during pregnancy. As such, around 25% of the depressed women continue SSRI use, and another 0.5% start using them during pregnancy (Ververs et al., 2006). However, SSRIs cross the placenta (Rampono et al., 2004) with SSRI transfers ranging between a ratio of 52 and 72% (Rampono et al., 2004). After birth, exposure of the offspring to SSRIs also occurs through breast milk during the neonatal period of breast feeding (Homberg et al., 2010; Capello et al., 2011). This is problematic, because SSRI-induced rises in 5-HT levels can affect neurodevelopmental programming. Clinical data have already emphasized the potential hazards of prenatal SSRI exposure [for review see (Alwan and Friedman, 2009; Gentile and Galbally, 2011)]. The symptoms that have been noted in SSRI exposed newborns of depressed mothers compared to non-exposed newborns of depressed mothers include tremor, hypoglycemia, sudden infant death, pulmonary hypertension, rigidity, low Apgar scores, startles, tremors, back-arching, and hypertonic reflexes (Laine et al., 2003; Moses-Kolko et al., 2005; Chambers et al., 2006; Salisbury et al., 2011; Colvin et al., 2012), lower birth weights and preterm births (Lee, 2009; Oberlander et al., 2009; Grzeskowiak et al., 2012), poor feeding, weaker or absent cry, tachypnea and an increase in motor activity (Zeskind and Stephens, 2004). Additionally, it has been reported that infants show blunted somatosensory responses upon prenatal SSRI exposure(Oberlander et al., 2005), poorer psychomotor development (Casper et al., 2003, 2011), an increased risk for ASD symptoms (Croen et al., 2011), and early death (Colvin et al., 2012). Furthermore, children exposed *in utero* to SSRIs that developed a neonatal abstinence syndrome were at an increased risk for social-behavioral abnormalities (Klinger et al., 2011). Given that 5-HTT$^{-/-}$ rodents display an impaired whisker dependent tactile perception and reduced social interactions (a core symptom of ASD) (see **Box 1**), there appears to be a striking resemblance between the neurodevelopmental consequences of prenatal SSRI exposure and genetic 5-HTT down-regulation. This is further supported by rodent perinatal SSRI exposure studies, as discussed in detail below.

PERINATAL SSRI EXPOSURE IN RODENTS

Whereas human studies are hampered by ethical and time-related limitations, rodents are well-suited to obtain insight in the long-term consequences of prenatal SSRI exposure. Notably, it has been reported that the placental transfer of fluoxetine is 70–80% in rodents, and thereby comparable to values reported in humans (Noorlander et al., 2008; Olivier et al., 2011). This has been shown to have profound consequences for the structural and functional organization of the developing brain of the fetus. In rats prenatal SSRI exposure does not only block 5-HTT activity but also reduces its expression. Furthermore, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor density is reduced, indicating an overall decrease of 5-HT activity. In addition, the expression of Tph2, which is crucial for the synthesis of 5-HT, is reduced in the raphe nuclei after neonatal SSRI treatment (Maciag et al., 2006). Also the number of 5-HTT and TpH2 density is reduced in the raphe nuclei of perinatally SSRI exposed rats (Simpson et al., 2011). Autistic children show decreases of α-methyl-l-tryptophan, which is an analogue to the 5-HT precursor tryptophan, in the left cortical hemisphere and exhibit a higher prevalence of severe language impairment, whereas those with decreases in the right cortical hemisphere more frequently display left and mixed handedness (Chandana et al., 2005). Additionally, depletion of tryptophan, the precursor of 5-HT, has been found to increase various stereotyped behaviors in autistic children (McDougle et al., 1996). Yet, early-life fluoxetine exposure resulted in the long-term up-regulation of BDNF expression in adult mice, which seemingly contrast the BDNF down-regulation in 5-HTT$^{-/-}$ rats (section 5-HTT Knockout Mice and Rats) and ASD patients (Correia et al., 2010; Al-Ayadhi, 2012).

Also comparable to the human situation is the finding that rats treated with fluoxetine during pregnancy delivered smaller pups (Vorhees et al., 1994). It is well-known that weight loss is a side-effect of fluoxetine in non-pregnant women and in men; therefore these results could be related to lowered maternal weight gain, which in turn could limit fetal growth. Furthermore, Noorlander and colleagues (2008) found that the majority of the mouse pups that were exposed to fluoxetine during pregnancy died postnatally of severe heart failure caused by dilated cardiomyopathy. Similar effects were found in rats that were exposed to paroxetine treatment during the last week of gestation, which led to a shortened gestational length, reduced birth weight and a 10-fold rise in neonatal mortality (Van den Hove et al., 2008). These findings may match the increased risk for mortality in prenatally SSRI exposed children (Colvin et al., 2012). Taking advantage of the relative short life time of rodents, it has been demonstrated that prenatal or early postnatal (P4-P21) SSRI treatment leads to anxiety- (Ansorge et al., 2004; Smit-Rigter et al., 2012) and depression-related (Hansen et al., 1997; Popa et al., 2008) phenotypes during adulthood. Furthermore, in correspondence with the repetitive behavior phenotype of ASD, prenatal SSRI exposure has been reported to increase grooming and digging behavior (Rodriguez Echandia et al., 1988), as was reported in 5-HTT$^{-/-}$ mice (section 5-HTT Knockout Mice and Rats). Besides, evidence is now accumulating showing that prenatal SSRI treatment leads to blunted somatosensory responses and decreases in social behavior, as reported for 5-HTT$^{-/-}$ rats. Regarding the

somatosensory responses, (Lee, 2009) showed that postnatal SSRI (fluoxetine) treatment from P0-P7 decreased performance in the whisker-dependent gap test. This effect could be mimicked by clipping the wishers, and matches the decreased gap test performance reported in 5-HTT$^{-/-}$ mice (Pang et al., 2011). Also prepulse inhibition was reduced in prenatally fluoxetine exposed rats (Olivier et al., 2011), indicative for an impairment in sensorimotor integration. Finally, prenatal (E11-delivery) and perinatal (P4/8-P21) SSRI treatment have been demonstrated to decrease social play behavior in juvenile rats (Olivier et al., 2011; Simpson et al., 2011), aggressive behavior (Manhaes de Castro et al., 2001), and sexual behavior (Maciag et al., 2006). Again, the reduction in social behavior across ages is consistent with findings in 5-HTT$^{-/-}$ rodents, as well as the effects of prenatal SSRI exposure in humans.

These robust behavioral alterations due to developmental SSRI exposure must be reflected by changes in the wiring of the brain. To provide a link with the blunted functioning of the whisker related somatosensory system, the structure and physiological properties of the barrel cortex and its afferent thalamocortical connections have been in the focus of several studies. Fluoxetine, when applied to rats during PND 0–6, leads to a reduction in the complexity of TCA projections into the barrel cortex. On the intracortical target side, excitatory spiny stellate cells within the layer IV barrel structures possess a reduced dendritic span and arborization (Lee, 2009). Comparable findings were obtained by Xu and coworkers (2004), who found that exposure to the SSRI paroxetine in rats from PND 0 till PND 8 affected the refinement, but not the formation, of dense clusters of the TCA's in the layer IV of the barrel cortex (Xu et al., 2004). Thus, developmental increases in 5-HT levels lead to substantial alterations in the somatosensory system and most likely explain the blunted tactile perception as reported in postnatal SSRI treated rats (Lee, 2009). Of further interest, it has been demonstrated that postnatal SSRI treatment in rat pups altered the myelination of axons in the corpus callosum and interfered with oligodendrocyte (OL) soma morphology. OLs showed hypo- and hypermyelination (Simpson et al., 2011), and the processes of OL progenitor cells were shortened, distorted, and/or polarized. Because the corpus callosum connects hemispheres, it was also investigated whether the aberrant morphology of OLs affected cortico-cortical connectivity. Retrograde tracer studies revealed a reduction in the connectivity between the primary somatosensory cortices across the hemispheres. This was more pronounced for layers II/III than for layer IV (barrel cortex) (Simpson et al., 2011). Although this connectivity has not been investigated in human 5-HTTLPR-s allele carriers, 5-HTT$^{-/-}$ rats, and ASD patients, the decrease in corpus callosum connectivity found in 5-HTT$^{-/-}$ rats and ASD patients (see section 5-HTT Knockout Mice and Rats) may suggest that developmental increases in 5-HT levels affects myelination, and thereby long-distance connectivity in the brain. Moreover, the structural uncoupling between the PFC and amygdala in 5-HTTLPR s-allele carriers (Pacheco et al., 2009; section 5-HTT Gene Variance in Humans and Rodents) implies that besides the corpus callosum other white matter tracks are altered by high developmental 5-HT levels, too. Because complex behaviors like social behavior requires the correct integration of information derived from several brain regions, it is conceivable that alterations in myelination and thereby the long ranging connectivity between hemispheres and brain regions contribute to the behavioral deficits seen in ASD.

PRENATAL VALPROIC ACID EXPOSURE

PRENATAL VALPROIC ACID EXPOSURE IN HUMANS

Besides prenatal SSRI exposure, prenatal exposure to VPA leads to ASD-related symptoms in humans and rats. VPA is a mood stabilizing drug primarily used in the treatment of bipolar disorder and epilepsy (Markram et al., 2007). It inhibits the enzyme histone deacetylase, which mediates epigenetic processes through acetylation of histone proteins. A decrease in histone acetylation, as may be induced by VPA treatment, makes the DNA less accessible to the transcriptional machinery and is hypothesized to be associated with a decrease in gene expression (Yildirim et al., 2003). Depending on the timing of decreased histone acetylation, this leads to a cascade of neuropathologies including ASD. Indeed, case studies have shown that prenatal VPA exposure is likely to induce ASD (Christianson et al., 1994; Williams and Hersh, 1997; Williams et al., 2001). An increased incidence of ASD is specifically found following fetal exposure to the agent around the time of neural tube closure. It is worth mentioning that this finding has led to the hypothesis that ASD may be caused by brainstem injury during embryonic development (Rodier et al., 1996; Arndt et al., 2005). Given that the raphe nuclei are located in the brainstem and start to develop at the time of neural tube closure, the serotonergic system is one possible target of VPA-mediated alterations in gene expression.

PRENATAL VALPROIC ACID EXPOSURE IN RATS

Based on the human case studies, the VPA rat model for ASD has been established. In rats, the neural tube closes at E9. A single dose of VPA (350 mg/kg) administered to pregnant dams on E12.5 results in a decrease in social interactions, increase in repetitive behavior, enhanced anxiety, impaired fear extinction, and impaired pre-pulse inhibition (Schneider and Przewlocki, 2005; Schneider et al., 2006, 2007; Markram and Markram, 2010). Remarkably, these behavioral manifestations resemble those found in 5-HTT knockout (section 5-HTT Knockout Mice and Rats) and prenatally SSRI exposed (section Perinatal SSRI Exposure in Rodents) rodents. It has also been reported that prenatally VPA exposed rats failed to emit the characteristic 70 kHz USV preceding mating, and that pups show a reduction in distress calls (Gandal et al., 2010). Possibly this matches the finding that 5-HTT$^{+/-}$ mice show decreased ultrasonic vocalization (section 5-HTT Knockout Mice and Rats). However, results are diverse since Felix-Ortiz and coworkers observed an increase of three specific forms of USVs on PND5 of VPA treated mice (Felix-Ortiz and Febo, 2012). Furthermore, VPA exposed rats show reduced pain sensitivity (Schneider et al., 2001; Schneider and Przewlocki, 2005), which may match the reduced pain perception observed in 5-HTT knockout mice (section 5-HTT Knockout Mice and Rats) as well as the blunted somatosensory responses as reported in prenatally SSRI exposed infants (section Perinatal SSRI Exposure in Humans) and rats (section Perinatal SSRI Exposure in Rodents) and 5-HTT knockout rats (section 5-HTT Knockout Mice and

Rats). Recent studies provided direct evidence for VPA interfering with the serotonergic system. Kuwagata et al. (2009) showed that a VPA challenge at E11 was associated with abnormal migration of serotonergic neurons at the level of the pons, which coincidences with the appearance of serotonergic neurons at E10.5. Yet, others reported that the serotonergic system was pertubated after administration of VPA at E9, thus at an earlier developmental time point. It was found that VPA exposure at E9 was associated with an increase in 5-HT levels in the blood as well as the frontal cortex, hippocampus and cerebellum (Miyazaki et al., 2005; Dufour-Rainfray et al., 2010). VPA exposure in rats does not alter the number of serotonergic neurons, but their location is shifted more caudally within the dorsal raphe nucleus, probably caused by abnormal serotonergic neuronal differentiation and migration (Miyazaki et al., 2005; Tsujino et al., 2007). Finally, *in situ* hybridization experiments revealed lower cortical expression of BDNF mRNA in VPA exposed rats (Roullet et al., 2010), like 5-HTT$^{-/-}$ rats (section 5-HTT Knockout Mice and Rats). In sum, the VPA rat model shares phenotypic similarities with the prenatal SSRI and 5-HTT knockout models, and possibly these models even share 5-HT-mediated structural changes (**Figure 2**).

From a mechanistic point of view it is striking that both 5-HTT knockout rats and VPA exposed rats show hyper-reactivity in cortical layers II/III, as revealed by electrophysiological recordings using multi electrode arrays (Rinaldi et al., 2007; Miceli et al., submitted). This can be caused by increased synaptic efficiency, hyper-connectivity, lack of proper inhibitory control, or by alterations in neuron density and morphology. Hyper-reactivity was also found in layers II/III of VPA exposed rats, as reflected by enhanced long-term potentiation (reflecting altered synaptic plasticity). Besides the cortex, amygdala hyper-connectivity and hyper-reactivity have been noted in VPA exposed rats. That is, neurons in the lateral amygdala were found to be hyper-reactive when electrically stimulated using the multi electrode array technology. It was also found that this was due to a reduction in inhibition (Markram et al., 2007). Furthermore, like in the cortex, long-term potentiation was increased in the amygdala of VPA exposed rats, indicative for hyper-plasticity. It is well-possible that this explains the enhanced fear memories (as reflected by impaired fear extinction) in VPA exposed rats. Although 5-HTT knockout mice show increased dendritic complexity of pyramidal neurons in the amygdala (Wellman et al., 2007) and increased cell density in the neocortex (Altamura et al., 2007), whether there are similarities at either of these levels in the 5-HTT knockout and VPA exposed rats remains to be established. *Vice versa*, the corpus callosum abnormalities reported in perinatally SSRI exposed rats (Simpson et al., 2011); section Perinatal SSRI Exposure in Rodents) and reduced corpus callosum connectivity found in 5-HTT rats (Van der Marel et al., 2013; section 5-HTT Knockout Mice and Rats) remain to be investigated in the VPA rat model.

As proposed by Markram et al. (2007), autism may be associated with excessive neuronal information processing and storage in (cortical) microcircuits. This may lead to hyper-perception, hyper-attention, and hyper-memory. Simultaneously, autism is associated with reduced long-distance cortical and subcortical connections, impairing the integration of different pieces of information, and thereby complex cognitive and social functions (Kana et al., 2011). In other words, autistic patients may experience the world intensely but fragmented. The impairments in social behavior may also arise from this fragmented intense world syndrome, as ASD patients may experience social cues overly intense while being unable to integrate these social cues as is needed for a proper understanding. This may lead to avoidance of eye and social contact. Of interest, we have suggested these behavioral manifestations also for 5-HTTLPR s-allele carriers, by using the term "hypervigilance" (Homberg and Lesch, 2011). We used this term—based on amygdala and prefrontal hyper-reactivity in fMRI studies—to explain why these individuals are supersensitive to adverse as well as rewarding environmental influences. 5-HTT knockout rats show stimulus-bound habitual-like behavioral responses (e.g., impaired goal-directed behavior in the reward devaluation task) (Nonkes et al., 2010, 2011, 2012), which also may be the consequence of a fragmented world: If the world is perceived as fragmented, it may be very effective to use conditioned cues as a hand-tight to behaviorally perform in a world consisting of a "chaos" of intense stimuli. The consequence, however, may be that this leads to behavioral persistence or repetitive behavior. In both 5-HTT knockout rodents and VPA exposed rats this for instance may be reflected by fear extinction failures. Also the lack of goal-directed behavior in 5-HTT knockout rats (Nonkes et al., 2010) implies that these animals are unable to update a previously acquired conditioned response. Possibly, this matches impairments in goal-directed behavior observed in ASD patients (Poljac and Bekkering, 2012). It would be intriguing to assess whether VPA exposed rats show similar phenotypes. There is, however, one paradox: Whereas the hypervigilance in 5-HTTLPR s-allele carriers (Jedema et al., 2010) and 5-HTT knockout rodents (Brigman et al., 2010; Nonkes et al., 2011) conveys increases behavioral flexibility, no changes in behavioral flexibility has been noted in prenatally SSRI treated animals (Ishiwata et al., 2005). Furthermore, VPA treatment during gestation caused a reduction in behavioral flexibility, (Stanton et al., 2007). Possibly, non-serotoninergic systems may be involved in the behavioral inflexibility observed in VPA exposed rats.

DISCUSSION

As we reviewed in this article, there is strong evidence that 5-HT plays a major role in the etiology of mood disorders and particularly ASD. Exposure to elevated levels of 5-HT over a long time period during crucial periods of brain development, or a fetal SSRI/VPA challenge during a critical developmental stage, causes alterations in the wiring of the brain, both at the microcircuit and macrocircuit level. This leads to rather consistent behavioral manifestations (**Table 1**), including decreased social interactions, increased anxiety-like behavior, and blunted (somato) sensory perception. The brain areas that mediate, at least in part, these behavioral manifestations include the (prefrontal/somatosensory) cortex and the amygdala. More specifically, it is likely that amygdala hyper-reactivity contributes to anxiety, enhanced fear memories, and social impairments in 5-HTTLPR s-allele carriers, 5-HTT knockout rodents, prenatally SSRI exposed animals and VPA exposed rats. Indeed, the amygdala is strongly implicated in signaling the emotional value or salience of

Table 1 | Neuroanatomical and behavioral alterations in 5-HTTLPR s-allele carriers and 5-HTT$^{-/-}$ rodents, perinatally SSRI exposed humans and rats, ASD patients, and VPA exposed rats.

		5-HTTLPR polymorphism	5-HTT$^{-/-}$	Perinatal SSRI exposure (human studies)	Perinatal SSRI exposure (rodent studies)	Autism spectrum disorder (ASD)	VPA exposure
Neurochemical/neuroanatomical changes	5-HT levels		Tissue 5-HT levels in PFC, hippocampus ↓ (Kim et al., 2005; Homberg et al., 2007)	5-HT and 5-HT metabolite serum levels ↓ (Laine et al., 2003)		Platelet 5-HT level ↑ (Betancur et al., 2002)	Tissue 5-HT levels in PFC, hippocampus, cerebellum ↑ (Narita et al., 2002)
			Extracellular 5-HT levels↑ (Mathews et al., 2004; Shen et al., 2004; Homberg et al., 2007)		Tryptophan hydroxylase expression in raphe nuclei ↓ (Maciag et al., 2006)	Cortical α-methyl-l-tryptophan ↓ (Chandana et al., 2005)	Platelet 5-HT level ↑ (Miyazaki et al., 2005)
			5-HT synthesis ↑ (Kim et al., 2005; Haenisch and Bonisch, 2011)				
	5-HT neurons		Number of 5-HT neurons in the dorsal raphe nucleus ↓ (Lira et al., 2003)		Number of 5-HT neurons in the dorsal raphe nucleus ↓ (Olivier et al., 2011)		Caudal shift of 5-HT neurons in the dorsal raphe nucleus (Tsujino et al., 2007)
					Number of serotonergic terminals in hippocampus ↓ (Silva et al., 2010)		Abnormal migration of 5-HT neurons at the pons (Kuwagata et al., 2009)
	5-HT receptors		Expression and function 5-HT1A,1B receptors ↓(Li et al., 1999; Fabre et al., 2000; Holmes et al., 2003b; Kim et al., 2005)		5-HT1A and 5-HT1B receptor sensitivity ↑ (Xu et al., 2004; Olivier et al., 2011)		
			Function 5-HT2A,2C ↓ (Li et al., 2003; Qu et al., 2005; Basselin et al., 2009)		5-HT2A/2C receptor density ↓ (Maciag et al., 2006)		
	5-HTT	5-HTT function ↓ (Hansen et al., 1997)	5-HTT-binding absent (Homberg et al., 2007)		5-HTT expression ↓ (Hansen et al., 1997)	5-HTT-binding and -density in (medial) frontal cortex ↓ (Azmitia et al., 2011)	
			5-HT uptake and clearance ↓ (Kim et al., 2005; Haenisch and Bonisch, 2011)				
	Neuro-anatomical		Corpus callosum connectivity ↓ (Van der Marel et al., 2013)		Altered myelination of axons in the corpus callosum (Simpson et al., 2011)	White matter density ↓ in corpus callosum (Hong et al., 2011; Schipul et al., 2012)	

(Continued)

Table 1 | Continued

		5-HTTLPR polymorphism	5-HTT$^{-/-}$	Perinatal SSRI exposure (human studies)	Perinatal SSRI exposure (rodent studies)	Autism spectrum disorder (ASD)	VPA exposure
			Barrel-like formation of somatosensory cortex nearly absent (Persico et al., 2000)		Connectivity between somatosensory cortices ↓ (Simpson et al., 2011)	Corpus callosum abnormalities (Schipul et al., 2012)	
			Barrel cortex function ↓ (Esaki et al., 2004)		Barrel size of rodents barrel cortex ↓ (Xu et al., 2004)	Brain connectivity↓ (Sato et al., 2012; von dem Hagen et al., 2012)	Barrel cortex hyper-reactivity (Rinaldi et al., 2008)
					Dendritic complexity in the barrel cortex ↓ (Lee, 2009)		
		Cerebral cortical gray matter volumes ↑ (Wassink et al., 2007)	Cell density in neocortex ↑ (Altamura et al., 2007)				
		Amygdalal hyper-reactivity ↑ (Hariri et al., 2002; Thomason et al., 2010)	Dendritic branching in amygdala and prefrontal cortex ↑ (Wellman et al., 2007)			Amygdala activity ↑ (Kleinhans et al., 2010; Weng et al., 2011)	Hyper-connectivity and hyper-plasticity in amygdala (Markram et al., 2007)
		Structural and functional uncoupling of amygdala and PFC (Pezawas et al., 2005; Pacheco et al., 2009)					
Behavioral changes	Anxiety/ depression like	Anxiety related traits (i.e., neuroticism) ↑ (Lesch et al., 1996; Munafo et al., 2009)	Anxiety-like behavior ↑ (Kalueff et al., 2007a; Holmes et al., 2003a; Olivier et al., 2008)		Anxiety like behavior ↑ (Ansorge et al., 2004; Noorlander et al., 2008; Olivier et al., 2011; Simpson et al., 2011)	Anxiety ↑ (van Steensel et al., 2011; Rieske et al., 2013)	Anxiety-like behavior ↑ (Dufour-Rainfray et al., 2010)
		Impaired fear extinction (Garpenstrand et al., 2001)	Impaired fear extinction (Wellman et al., 2007; Narayanan et al., 2011; Nonkes et al., 2012)				Impaired fear extinction (Markram et al., 2007)
		Risk to develop depression ↑ (Caspi et al., 2003; Karg et al., 2011)	Depression-like behavior ↑ (Lira et al., 2003; Olivier et al., 2008)		Depression like behavior ↑ (Lisboa et al., 2007; Schwandt et al., 2010; Azmitia et al., 2011; Olivier et al., 2011)		

(Continued)

Table 1 | Continued

	5-HTTLPR polymorphism	5-HTT$^{-/-}$	Perinatal SSRI exposure (human studies)	Perinatal SSRI exposure (rodent studies)	Autism spectrum disorder (ASD)	VPA exposure
Social/ communication	Aggressive behavior in social environment ↑ (Schwandt et al., 2010)	Social interaction and memory ↓ (Moy et al., 2009; Page et al., 2009; Kalueff et al., 2007b)	ASD-related behavior (Croen et al., 2011)	Social play behavior ↓ (Homberg et al., 2007; Simpson et al., 2011)	Sociability and communication ↓ DSM IV	Social behavior and memory ↓ (Schneider et al., 2006, 2007; Bambini-Junior et al., 2011)
		Social play ↓ (Homberg et al., 2007)		Sexual behavior ↓ (Maciag et al., 2006)		
		Aggression ↓ (Holmes et al., 2002; Homberg et al., 2007)		Aggressive behavior ↓ (Manhaes de Castro et al., 2001)		
		Distress vocalizations ↓ (Jones et al., 2010)				Distress vocalizations ↓ (Gandal et al., 2010)
Repetitive		Self-grooming ↑ (Kyzar et al., 2012)			Repetitive behavior ↑ DSM IV	Self-grooming and digging ↑ (Markram et al., 2007)
Somato-sensory		Sensorimotor integration ↓ (Page et al., 2009)	Somatosensory responses ↓(Oberlander et al., 2009)	Whisker-specific tactile function ↓ (Lee, 2009)	Sensory hyper- and hypo-responsiveness (Marco et al., 2012)	Sensorimotor integration ↓ (Schneider and Przewlocki, 2005)
		Whisker tactile function ↓ (Pang et al., 2011)			Tactile and auditory sensitivity ↑ (Marco et al., 2012)	Tactile function ↓ (Schneider and Przewlocki, 2005)
		Thermal/inflammatory pain reactivity ↓ (Vogel et al., 2003; Palm et al., 2008)	Altered pain responsivity (Oberlander et al., 2009)		Pain sensitivity ↓ (Militerni et al., 2002)	Pain sensitivity ↓ (Schneider and Przewlocki, 2005)

environmental stimuli, including social stimuli (Adolphs, 2010). Furthermore, ASD is characterized by amygdala hyper-reactivity in response to adverse stimuli (Kleinhans et al., 2010; Weng et al., 2011). Changes in the organization of the "labeled line" of the somatosensory system are responsible for the alterations in sensory performance of 5-HTT knockout rodents, prenatally SSRI exposed animals, and possibly also in VPA exposed rats. Given that social behavior is strongly dependent on how social stimuli are perceived, for instance through the whiskers in rodents, hyper-reactivity in the somatosensory cortex may contribute to the social impairments in these models as well. Yet, it appears counterintuitively that cortical hyper-reactivity would contribute to blunted somatosensory responses and reduced social interactions, unless it represents a compensatory mechanism for reduced or diffuse sensory input, as described by Miceli et al. (submitted) based on neuroanatomical findings in 5-HTT knockout rats. As speculated (section Prenatal Valproic Acid Exposure in Rats), if environmental stimuli are perceived overly intense, these responses are driven by avoidance or withdrawal, as a self-protective mechanism.

To increase our understanding of the role of 5-HT in brain development, with important implications for ASD, it would be essential to fill in the "gaps" in **Table 1**, and to link structural phenotypes to behavior. To this end, new *in vivo* technologies like optogenetics will significantly increase the understanding of the physiological properties of specific cell-types in relation to behavior. E.g., if cortical layer II/III neurons show hyper-connectivity, optogenetically-mediated modulation in firing of specific excitatory or inhibitory neuron classes upon their activation in response to specific environmental stimuli (e.g., whisker stimulation) may help to remediate the somatosensory part of the intense world syndrome. Furthermore, using *in utero* electroporation, a technique that allows region specific gene manipulation in embryo's (Kolk et al., 2011), we might be able to understand through which 5-HT receptors 5-HT mediates its developmental effects. This information may eventually lead to pharmacological targets to steer the structural and behavioral consequences of high 5-HT levels during embryonic development.

In sum, comparing distinctive human conditions and animal models characterized by early life perturbations of the serotonergic system is a powerful approach to unravel the structural and behavioral consequences of high 5-HT levels during development. Indeed, similarities in brain and behavior in human subjects and animal models characterized by high 5-HT levels during development will strengthen the value of experimental findings and bring us closer to the answer how 5-HT during development contributes to ASD-related symptoms.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 January 2013; accepted: 30 April 2013; published online: 12 June 2013.

Citation: Kinast K, Peeters D, Kolk SM, Schubert D and Homberg JR (2013) Genetic and pharmacological manipulations of the serotonergic system in early life: neurodevelopmental underpinnings of autism-related behavior. Front. Cell. Neurosci. 7:72. doi: 10.3389/fncel.2013.00072

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 21 May 2013
doi: 10.3389/fncel.2013.00073

The effects of maternal depression and maternal selective serotonin reuptake inhibitor exposure on offspring

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It has been estimated that 20% of pregnant women suffer from depression and it is well-documented that maternal depression can have long-lasting effects on the child. Currently, common treatment for maternal depression has been the selective serotonin reuptake inhibitor medications (SSRIs) which are used by 2–3% of pregnant women in the Nordic countries and by up to 10% of pregnant women in the United States. Antidepressants cross the placenta and are transferred to the fetus, thus, the question arises as to whether children of women taking antidepressants are at risk for altered neurodevelopmental outcomes and, if so, whether the risks are due to SSRI medication exposure or to the underlying maternal depression. This review considers the effects of maternal depression and SSRI exposure on offspring development in both clinical and preclinical populations. As it is impossible in humans to study the effects of SSRIs without taking into account the possible underlying effects of maternal depression (healthy pregnant women do not take SSRIs), animal models are of great value. For example, rodents can be used to determine the effects of maternal depression and/or perinatal SSRI exposure on offspring outcomes. Unraveling the joint (or separate) effects of maternal depression and SSRI exposure will provide more insights into the risks or benefits of SSRI exposure during gestation and will help women make informed decisions about using SSRIs during pregnancy.

Keywords: 5-HTT, maternal depression, neurodevelopment, serotonin, SSRI

The number of women using selective serotonin reuptake inhibitors (SSRIs) during pregnancy is increasing, although knowledge on long-term neurodevelopmental effects to the child is lacking. This review summarizes clinical and preclinical findings of how SSRI exposure during pregnancy affects child outcomes. Many clinical findings parallel aspects of the preclinical data, such as decreased gestational length, birth weight, pain responses, and social behavior, increased spontaneous abortion/mortality rate, risk of cardiac anomalies, anxiety, depression, and rapid eye movement (REM) sleep, and affected 5-HT metabolism, motor development, and hypothalamic-pituitary-adrenal (HPA) stress reactivity. However, antenatal depression also has been associated with long-term neurodevelopmental outcomes. This review therefore starts by describing effects on the offspring exposed to antenatal depression and will then focus on outcomes of SSRI exposure during pregnancy.

MATERNAL DEPRESSION

Women are at an increased risk of becoming depressed during pregnancy and in the postpartum period, especially when they have pre-existing psychiatric illnesses. In fact, depressive symptoms may occur more frequently during pregnancy than in the postpartum period (Suri et al., 2007). During pregnancy, ∼20% of women report symptoms of depression (Patkar et al., 2004), and 4–7% of pregnant women suffer from major depressive disorder (Andersson et al., 2003; Gorman et al., 2004; Melville et al., 2010). Among women who experience postpartum depression, nearly 40% develop their symptoms during pregnancy (Johnson, 1997). Biological and psychosocial factors, such as the genetic setup of the mother, hormonal/reproductive history, current stressors, and life experiences, are known to be risk factors for development of antenatal depression (Miller and LaRusso, 2011). Antenatal depression has been associated with higher rates of poor pregnancy outcomes (such as pre-eclampsia and premature delivery), impaired fetoplacental function, decreased fetal growth, and neonatal complications (Orr and Miller, 1995; Kurki et al., 2000; Bonari et al., 2004; Jablensky et al., 2005; Wisner et al., 2009; El Marroun et al., 2012). However, while premature delivery and decreased fetal growth are established outcomes of antenatal depression (Henrichs et al., 2010), the influence is most profound in low-income countries and countries with great health inequalities (Grote et al., 2010). Antenatal depression is also associated with poor nutrition, obesity, smoking, alcohol, and drug abuse which all can have negative effects on the developing child (Andersson et al., 2004; Bonari et al., 2004).

Several neurodevelopmental outcomes have been reported in children exposed to antenatal or postpartum depression. While it has long been known that postpartum depression is associated

with poor maternal-child attachment with long-term repercussions (Murray and Cooper, 1997), fewer studies have addressed the effects of antenatal depression. DiPietro et al. (2006) reported that antenatal depression improved the mental and motor development in 2-year-old children, indicating that moderate amounts of maternal adversity may optimize early child development. However, most other studies have found negative associations between antenatal depression and neurodevelopmental outcomes in children. For instance, antenatal depression has been associated with developmental delays in 18-month-old children (Deave et al., 2008), increased behavioral and emotional problems in 4-year-old children (O'Connor et al., 2002), increased anxiety in 6- to 9-year-old children (Davis and Sandman, 2012), and attention problems in children aged 3 and 4 (Van Batenburg-Eddes et al., 2012). Later on, also gender-related offspring effects have been reported. Hay et al. (2008) tested the effects of antenatal and postpartum depression on children's outcomes during adolescence and found that 42% of the antenatally depression-exposed and 46% of the postpartum depression-exposed adolescents displayed emotional disorders. Interestingly, the association between antenatal depression and emotional disorders was only significant in adolescent girls. Parenthetically this gender-related offspring differences hold true for postpartum depression as well. Following exposure to maternal postpartum depression increased internalizing and externalizing problems in 12-year-old children have been reported (Agnafors et al., 2012), where girls expressed more internalizing problems, and boys expressed more externalizing problems. Hay et al. (2008) conclude that the greater the extent of exposure to maternal depression, the more likely it was for the child to develop a broader range of problems.

It should also be emphasized that paternal depression is of relevance for offspring developmental outcomes. Paulson et al. (2009) studied language development in children whose mother or father were depressed 9 and 24 months after birth. Depressive symptoms in either the mother or the father lowered the frequency of reading to their child. However, only fathers' depression predicted lower frequencies of reading to the child at the age of 24 months and reduced expressive language at the age of 2 years. Furthermore, van den Berg et al. (2009) showed that paternal depression also has an influence on excessive infant crying.

Thus, antenatal maternal depression poses a threat to maternal both well-being and healthy development in the offspring. These effects are likely due to a number of factors such as the physiology of the intrauterine environment, perinatal maternal and paternal mood disorders, current stressors, social support, timing, intensity, and genetic background. Therefore, understanding the influence of antenatal depression during pregnancy on child outcomes is rather complex. Incorporating methods of studying the fetus that has been exposed to antenatal depression provides the opportunity to examine the intrauterine milieu as the developmental niche of the fetus and will help us to unravel the mechanisms underlying maternal psychological factors that may have long-lasting developmental effects (DiPietro, 2012; Sandman and Davis, 2012).

ANTIDEPRESSANT MEDICATION USE DURING PREGNANCY

Continuing or starting pharmacological therapy during pregnancy is often unavoidable. Cohen et al. (2006) showed that 68% of depressed women who discontinued treatment relapsed during pregnancy, while only 26% of those who continued treatment did so. Currently, 1–3% of pregnant women in Europe are using antidepressant medications (El Marroun et al., 2012; ADs; Kieler, 2012), while user frequencies in the U.S. are 4–13% (Cooper et al., 2007; Hayes et al., 2012). Twenty-five percent of women on antidepressants continue treatment during pregnancy and 0.5% of pregnant women who have not been treated with antidepressants previously begin treatment (Ververs et al., 2006). As antidepressant medications cross the placenta and are evident in breast milk, questions have been raised about their developmental safety (Heikkinen et al., 2003; Noorlander et al., 2008). However, exposure to antenatal depression similarly increases the risk of child psychopathology (affective, anxiety, and disruptive behavior disorder; Weissman et al., 2006). Therefore, the question arises as to whether children exposed to maternal antidepressants are at risk and, if so, whether the risks are due to medication or to the underlying depression.

SELECTIVE SEROTONIN REUPTAKE INHIBITORS

SSRIs are the most widely prescribed antidepressants worldwide because of their efficacy, relatively few (adverse) side-effects, and therapeutic safety (Barbey and Roose, 1998). SSRIs do not cause gross structural neuroteratogenic effects and are often considered to be safe for antenatal use (Gentile, 2005). Therefore, prescription of SSRIs during pregnancy, to promote the psychological health of the mother, has increased (Oberlander et al., 2006). By blocking the serotonin transporter (5-HTT) SSRIs inhibit the reuptake of serotonin (5-HT) into presynaptic nerve terminals resulting in an increase in the synaptic concentration of 5-HT (see **Figure 1**). During adulthood 5-HT mainly acts as a modulatory neurotransmitter regulating emotion, stress responses, arousal, sleep, learning, cognition, and attention. However, during brain development 5-HT also acts as a neurotrophic factor, regulating cell division, differentiation, migration, growth cone elongation, myelination, synaptogenesis, and dendritic pruning (Gaspar et al., 2003). Thus, changes in the 5-HT levels during neurodevelopment have the potential to affect a number of processes (Ansorge et al., 2007). While human studies are hampered by time and ethical constraints, animal models offer the possibility to study both the short- and long-term consequences of maternal SSRI exposure. Therefore, both **clinical** and **preclinical** data on the effects of maternal SSRI exposure on the offspring are described in this review.

CLINICAL FINDINGS

Antidepressants are able to cross the placenta and relevant concentrations have been detected in umbilical vein blood (Hendrick et al., 2003b). Fluoxetine and citalopram have a high ratio of umbilical vein-to-maternal serum concentration, while sertraline and paroxetine have a low ratio. Maternal plasma levels of fluoxetine and its metabolite, norfluoxetine, decrease drastically during pregnancy (Heikkinen et al., 2003), probably due to the normal physiological changes during pregnancy. At birth, neonatal

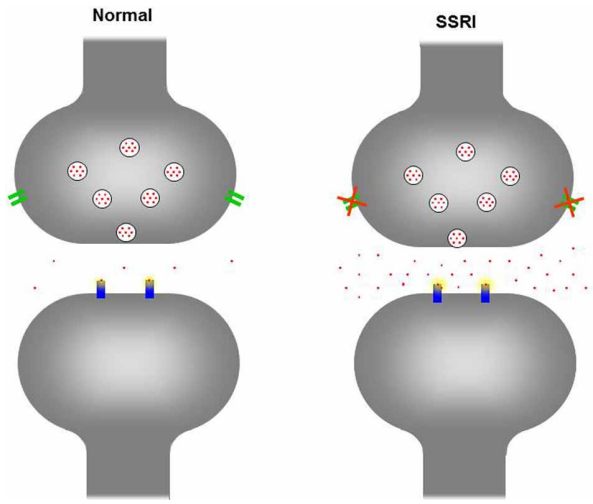

FIGURE 1 | Schematic figure of the serotonergic neuron in a normal situation (left) and when exposed to SSRIs (right). Upon neuronal activation 5-HT (red dots) is being released in the extracellular cleft, activating receptors (blue) at the postsynaptic neuron. To end the signal, excessive 5-HT is being re-uptaken by the serotonin transporter (green) from the extracellular cleft into the presynaptic neuron. In the presynaptic neuron 5-HT is degraded and/or stored in vesicles for future release. In the picture on the right, the serotonin transporter has been blocked by a SSRI and is no longer capable to reuptake 5-HT in the presynaptic neuron, increasing the 5-HT in the extracellular cleft.

plasma levels of fluoxetine and norfluoxetine have been shown to be 65 and 72% of the maternal levels (Heikkinen et al., 2003).

Pregnancy complications

Two meta-analyses have revealed that SSRIs and other antidepressant medications may increase the risk of miscarriage (Hemels et al., 2005; Rahimi et al., 2006). However, this may not always be the case (reviewed in Ellfolk and Malm, 2010), while a recent meta-analysis showed only a borderline association (Ross et al., 2013). Women continuing SSRI use after the first trimester also have an increased risk of preeclampsia compared with women who discontinue treatment or non-users (Qiu et al., 2009; Toh et al., 2009b; Reis and Källén, 2010). Recently, Palmsten et al. (2012) found that the risk of developing preeclampsia was similar in non-depressed and depressed women (2.3 and 2.4%, respectively). Furthermore, compared to depressed women, the relative risk of preeclampsia after SSRI exposure in GW10 and 20 was 3.3 for monotherapy and 4.5 for polytherapy [and even greater for selective noradrenalin reuptake inhibitors (SNRI) and tricyclic antidepressants (TCA)]. In conclusion, antidepressant use during pregnancy increases the risk of preeclampsia, with modest effects after use of SSRIs and much higher effects after use of SNRIs and TCAs.

Pregnancy outcomes

As with maternal antenatal depression, SSRI use during pregnancy has often been associated with increased rate of preterm birth (Chambers et al., 1996; Costei et al., 2002; Simon et al., 2002; Källén, 2004; Wen et al., 2006; Davis et al., 2007; Lund et al., 2009; Wisner et al., 2009; Reis and Källén, 2010; Yonkers et al., 2012), decreased birth weight (Chambers et al., 1996; Källén, 2004; Wen et al., 2006), being born small for gestational age (Oberlander et al., 2006; Toh et al., 2009a), and reduced fetal head growth (El Marroun et al., 2012). However, several studies did not find an effect of SSRIs on preterm birth (Kulin et al., 1998; Ericson et al., 1999; Suri et al., 2004; Malm et al., 2005; Oberlander et al., 2006; Toh et al., 2009a) and birth weight (Ericson et al., 1999; Suri et al., 2004; Malm et al., 2005; Lund et al., 2009; Reis and Källén, 2010). Nevertheless, an inverse relationship was found between lower gestational age and high doses of SSRIs in late pregnancy (Suri et al., 2007). Several theories have been postulated for low birth weight after exposure to SSRIs; for example, fluoxetine reduces maternal appetite and weight gain, which may affect fetal growth (Chambers et al., 1996). However, other SSRIs have been associated with weight gain, rather than weight loss. Another theory is that the altered 5-HT levels, caused by SSRIs use, increase the risk of intrauterine growth retardation and preterm delivery by impairing placental blood flow (Wen et al., 2006). Whether or not these factors play a role in gestational age and weight remains to be elucidated.

Umbilical cord blood monoamine and metabolite concentrations

SSRI treatment during pregnancy reduces whole blood 5-HT (−69%), 5-hydroxyindoleacetic acid (−18%; 5-HIAA; main metabolite of 5-HT) and homovanillic acid (−23%; a major catecholamine metabolite) concentrations in the umbilical vein (Laine et al., 2003). In infants, lower 5-HIAA concentrations are inversely correlated with 5-HTergic symptom scores (such as myoclonus, restlessness, tremor, shivering, hyperreflexia, incoordination, and rigidity) and there is a positive correlation between cerebrospinal fluid and peripheral blood 5-HT/metabolite concentrations (Sarrias et al., 1990). This suggests an association between the central 5-HTergic effects and the cord blood 5-HIAA concentration. Similarly, plasma levels of noradrenalin were decreased in the umbilical vein of SSRI-exposed infants and there was also a tendency for reduced dihydroxyphenylglycine (DHPG; group I metabotropic glutamate receptor selective agonist) and 3,4-Dihydroxyphenylacetic acid (DOPAC; metabolite of dopamine) in SSRI-exposure infants (Laine et al., 2003). Not surprisingly, pharmacokinetic differences exist between antidepressants. DHPG concentrations were significantly lower (−40%) in fluoxetine-exposed infants compared with citalopram-exposed infants. This effect may be due to the lower affinity of citalopram, compared to fluoxetine, for the noradrenaline reuptake pump (Hyttel, 1994). On the other hand, citalopram, but not fluoxetine, significantly reduces cord blood DOPAC concentrations compared with controls. Thus, maternal use of SSRIs induces significant changes in the cord blood 5-HT and metabolite concentrations. However, it remains to be determined how these changes in 5-HT and its metabolite impact the outcome of the offspring.

Neonatal adaptation

In the first 2 weeks after birth up to 30% of antenatal SSRI-exposed neonates display poor neonatal adaption such as respiratory distress, temperature instability, feeding difficulties, jitteriness, irritability, sleep problems, tremors, shivering,

restlessness, convulsions, rigidity, hypoglycaemia, and jaundice (Chambers et al., 1996; Cohen et al., 2000; Costei et al., 2002; Casper et al., 2003; Laine et al., 2003; Källén, 2004; Oberlander et al., 2006; Davis et al., 2007). These effects occur more often in neonates who were exposed to SSRIs in late pregnancy, and symptoms arise earlier and more often in neonates exposed to higher SSRI doses (Costei et al., 2002; Källén, 2004; Davis et al., 2007). A dose-response effect of paroxetine on neonatal adaptation problems has been reported (Levinson-Castiel et al., 2006) with higher doses of paroxetine being related to greater neonatal adaptation problems. In addition to the dose, the duration of SSRI exposure plays a significant role on neonatal outcomes with respiratory distress being linked to longer prenatal SSRI exposure (Oberlander et al., 2008c). It is unclear whether the neonatal adaptation symptoms are a result of neonatal withdrawal from the SSRIs or overstimulation of the 5-HTergic system (Isbister et al., 2001). Nevertheless, these symptoms are usually mild and disappear 2 weeks postpartum (Moses-Kolko et al., 2005).

Another way to measure neonatal adaptation is to measure gross outcome markers such as Neonatal Intensive Care Unit (NICU) admission and neonatal seizures. Several studies report an increased risk of neonatal seizures, longer hospital stays, and NICU admissions after SSRI use during pregnancy (Simon et al., 2002; Källén, 2004; Lattimore et al., 2005; Oberlander et al., 2006; Wen et al., 2006; Cole et al., 2007; Davis et al., 2007), although an increased risk for NICU admissions have also been found after prenatal depression (Chung et al., 2001). Malm et al. (2005) found that 11.2% of neonates exposed to SSRIs in the first trimester and 15.7% of infants exposed to SSRIs during the third trimester of pregnancy were treated in specialized or intensive care units. There is also a 2- to 8-fold increase risk for low Apgar scores in SSRI-exposed neonates (Källén, 2004; Lund et al., 2009; Wisner et al., 2009). Neonates of depressed mothers also often display low Apgar scores (Wisner et al., 2009). Therefore, it is difficult to disentangle whether the low Apgar scores and NICU admissions are due to the SSRI exposure or to the underlying depression.

Congenital malformations in the neonate

SSRI use during pregnancy may increase the risk for congenital malformations and cardiac anomalies. A Danish study reported that 4.9% of infants exposed to SSRIs during the first trimester of pregnancy, and 6.8% exposed to SSRIs during late pregnancy display congenital malformations, while corresponding the figure in non-exposed infants was 3.4% (Wogelius et al., 2006). Chambers et al. (1996) found more minor anomalies in infants exposed to SSRIs during the first trimester of pregnancy compared with non-exposed infants, while no differences were found in the number of major anomalies. Alwan et al. (2007) report that first trimester SSRI exposure increases the risk for anencephaly, craniosynostosis, and omphalocele. Louik et al. (2007) also found an increased risk for omphalocele and for septal defects after first trimester exposure to sertraline and an association between paroxetine exposure and right ventricular outflow tract obstruction defects. Moreover, sertraline was associated with anal atresia and limb-reduction defects and paroxetine was associated with neural tube defects, club foot, and undescended testes (Louik et al., 2007). Cardiac malformations were also reported by Malm et al. (2005) and Diav-Citrin et al. (2008), who found a 3- to 4-fold increased in cardiac malformations in infants of fluoxetine-exposed women. However, there are also several studies that do not report an association with maternal prenatal SSRI exposure and neonatal congenital malformations (Altshuler et al., 1996; Ericson et al., 1999; Simon et al., 2002; Hendrick et al., 2003a; Einarson and Einarson, 2005; Källén and Otterblad, 2007). Overall, the effects of prenatal SSRI exposure on congenital malfunction appear small and seem to be most apparent when SSRIs are used in the first trimester of pregnancy. However, the effects of prenatal SSRIs on congenital heart disease becomes more severe if SSRIs are taken with other medications, such as benzodiazepines (Oberlander et al., 2008d).

Persistent pulmonary hypertension in the neonate

In the condition of persistent pulmonary hypertension (PPHM) the pulmonary vasculature fails to relax after birth, which results in hypoxemia. The occurrence of PPHN is ∼0.2% in live-born infants and it is associated with substantial infant mortality and morbidity. Several studies have shown an increased risk for PPHM in SSRI-exposed infants. Exposure during the first trimester (Källén and Olausson, 2008), as well as during late pregnancy (Chambers et al., 1996, 2006), significantly increases the risk for PPHM. This result was confirmed in a large Nordic study, where the risk for PPHM in neonates after SSRI exposure was shown to be at least doubled (Kieler, 2012). However, several studies did not find any association between prenatal SSRI use and PPHM (Andrade et al., 2009; Wichman et al., 2009; Wilson et al., 2011). Moreover, both maternal depression and SSRI usage have been linked to increased risk of premature birth (Wisner et al., 2009), with the risk of PPHN being four times higher in babies born at 34–36 weeks compared to those with full-term gestation (Källén and Otterblad, 2007; Hibbard et al., 2010). Therefore, it is difficult to state whether maternal SSRI exposure truly increases the risk for PPHM, or if other, secondary, factors contribute to the increased risk for PPHM.

Neurodevelopmental outcomes

Within the first week after birth, infants are exposed to a routine heel lance (blood sampling for screening of metabolic diseases). Oberlander et al. (2002) used this acute noxious event to study the effect of maternal SSRI exposure on neonatal responses to pain. In response to the heel lance, SSRI-exposed newborns show significantly less facial activity and a reduced heart rate, indicating that prenatal exposure to SSRIs attenuates the response to acute pain in newborns. When the heel lance was repeated after 2 months, the pain response was still attenuated in SSRI exposed infants (Oberlander et al., 2005). The attenuated pain response may be due to increased 5-HT and GABA agonist levels caused by SSRIs, as 5-HT and GABA agonists are known to play a role in pain modulation and are active during early fetal neurologic growth (Whitaker-Azmitia, 2001; Oberlander et al., 2002). Zeskind and Stephens (2004) found that SSRI-exposed infants displayed increased tremulousness, fewer changes in behavioral state, fewer different behavioral states and greater amounts of uninterrupted REM-sleep. Together, these results suggest that

prenatal SSRI exposure has an effect which already appears early after birth.

Although some studies exist, the long-term neurodevelopmental outcomes of prenatal SSRI exposure have not been extensively studied. With regards to language development, Nulman et al. (1997); Nulman et al. (2002) studied the IQ, temperament and language development in children (16 and 86 months old) who were exposed to SSRIs during pregnancy but did not find any effects of prenatal SSRI exposure on the neurodevelopmental outcomes measured. Prenatal SSRI exposure also appears to have no effect on motor or speech development during the first 2 years of life (Simon et al., 2002). Interestingly, Weikum et al. (2012) compared infants of healthy mothers, with infants exposed to SSRIs and infants exposed to antenatal depression and found that SSRI-exposed infants showed accelerated perceptual development by discriminating both vowels and consonants at 36 weeks gestation (while *in utero*). These data indicate that SSRI-exposure may alter the developmental time course of language perception.

However, there are several studies which describe an effect of prenatal SSRI exposure on neurobehavioral outcomes. Oberlander et al. (2007) studied externalizing behaviors (attention, aggression, attention/hyperactivity, and oppositional or defiant behaviors) in 4 year olds and found that SSRI-exposed children had greater externalizing scores than the clinical cutoff. Data on internalizing behaviors is more conflicting. Whereas prenatal SSRI exposure and/or maternal depression have been associated with increased internalizing behaviors (e.g., depression, anxiety, withdrawal) in 3- and 4-year-old children (Oberlander et al., 2010), other studies have found no such effects (Misri et al., 2006). Additional studies report that 6–40 month old SSRI-exposed children show mild effects on motor development and control (tremulousness and fine motor movements), and lower Psychomotor Developmental Index (PDI) scores on the Bayley Scales of infant development (Casper et al., 2003). Mortensen et al. (2003) studied psychomotor development in 7- to 10-month-old children by means of the Boels test and found that in children prenatally exposed to antidepressants (not specific for SSRIs) had an increased risk for abnormal Boels test, indicating that the risk for abnormal psychomotor development (such as hearing, sight, and motor attention) is higher in children exposed to antidepressants. Recently prenatal SSRI exposure, especially during the first trimester, has been associated with an increased risk for autism spectrum disorders (Croen et al., 2011). Together these data suggest that prenatal SSRI exposure has effects on neurodevelopmental outcomes, at birth and also later in childhood.

Stress regulation

Apart from its role in neurodevelopment, 5-HT is implicated in the development and function of the HPA axis (Meaney et al., 1994; Laplante et al., 2002; Andrews and Matthews, 2004) and prenatal SSRI exposure has been suggested to affect aspects of HPA function. Previous work has shown that prenatal SSRI exposure results in attenuated basal salivary cortisol levels (Brennan et al., 2008; Oberlander et al., 2008b) and attenuated facial action and heart rate in response to an acute painful stressor in infants (Oberlander et al., 2002, 2005). Corticosteroid binding globulin (CBG), a transporter and regulator of circulating cortisol levels (Siiteri et al., 1982), has been shown to be increased in SSRI-exposed neonates, particularly after vaginal delivery (Pawluski et al., 2012a). This increase in neonatal CBG levels was negatively associated with diurnal changes in salivary cortisol at 3 months of age. Furthermore, infants prenatally exposed to SSRIs have lower evening basal cortisol levels and there are lower post-stress cortisol levels in non-SSRI exposed and non-breastfed infants compared with SSRI-exposed and non-SSRI exposed infants who were breastfed at 3 months of age (Oberlander et al., 2008b). These findings suggest that the effect of prenatal SSRI exposure is present, but may only become apparent in a particular maternal caregiving context (Hanley and Oberlander, 2012).

Serotonin transporter gene

The 5-HT transporter (5-HTT) plays a critical role in moderating environmental influences and developmental risks (Homberg and Lesch, 2011). Humans carry a polymorphism in the promoter region of the 5-HTT gene (5-HTTLPR), which involves a common 44-base pair insertion/deletion of a repetitive sequence (Lesch et al., 1996). The dominant short (S) allelic variant reduces transcriptional efficiency of the SERT as compared with the long (L) allelic variant (Lesch et al., 1996). Allelic variation of 5-HTTLPR may contribute to the responsiveness of SSRIs in depressed patients. Pollock et al. (2000) showed that paroxetine reduced depressive symptoms more rapidly in patients with the LL genotype compared with S-allele carriers. Even early in life allelic variation of the 5-HTTLPR can influence neonatal behavior, especially in combination with environmental factors. For example, when maternal anxiety levels were high, more negative emotionality was found in infants carrying the S-allele, whereas no effect of the 5-HTTLPR was found in circumstances with low maternal anxiety (Pluess et al., 2011). Tiemeier et al. (2012) also showed that the effect of maternal anxiety during fetal life and early adulthood is moderated by the 5-HTTLPR of the child. Children with the S-allele were at increased risk of developing emotional problems and were less accurate in emotion-matching, indicating affected ability to process emotions. Adults with two S-alleles may be at increased risk for depression following early life adversity (Caspi et al., 2003; Kendler et al., 2005; Lesch, 2007); however, under positive environments S-allele carriers might benefit more compared to L-allele carriers. Hankin et al. (2011) showed that positive parenting resulted in higher levels of positive affect in S-allele infants. These data are in agreement with the theory of Belsky et al. (2009) who suggested that S-allele carriers are more vulnerable in general, not only negatively, but also positively. Thus, vulnerability genes, or risk alleles, seem to make individuals more susceptible to environmental influences.

The combination of the allelic variation in 5-HTTLPR and prenatal SSRI exposure may compound risks associated with altered 5-HT levels. Recently an association was found, after prenatal SSRI exposure, between (1) SS-allele carriers and lower 5-min Apgar score and risk for neuromotor symptoms; (2) LS-allele carriers and low birth weight; and (3) LL-allele carriers and respiratory distress and tachypnea (Oberlander et al., 2008a). In 3-year old SS-allele carriers, prenatal exposure to maternal anxiety was associated with increased internalizing behaviors and in 3-year old LL carriers, prenatal maternal anxiety was

associated with more externalizing behaviors, regardless of prenatal SSRI exposure (Oberlander et al., 2010). Thus, 5-HTTLPR genotype influences the effect of antenatal mood on child behavior (Oberlander et al., 2010) and may modulate the outcome of adverse neonatal effects following maternal SSRI exposure. However, much more research is necessary to understand how perinatal exposure to SSRIs affect developmental outcomes and how these effects differ from the effects of exposure to perinatal maternal mood disorders.

PRECLINICAL FINDINGS

In order to better understand the neurobehavioral and long-term effects of perinatal exposure to SSRIs animal models have been used. In particular, much research has investigated these effects using rodents. At birth rats and mice are at a relative early stage of maturation and their brain maturation occurs after birth. This makes rodents highly suitable as a model for studying the direct effects of SSRI exposure on early brain development. When rats and mice are between 12 and 13 days old, the maturation of the cerebral cortex is comparable to the human neocortex around birth (Romijn et al., 1991; Homberg et al., 2010). The first and second trimester of pregnancy in humans is comparable to the prenatal period in rats, while the third trimester in humans is comparable to the period right after birth (until PND12–13) in rats. In the following studies both prenatal exposure and postnatal exposure to SSRIs are described.

Pregnancy outcomes

SSRIs are able to cross the placenta in rodents at a similar transfer rate to humans. Noorlander et al. (2008) exposed mice (i.p. injection) from embryonic day (E)8–E16 of gestation with either fluoxetine or fluvoxamine and collected blood plasma 5 h after the last injection. The transfer rate of fluoxetine across the placenta in mice (69%) was similar to the transfer rate of fluoxetine across the placenta in women (73%). A lower placental transfer rate was found for fluvoxamine in both mice (30%) and humans (35%). When pregnant rats were injected daily with fluoxetine from gestational day (G)11 until birth the placental transfer rate 5 h after the last injection was 83% for fluoxetine and 78% for norfluoxetine (Olivier et al., 2011). The norfluoxetine/fluoxetine ratio was 1.44 in mothers and 1.39 in pups, which is similar to the ratios found in humans (Lundmark et al., 2001). SSRIs are able to pass the blood brain barrier (Baumann and Rochat, 1995) and this was confirmed in the study of Olivier et al. (2011). Both fluoxetine and norfluoxetine have been detected in whole brain samples of rat pups (Olivier et al., 2011). Although differences exist between transfer rates of different SSRIs, they are transferred from mother to pup, altering both the periphery and the central nervous systems. At the highest dose of fluoxetine tested (0.8 mg/kg/day), an 81% mortality rate was found after prenatal exposure, while fluvoxamine did not affect the survival rate in mice (Noorlander et al., 2008). A 10-fold higher mortality rate of neonatal rats was also found after prenatal paroxetine exposure (van den Hove et al., 2008). Interestingly, rats that were prenatally exposed to fluoxetine (12 mg/kg/day; orally) from E11 until birth did not show increased mortality (Olivier et al., 2011). However, litters that were prenatally exposed to fluoxetine were smaller, therefore prenatal mortality is possible. Prenatal paroxetine exposure in rats did not influence the litter size at birth, but did reduce the gestational length and birth weight (van den Hove et al., 2008). Prenatal fluoxetine exposure from E11 until birth did not affect the gestational length, but did reduce the weight of pups early after birth (Olivier et al., 2011). Interestingly, Vorhees et al. (1994) have found increased neonatal mortality after prenatal fluoxetine exposure. Days of exposure and the use of different rat strains may account for differences between studies. No effects were found on long-term growth or survival (Vorhees et al., 1994; Olivier et al., 2011). In conclusion, differences types of SSRIs, doses, time-periods of SSRI exposure, and animal strains likely influence the birth and neonatal outcomes.

Monoamine and biochemical concentrations

Prenatal exposure to fluoxetine from E11 to E21 significantly reduced placental levels of 5-HT in rats (Fornaro et al., 2007). Postnatal exposure to Zimelidine (SSRI) to rat pups 2–3 weeks after birth significantly increased the 5-HIAA/5-HT ratio in the brain stem and cortex of 2 month old offspring (Hilakivi et al., 1995). In prenatally stressed mice, treatment with fluoxetine during postnatal weeks 1–3 also lowered the 5-HT turnover rate in offspring (Ishiwata et al., 2005). These data indicate that the 5-HT metabolism is affected by early SSRI exposure both in the periphery and the central nervous system. Limited amounts of information are available on the biochemical profile in rodents prenatally exposed to SSRIs. The neonatal behavioral syndrome, which is often seen after withdrawal of SSRIs, is associated with hypoglycemia (Favreliere et al., 2010). For this reason Dubovický et al. (2012) studied glucose, lactate dehydrogenase, aspartate aminotransferase/alanine aminotransferase ratio and antioxidant status in blood from prenatally (E15–E20) venlafaxine-exposed (SNRI) rats. However they report no differences between venlafaxine-exposed and non-exposed rat offspring on postnatal day (PND)21.

Congenital malformations

A higher mortality rate has been found in neonatal rodents after prenatal SSRI exposure (Noorlander et al., 2008; van den Hove et al., 2008) and it has been postulated that heart malformations may be one reason for this increase in mortality. Noorlander et al. (2008) found that the majority of fluoxetine-exposed offspring died postnatally because of severe dilated cardiomyopathy. Moreover, the ratio of thickness of the left ventricle to the radius of the left ventricle cavity was significantly decreased in prenatal fluoxetine-exposed mouse offspring both at PND20 and during adulthood. These data clearly show that prenatal fluoxetine exposure (0.8 mg/kg/day; i.p.) severely affects heart development, resulting in an increased death rate in offspring. *In vitro*, (Sari and Zhou, 2003) found that paroxetine significantly decreased the rate of proliferation of fetal heart cells (E13) from rats, particularly cardiac myocytes and, to a lesser degree, non-muscle cells. Fluoxetine and sertraline also have similar influences on the proliferation of cardiac cells in the mouse embryo (Yavarone et al., 1993). These data indicate that changes in prenatal 5-HT levels influence the proliferation of the embryonic heart cells, at least *in vitro*. Fluoxetine has furthermore been shown

to affect cell viability and differentiation from undifferentiated ES cells to cardiomyocytes in a dose-dependent manner. Analysis of tissue-specific markers showed also that fluoxetine inhibits mesodermal development but it promotes ectodermal differentiation (Kusakawa et al., 2008). In another study, late two-cell stage embryos incubated with fluoxetine for 6 h were more likely to develop into blastocysts compared to the controls. Exposure to fluoxetine for 24 h showed a reduction in blastocyst formation, suggesting a time dependent effect of fluoxetine on blastocyst formation. It also appears that these effects are, in part, due to altered TREK signaling (Kim et al., 2012). In humans, the cardiomyocyte proliferation is essentially complete at birth, whereas in rodents cardiomyocyte growth and proliferation is robust for the first 14 days after birth (Clubb and Bishop, 1984; Walsh et al., 2010). Haskell et al. (2012) injected mouse offspring with sertraline from PND1 to PND14, reflecting the third trimester in humans, and found that sertraline-exposed offspring showed increased heart rate and activity levels, as well as smaller left ventricular internal diameters in diastole and decreased stroke volumes, indicating changes in the cardiac morphology. Taken together, both *in vitro* and *in vivo* early-exposure to SSRIs have adverse consequences for the developmental outcomes of the heart.

Pulmonary hypertension

As far as we know, only one study has investigated the effects of prenatal SSRI exposure on pulmonary hypertension in animal models (Fornaro et al., 2007). Fluoxetine exposure during late gestation resulted in abnormal oxygenation and a higher mortality rate in new-born rat pups compared to non-exposed controls. Moreover, the right ventricular mass of the lung was higher in prenatal fluoxetine-exposed rats compared to controls. Interestingly the effects seem to be sex-dependent; the right ventricular hypertrophy after prenatal fluoxetine exposure was only significant in female pups (Belik, 2008). Moreover, the thickness of the medial smooth muscle layer of the small and large pulmonary arteries (used as magnitude of pulmonary vascular modeling) tended to be thicker in the female, compared to male, pups. These sex-differences in rats are interesting as the prevalence for PPHN in humans is higher in male infants (Hernandez-Diaz et al., 2007).

Rodents that constitutively lack the 5-HTT could be seen as a model for life-long SSRI exposure from conception. In 5-HTT knockout (5-HTT$^{-/-}$) mice that were exposed to hypoxia for several weeks, the number and wall thickness of pulmonary vessels decreased compared with controls (Eddahibi et al., 2000). Moreover, compared with wild-type controls the right ventricular systolic pressure was lower and the right ventricle hypertrophy was less hypertrophied in hypoxic 5-HTT$^{-/-}$ mice. In mice that overexpress the 5-HTT (5-HTT+) there is a 3-fold increase in right ventricle pressure compared to wild-type mice (MacLean et al., 2004). Moreover, when 5-HTT+ mice were exposed to hypoxia, right ventricular hypertrophy and pulmonary vascular remodeling were doubled compared to wild-types (MacLean et al., 2004).

In summary, SSRI exposure during development increases the risk for pulmonary hypertension in rodent models. Moreover, overexpression of the 5-HTT from conception on increases the risk, while disruption of the gene lowers the risk, for pulmonary hypertension. It appears that the imbalance of the 5-HTT during development contributes to the development of pulmonary hypertension.

Neurodevelopmental outcomes

Prenatal fluoxetine exposure (G6–G20) has been reported to cause a transient delay in motor development in rats on PND10 and PND12; decreased horizontal activity in an open arena on PND8, but increased retention time on a rotating rod on PND22 and PND49 (Bairy et al., 2007). With respect to pain, the sensitivity in response to a hot-plate test on PND30, PND45, and PND70 was not altered by early fluoxetine exposure (G0-PND21) in mice (Lisboa et al., 2007) or after fluoxetine exposure (PND1–21) in 8-week-old male rat offspring (Knaepen et al., 2013). However, in adolescent rat offspring postnatal fluoxetine exposure (PND0–PND6) did reduce pain sensitivity (Lee, 2009). Moreover, sensorimotor learning deficits were found in adolescence offspring exposed to fluoxetine, as well as reduced dendritic complexity of thalamocortical afferents and in layer IV of the barrel cortex on PND7 (Lee, 2009). In line with this, Xu et al. (2004) showed that early postnatal paroxetine exposure (PND0–PND8) in rats disrupts the organization of thalamocortical somatosensory barrels on PND8. Recent work has also shown that adult male offspring exposed postnatally (PND1–21) to fluoxetine has increased post-operative pain, measured as hypersensitivity to mechanical stimuli after hind paw incision (Knaepen et al., 2013). However, fluoxetine exposure to prenatally stressed offspring normalized post-operative pain. This suggests that the actions of fluoxetine likely differ in the presence of maternal adversity (Knaepen et al., 2013). Taken together, these data suggest that early SSRI exposure alters cortical development resulting in impaired transmission of tactile information to the primary somatosensory cortex.

Sleep-wakefulness patterns are also altered by early SSRI exposure. Escitalopram exposure (PND5–PND19) increased REM-sleep duration and decreased REM latency in mouse offspring (Popa et al., 2008). In rat offspring, postnatal chlorimipramine exposure (week 1–3) resulted into reduced active sleep, compensated with quiet sleep (Mirmiran et al., 1981). Apart from altered sleep patterns, chlorimipramine-exposed animals also performed less efficiently on a temporal learning task but responded more rapidly in a spatial alternation learning task. Prenatal exposure to fluoxetine (G6–G20) increased cognitive performance; fluoxetine-exposed rat offspring found a hidden platform in a water maze faster compared with controls and had an increased latency to enter a compartment where they previously received a shock (Bairy et al., 2007). Using a model of prenatal stress, Ishiwata et al. (2005) found that postnatal fluoxetine treatment (postnatal weeks 1–3) to mouse offspring reduced the deficits in spatial learning and memory seen after prenatal stress. Moreover, postnatal SSRI exposure reversed the prenatal stress-induced reduction in spine and synapse density in CA3 pyramidal cells of the hippocampus (Ishiwata et al., 2005). As the learning ability strongly correlates with the spine or synapse density in hippocampal neurons, these data indicate that the increased synapse density found after early fluoxetine exposure is the cellular basis of restoring learning deficits induced by prenatal stress. Together these

data indicate a favorable effect of early SSRI exposure on learning and memory.

With respect to social and reproductive behaviors, early (G0 to PND21) fluoxetine exposure (Lisboa et al., 2007) as well as postnatal (PND1–PND19) citalopram exposure (Manhães de Castro et al., 2001) increased the latency to the first attack of an intruder, indicating reduced aggression. Postnatal treatment (PND8–PND21) with chlorimipramine, a tricyclic antidepressant, clearly disturbed the performance of sexual behavior in male offspring with fewer mice ejaculating (Mirmiran et al., 1981). The offspring that did ejaculate showed an increased latency to the first ejaculation. Nevertheless, the number of mounts and intromissions were similar between groups, although the mount/intromission ratio was higher in chlorimipramine-exposed animals indicating that these animals were less efficient. Maciag et al. (2006) found that postnatal citalopram exposure (PND8–PND21) significantly impaired mounting behavior, reduced the number of intromissions and the number of ejaculations. Interestingly, when rats were prenatally (G11 till birth) exposed to fluoxetine no effects were found on the sexual performance (Olivier et al., 2011). However, developmental fluoxetine treatment (PND1–21) decreased the anogenital distance in juvenile male offspring, decreased the number of intromissions, increased the latency to the first intromission, and increased the latency to the first ejaculation in sexually naive male offspring (Rayen et al., 2013). These effects were not evident if postnatal fluoxetine exposure occurred after prenatal stress. Furthermore, developmental fluoxetine and/or prenatal stress decreased the area of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in these offspring (Rayen et al., 2013). Prenatal fluoxetine exposure significantly affected juvenile play behavior and, during adulthood, prenatal fluoxetine-exposed animals still tended to make less contact with other rats (Olivier et al., 2011). Postnatal exposure (PND8–PND21) to citalopram also decreased the interest to play in male, but not female, juvenile rats (Simpson et al., 2011). In conclusion, social and reproductive behaviors appear to be most affected when 5-HT levels are disturbed during the postnatal period in rodent models.

Affective behaviors in offspring are also altered by early SSRI exposure. When rats were postnatally (PND8–PND21) exposed to citalopram a neophobic response to an auditory stimulus, as well as reduced exploration to a novel object, were found (Simpson et al., 2011). In addition, citalopram exposure led to abnormal myelin formation and a reduction in callosal connectivity, indicating the importance of normal 5-HT homeostasis for a proper maturation of the brain. Both prenatal (Bairy et al., 2007; Olivier et al., 2011) and postnatal (Mirmiran et al., 1981; Ansorge et al., 2004; Lisboa et al., 2007; Ansorge et al., 2008; Popa et al., 2008; Simpson et al., 2011) SSRI exposure increased anxiety-like behaviors in adult mice and rats. Also depression-like behavior was increased after prenatal (Olivier et al., 2011) and postnatal (Hansen et al., 1997; Lisboa et al., 2007; Popa et al., 2008) SSRI exposure in adulthood. In adolescence, recent work has shown that postnatal fluoxetine exposure (PND1–21) does not significantly alter depressive-like behavior in male and female rat offspring (Rayen et al., 2011). In addition, postnatal fluoxetine exposure reversed effects of prenatal stress on depressive-like behavior in adolescent offspring, thus normalizing this behavior (Rayen et al., 2011). Similarly, postnatal fluoxetine exposure reversed the effects of prenatal stress on hippocampal neurogenesis in adolescence (Rayen et al., 2011). This suggests that the long-term effects of fluoxetine may vary with age and previous exposure to maternal stress.

The 5-HT_{1A} receptor might be an important factor contributing to the altered affective behaviors. During early brain development, the 5-HT_{1A} receptor is involved in neurite branching (Sikich et al., 1990), neurite outgrowth and neuronal survival (Fricker et al., 2005). Moreover, 5-HT_{1A} autoreceptors in raphe 5-HTergic neurons are important in regulating central 5-HT neurotransmission by their negative feedback of 5-HT neuron firing. Functional desensitization of the 5-HT_{1A} autoreceptors is one of the mechanisms that **is** thought to play a role in the therapeutic action of SSRIs (Pineyro and Blier, 1999). Interestingly, both prenatal (Olivier et al., 2011) and postnatal (Popa et al., 2008) SSRI exposure increased the 5-HT_{1A} agonist-induced hypothermia, indicating increased sensitivity of the 5-HT_{1A} receptor. Besides changes in the 5-HT_{1A} receptor functioning, embryonic SSRI exposure has also been shown to reduce 5-HTT expression (Hansen and Mikkelsen, 1998) and 5-HT_2 receptor density and function (Cabrera and Battaglia, 1994). Thus, early exposure to SSRIs affects the 5-HTergic system, however, processes downstream of 5-HT receptors also mediate the neurotrophic effect of 5-HT. Moreover epigenetic modifications may contribute to developmental outcomes (Kinnally et al., 2010). Overall, early exposure to SSRIs has an effect on brain development and neuroplasticity (for review see: Pawluski, 2012) which can markedly alter the behavior of the offspring.

Stress regulation

Prenatal SSRI exposure has been shown to affect the developing HPA system in animal models. For example, prenatal exposure to fluoxetine increased cortisol levels in fetal lambs (Morrison et al., 2004). Moreover, postnatal exposure to SSRIs decreased the serum corticosterone levels and reduced the expression of CA3 hippocampal glucocorticoid receptor (GR) and its co-activator GR interacting protein 1 (GRIP1) in adolescent rat offspring (Pawluski et al., 2012b). These results were only found in male adolescent offspring, indicating a sex difference in the neurodevelopmental outcome. Postnatal exposure to fluoxetine (weeks 1–3) was also shown to reverse the effects of prenatal stress on the corticosterone response to stress in adult mouse offspring (Ishiwata et al., 2005). Postnatal fluoxetine exposure to prenatally stressed rats also increased CBG levels during adolescence, suggesting significant alterations in circulating levels of free corticosterone (Pawluski et al., 2012b). Of interest is the fact that these results were sex specific with long-term effects of combined early-life stress and fluoxetine exposure on the HPA system existing only in male offspring. These sex differences are likely due to differences in circulating sex steroid hormone levels, as estradiol has been shown to modulate the HPA system (Viau and Meaney, 1991; Atkinson and Waddell, 1997; Viau, 2002). Much more research is necessary to unravel the mechanisms underlying these sex differences in HPA development and the role of steroid hormones and monoamines in regulating these effects.

Table 1 | Overview of clincal and preclinical findings after early SSRI exposure.

	Humans	Prenatal exposure in rodents	Postnatal exposure in rodents
Pregnancy complications	↑ spontaneous abortion[1,2] ↑ risk of preeclampsia[3–6]	↑ mortality rate in newborn rats[7]	↑ mortality rate in mice[8] ↑ mortality rate in rats[9]
Pregnancy outcomes	↑ rate of preterm birth[5,10–17]and ↓ gestational length[18] ↓ birth weight[10,13,16], ↑ small for gestational age[19,20], and ↓ fetal head growth[21]	↓ litter size and ↓ weight of the pups in rats[22]	↓ gestational length and birth weight in rats[9]
Monoamines and metabolites	↓ concentrations of 5-HT, 5-HIAA, and HVA in whole blood[23] ↓ concentration of noradrenalin, DHPG and DOPAC in infants[23]	↓ 5-HT levels in the placenta of rats[7]	affected 5-HT metabolism in the periphery and central nervous system[24,25]
Congenital malfunctions	↑ risk for congenital malformations like anencephaly, craniosynostosis, omphalocele, and septal defects[10,26–28] ↑ risk of cardiac anomalies[27,29–31]	↓ proliferation rate of embryonic heart cells in rat and mice[32,33]	severe dilated cardiomyopathy and ↓ ratio of the left ventricle thickness/the left ventricle cavity radius in mice[8] ↑ heart rate and activity levels in mice, ↓ left ventricular internal diameters in diastole and ↓ stroke volumes in mice[34]
Persistent pulmonary hypertension (PPHM)	↑ risk of PPHM in infants[10,35–37]	abnormal oxygenation[7] ↑ right ventricular mass of the lung and thicker medial smooth muscle layer of the small and larger pulmonary arteries of female rats[38]	↓ number and wall thickness of pulmonary vessels and ↓ right ventricle hypertrophy in hypoxic 5-HTT$^{-/-}$ mice[39] ↑ in right ventricular hypertrophy and pulmonary vascular remodeling in hypoxic 5-HTT+ mice[40]
Neurodeve-lopmental outcomes	↓ response to acute pain in newborns[41] ↑ tremulousness, ↓ changes in behavioral state and ↓ different behavioral states in infants[42] ↑ amounts of uninterrupted REM sleep in infants[42] mild effects on motor development and motor control[43] affected psychomotor development in younger children[44] ↑ internalizing behaviors in early adulthood[45] ↑ risk for autism spectrum disorders[46]	influence motor development transiently in rats[47] ↑ cognitive performance and ↓ impulsivity (treatment from G0 until PND21)[47,48] ↓ juvenile play behavior[22] ↓ contact making with other rats and ↑ self-grooming behavior[22] ↑ anxiety-like behavior[22,47] ↑ depression-like behavior[22] ↑ 5-HT1A agonist-induced hypothermia[22] ↓ 5-HTT expression[49] and 5-HT2 receptor density and function[50]	↓ pain response[57] ↑ REM sleep duration, ↓ REM latency and ↓ active sleep in rodents[52,53] ↓ efficient performance on a temporal learning task, but more rapidly in the spatial alternation learning task[52] recovered learning deficits in MWM and reversed altered hippocampal spine and synapse density in prenatally stressed mice[25] ↑ sensorimotor learning deficits[57] ↓ juvenile play behavior in male rats[54] ↓ aggressive behavior in rats[55] ↓ sexual activity and performance[52,56] ↑ anxiety-like behavior[48,52–54,57,58] ↑ depression-like behavior[48,53,59] ↑ the 5-HT$_{1A}$ receptor agonist-induced hypothermia[53] ↓ dendritic complexity of thalamocortical afferents and layer IV of the barrel cortex[51] disrupts the organization of thalamocortical somatosensory barrels[60]

(Continued)

Table 1 | Continued

	Humans	Prenatal exposure in rodents	Postnatal exposure in rodents
Stress regulation	↓ basal salivary cortisol levels, diurnal changes in salivary cortisol and altered HPA stress reactivity patterns[61,62] ↓ increased heart rate in response to acute painful stressors[41,63] ↑ CBG levels in serum of neonates[64]		↓ serum corticosterone levels and ↓ expression of CA3 hippocampal GR and GRIP1 in healthy male rats[65] ↑ CBG levels and normalized corticosterone response to stress in postnatal SSRI-treated prenatally stressed rodents[25,65]

References: [1]Hemels et al., 2005; [2]Rahimi et al., 2006; [3]Palmsten et al., 2012; [4]Qiu et al., 2009; [5]Reis and Källén, 2010; [6]Toh et al., 2009a,b; [7]Fornaro et al., 2007; [8]Noorlander et al., 2008; [9]van den Hove et al., 2008; [10]Chambers et al., 1996; [11]Costei et al., 2002; [12]Davis et al., 2007; [13]Källén, 2004; [14]Lund et al., 2009; [15]Simon et al., 2002; [16]Wen et al., 2006; [17]Wisner et al., 2009; [18]Suri et al., 2007; [19]Oberlander et al., 2006; [20]Toh et al., 2009a,b; [21]El Marroun et al., 2012; [22]Olivier et al., 2011; [23]Laine et al., 2003; [24]Hilakivi et al., 1995; [25]Ishiwata et al., 2005; [26]Alvan et al., 2007; [27]Louik et al., 2007; [28]Wogelius et al., 2006; [29]Diav-Citrin et al., 2008; [30]Malm et al., 2005; [31]Oberlander et al., 2008a,b,c,d; [32]Sari and Zhou, 2003; [33]Yavarone et al., 1993; [34]Haskell et al., 2012; [35]Chambers et al., 2006; [36]Kieler, 2012; [37]Källén and Olausson, 2008; [38]Belik, 2008; [39]Eddahibi et al., 2000; [40]MacLean et al., 2004; [41]Oberlander et al., 2002; [42]Zeskind and Stephens, 2004; [43]Casper et al., 2003; [44]Mortensen et al., 2003; [45]Oberlander et al., 2010; [46]Croen et al., 2011; [47]Bairy et al., 2007; [48]Lisboa et al., 2007; [49]Hansen and Mikkelsen, 1998; [50]Cabrera and Battaglia, 1994; [51]Lee, 2009; [52]Mirmiran et al., 1981; [53]Popa et al., 2008; [54]Simpson et al., 2011; [55]Manhães de Castro et al., 2001; [56]Maciag et al., 2006; [57]Ansorge et al., 2004; [58]Ansorge et al., 2008; [59]Hansen et al., 1997; [60]Xu et al., 2004; [61]Brennan et al., 2008; [62]Oberlander et al., 2008a,b,c,d; [63]Oberlander et al., 2005; [64]Pawluski et al., 2012a; [65]Pawluski et al., 2012b.

Serotonin transporter gene

The polymorphism in the promoter of the 5-HTT is unique for primates and not present in rodents (Caspi et al., 2010), but the role of the 5-HTT has been extensively studied in rodent models with genetic deletion of the 5-HTT (Murphy and Lesch, 2008; Kalueff et al., 2010; Homberg and Lesch, 2011). The phenotypes observed in these 5-HTT knockout (5-HTT$^{-/-}$) rodents mimic the long-term behavioral outcomes of early SSRI exposure. 5-HTT$^{-/-}$ rodents display reduced pain, exploratory behavior, social behavior, and increased anxiety-like and depression-like behavior (Kalueff et al., 2010). Moreover, 5-HTT$^{-/-}$ rodents have improved cognitive performance (Brigman et al., 2010; Nonkes et al., 2011; Van den Hove et al., 2011; Nonkes et al., 2012). Regarding neuronal plasticity, SERT$^{-/-}$ rodents have reduced brain-derived neurotrophic factor and activity-regulated cytoskeleton associated protein expression levels in hippocampus and prefrontal cortex (Molteni et al., 2009, 2010). Moreover, neuronal PAS domain protein 4, regulating activity-dependent genes and neuroprotection, is reduced in SERT$^{-/-}$ rodents and this effect could be mimicked by prenatal fluoxetine exposure (Guidotti et al., 2012). Reduced densities and functional alterations of 5-HT receptors have been found in SERT$^{-/-}$ rats, as well as changes in neurodevelopment (reviewed in: Kalueff et al., 2010). The overlapping findings of life-long 5-HTT ablation and early-life exposure to SSRIs in rodents suggest that neurodevelopmental changes are responsible for the phenotypes observed. Therefore, the 5-HTT$^{-/-}$ model is of heuristic value in studying the neurodevelopmental outcome of SSRI exposure.

CONCLUDING REMARKS

This review summarized clinical and preclinical findings of how SSRI exposure during pregnancy affects child outcomes. Although many clinical findings parallel aspects of the preclinical data (**Table 1**), in preclinical studies SSRIs are often administered to healthy animals, while in the clinic SSRIs are only administered to depressed women. Moreover, preclinical models are often tested during adulthood, whereas most clinical data comes from children. These factors should be taken into account.

In addition there are often discrepancies between clinical findings and this may be due the trimester when SSRIs are taken, whether other medications were also administered, variety of other diagnoses (e.g., anxiety), the dose of the medication and the gestational age of the infant. In preclinical studies, discrepancies between findings may be due to the timing of SSRI exposure (prenatal or postnatal), the duration of exposure, the dose administered and the SSRI used, as well as rodent strain.

Both genetic and environmental factors contribute to the well-being of a child. In humans, it is impossible to study the effects of SSRI exposure without taking the underlying depression into account. In animals, it is possible to disentangle the effects of maternal depression from the effects of maternal SSRI exposure. Moreover, the timing of maternal adversity and SSRI exposure (duration and dosing) can be studied during the prenatal or postnatal period or during both periods. The additional advantages of using animal models are that one can readily examine long-term neurodevelopmental outcomes, specific roles of maternal care,

and neural plasticity. Unfortunately, most preclinical research to date has studied the effects of SSRIs in healthy animals. In order to make preclinical findings translational, it is important to study the effects of SSRIs in a model of maternal depression or adversity, as the actions of developmental exposure to SSRIs can significantly vary with exposure to maternal adversity. Finally, preclinical studies reveal sexually dimorphic responses which likely apply to humans as well. It is, therefore, important to take the sex of the offspring into account.

It remains to be determined whether maternal SSRI use is more beneficial or has adverse effects beyond the underlying depression. Much more research is needed to understand the risks and benefits of perinatal exposure to SSRIs on the developing child. Future research should focus on the effects of maternal depression alone, and compare it to offspring exposed to SSRIs, and offspring exposed to SSRIs combined with maternal adversity. Unraveling the different underlying mechanisms (which can be environmental, genetic, or epigenetic) in these three different groups will provide the answer for the risks and benefits of SSRI use during pregnancy.

ACKNOWLEDGMENTS

This work was funded by research grants from the Swedish Research Council and the Marianne and Marcus Wallenberg Foundation. J. L. Pawluski is funded by the Fonds de la Recherche Scientifique (FNRS-FRS).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 February 2013; paper pending published: 02 March 2013; accepted: 01 May 2013; published online: 21 May 2013.

Citation: Olivier JDA, Åkerud H, Kaihola H, Pawluski JL, Skalkidou A, Högberg U and Sundström-Poromaa I (2013) The effects of maternal depression and maternal selective serotonin reuptake inhibitor exposure on offspring. Front. Cell. Neurosci. 7:73. doi: 10.3389/fncel.2013.00073

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 11 October 2013
doi: 10.3389/fncel.2013.00180

Prenatal serotonin reuptake inhibitor (SRI) antidepressant exposure and serotonin transporter promoter genotype *(SLC6A4)* influence executive functions at 6 years of age

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Prenatal exposure to serotonin reuptake inhibitor (SRI) antidepressants and maternal depression may affect prefrontal cognitive skills (executive functions; EFs) including self-control, working memory and cognitive flexibility. We examined long-term effects of prenatal SRI exposure on EFs to determine whether effects are moderated by maternal mood and/or genetic variations in *SLC6A4* (a gene that codes for the serotonin transporter [5-HTT] central to the regulation of synaptic serotonin levels and behavior). Children who were exposed to SRIs prenatally (SRI-exposed $N = 26$) and non-exposed ($N = 38$) were studied at age 6 years ($M = 6.3$; $SD = 0.5$) using the Hearts & Flowers task (H&F) to assess EFs. Maternal mood was measured during pregnancy (3rd trimester) and when the child was age 6 years (Hamilton Depression Scale). Parent reports of child behavior were also obtained (MacArthur Health & Behavior Questionnaire). Parents of prenatally SRI-exposed children reported fewer child externalizing and inattentive (ADHD) behaviors. Generalized estimate equation modeling showed a significant 3-way interaction between prenatal SRI exposure, *SLC6A4* variant, and maternal mood at the 6-year time-point on H&F accuracy. For prenatally SRI-exposed children, regardless of maternal mood, the H&F accuracy of children with reduced 5HTT expression (a short [S] allele) remained stable. Even with increasing maternal depressive symptoms (though all below clinical threshold), EFs of children with at least one short allele were comparable to children with the same genotype whose mothers reported few if any depressive symptoms—in this sense they showed resilience. Children with two long (L) alleles were more sensitive to context. When their mothers had few depressive symptoms, LL children showed extremely good EF performance—better than any other group. When their mothers reported more depressive symptoms, LL children's EF performance was worse than that of any other group. In the face of a mother with a more depressed mood, EFs were best preserved in children prenatally exposed to SRIs and with at least one short *SLC6A4* allele. Yet, prenatally-exposed LL children hold out promise of possibly superior EF if their mother's mood remains euthymic or improves.

Keywords: serotonin, executive function, childhood, prenatal exposure, SRI, *SLC6A4* genotype, depression

INTRODUCTION

Serotonin (5-HT) and its multiple receptors are highly expressed in prefrontal cortex (PFC) and play key roles in influencing complex cognition and resilience to stress (Canli et al., 2005; Lesch, 2007; Reuter et al., 2007; Homberg and Lesch, 2011). Dense projections of 5-HT neurons into prefrontal regions (Preece et al., 2004), and a wide distribution of 5-HT receptors and 5-HT transporter sites in PFC (Varnäs et al., 2004) contribute to 5-HT's role in cognition (King et al., 2008). Critical cognitive capacities that rely on PFC and related structures (Miller and Cohen, 2001; Braver et al., 2002; Petrides, 2005; Champod and Petrides, 2007; Zanto et al., 2011) are termed executive functions (EFs), and include abilities to (1) focus, sustain and shift attention (executive attention), (2) resist the pulls and temptations of external stimuli, our emotions, or engrained behavioral tendencies, inhibit acting impulsively, taking a moment to make a more considered response (inhibitory control), (3) hold information in mind and work with it, such as updating one's thinking or planning when given new information, considering alternatives, or mentally relating pieces of information to one other (working memory), and (4) creative problem-solving, flexibly adjusting to changed demands, priorities, new obstacles or opportunities (cognitive flexibility; Miyake et al., 2000; Diamond, 2013). Not surprisingly, good EFs are critical for all aspects of life, including mental and physical health and success in school and in life (Moffitt et al., 2011; Diamond, 2013). For example, childhood EFs predict school readiness and success in math and reading throughout all school years from kindergarten through university

better than does IQ, even when controlling for SES (Bull and Scerif, 2001; Blair, 2002; Riggs et al., 2004; Blair et al., 2005; Gathercole et al., 2005; Blair and Razza, 2007).

5-HT plays a critical role in brain development (Kalueff et al., 2010; Olivier et al., 2011). In animal models, developmental shifts in central 5-HT signaling shape early cognitive capacities setting pathways for learning and behavior later in life (see for review Kalueff et al., 2010). Little, however, is known about how developmental changes in 5-HT influence early cognitive development in humans during childhood.

The increasing use of serotonin reuptake inhibitor (SRI) antidepressants to manage maternal mood disorders during pregnancy (Cooper et al., 2007) raises critical questions about the impact of prenatal altered central 5-HT levels on the development of systems that regulate attention, working memory, and self-control (i.e., EFs) in childhood (Kalueff et al., 2010; Hanley and Oberlander, 2012). SRIs primarily act by blocking reuptake of serotonin transporter protein (5-HTT), thereby increasing how much, and how long, extracellular 5-HT remains active and available. SRIs readily cross the placenta and the blood-brain barrier (Kim et al., 2006) altering fetal central 5-HT levels (Laine et al., 2003). Prenatal SRI exposure affects (1) fetal (Salisbury et al., 2009; Mulder et al., 2011) and newborn neurobehavior (Moses-Kolko et al., 2005), (2) neonatal stress regulation (Oberlander et al., 2002, 2005), (3) shifts language perception during the first year of life (Weikum et al., 2012), and (4) is associated with emotional regulation in toddlers (Oberlander et al., 2010).

Why some, but not all, children are affected by prenatal SRI exposure is still a central and pressing question (Hanley and Oberlander, 2012). In the early school years prenatally exposed children appear to have typical language development, behavior and IQ (Nulman et al., 2002). However, not all outcomes can be specifically attributed to prenatal antidepressant exposure. Distinguishing the concurrent impact of pre and postnatal maternal mood disturbances remains challenging (Oberlander et al., 2010).

The pre-synaptic membrane-bound serotonin transporter protein (5-HTT)—the very target of SRI antidepressants—is central to the regulation of intra-synaptic 5-HT. Allelic variations in *5-HTTLPR* (*SLC6A4*) influences gene transcription and the amount of 5-HT available at postsynaptic sites (Lesch et al., 1996). The short (S) variant is associated with reduced gene transcription and reduced levels of 5-HTT protein, with an ∼50% reduction in 5-HT reuptake compared to the long (L) variant (Heils et al., 1996; Homberg et al., 2007a). Reduced 5-HTT protein availability and 5-HT reuptake results in a higher effective "serotonin dose."

Homozygosity for the short (S) allele is associated with increased stress sensitivity and risk for emotional disturbances including anxiety and depression but better EFs (not unlike what has been found for the COMT- MET genotype (Goldman et al., 2005; Diamond, 2011). In combination with early life stressors, the short allele has been widely studied as an important risk factor for mental illness later in life (Caspi et al., 2003; Kendler et al., 2005; Lesch, 2007). For example, adolescents who encountered adversity in childhood and are homozygous for the short allele of *5-HTTLPR* have a heightened sensitivity to potential negativity and threat in the environment and are more prone to anxiety and depression (Owens et al., 2012). In animal models, increased 5-HT levels secondary to 5-HTT blockade at developmentally sensitive time periods (akin to a human 3rd trimester) causes permanent axonal connection deficits in the somatosensory cortex (Homberg et al., 2010), the lateral geniculate nucleus (Gaspar et al., 2003), and altered neuronal dendritic branching, elongation and pruning (Homberg et al., 2010; Liao and Lee, 2011; Olivier et al., 2011; Simpson et al., 2011; Zheng et al., 2011). Beyond the newborn period, SRI-exposed animals demonstrate *decreased* 5-HT levels—possibly via prolonged activation of inhibitory receptors (i.e., 5-HT_{1a}; Hensler, 2006). This might underlie the reduced novelty investigation, poorer motor performance (Lee and Lee, 2012), increased anxiety in conflict tasks and anhedonia (Ansorge et al., 2004, 2008; Popa et al., 2008) reported in fluoxetine-exposed mice. Adults with two short *5-HTTLPR* alleles consistently outperform those with one or two long alleles on measures of EFs such as the Wisconsin Card Sorting test (Borg et al., 2009) and go/no-go tests (Roiser et al., 2007), they also show brain patterns consistent with better EFs (Enge et al., 2011). Conversely, the L-allele of the 5-*HTTLPR* gene is associated with poor EFs including impulsivity, inattention, and working memory deficits (see the meta-analysis by Gizer et al., 2009). Together these findings support the notion that changes in transcriptional activity associated with allelic variations in the 5-*HTTLPR* gene and presumably reflecting alterations in central serotonin levels, influence EFs in the mature adult brain.

Beyond genetic variations, experimental manipulations of central serotonin levels in adults also appears to affect cognitive functions. Acute SRI administration to healthy adults has been shown to improve verbal fluency, a measure of EFs requiring memory of words, inhibitory control to avoid repeating words, and cognitive flexibility to switch to different paths and strategies for coming up with words (Schmitt et al., 2001). Although reduced 5-HT, using an acute tryptophan depletion (ATD) model with healthy volunteers, has been found to improve focused attention (Schmitt et al., 2000; Evers et al., 2006), enhanced EF performance and reduced impulsivity have also been found in some animal models of SRI exposure (e.g., Sasaki-Adams and Kelley, 2001) but not all (e.g., Valluzzi and Chan, 2007). Importantly, *5-HTTLPR* genotype and SRI exposure do not affect, or inconsistently affect, non-EF cognitive abilities such as recall and recognition memory and mental rotation (e.g., 5-*HTTLPR* genotype: Roiser et al., 2006; Mannie et al., 2009, SRI exposure: Harmer et al., 2002; Siepmann et al., 2003; Riedel et al., 2005).

An acute pharmacological exposure to an SRI or dietary depletion of tryptophan in a mature brain may not result in the same consequences as chronic prenatal SRI exposure and the associated long-term changes in prenatal 5-HT signaling that occurs with such exposure across developmentally sensitive periods of brain growth (Ansorge et al., 2004). To date, studies focusing on the effects of prenatal SRI exposure have typically sought to examine the consequences of what is generally considered increased developmental serotonergic tone. However, in humans the developmental course or behavioral consequences that might follow prenatal SRI exposure (i.e., downstream lower serotonergic tone) is not known. Given the developmental role of 5-HT, it is

conceivable that prenatal changes in 5-HT either via genetic variations or prenatal SRI exposure might influence early cognitive development in humans.

To further understand the developmental impact of prenatal SRI exposure on early cognitive development, we studied whether prenatal exposure to SRI antidepressants or maternal mood affects core cognitive skills (EFs) in early childhood, controlling for prenatal maternal mood. Secondarily, we also sought to examine whether changes in EFs are moderated by the child's *SLC6A4* genotype, reflecting genetic variations in the capacity to control serotonergic tone that may influence the impact of exposure to maternal mood or SRIs. Given fetal changes in 5-HT signaling secondary to prenatal SRI exposure, and the literature showing improved cognitive function among S carriers, we expected that antidepressant exposure and reduced *SLC6A4* transcription (at least one short [S] allele) at a developmentally sensitive time (i.e., *in utero*) would be associated with improved EF capacity in early childhood, while elevated maternal depressive symptoms would have an opposing effect at 6 years of age.

MATERIALS AND METHODS

PARTICIPANTS

Children in this study are part of a longitudinal cohort study examining the effects of prenatal exposure to SRIs and maternal mood disturbances in 98 mothers recruited during their second trimester of pregnancy. Approval was obtained from the University of British Columbia Ethics Board and the Children's and Women's Health Centre of British Columbia Research Review Committee. Written informed parental consent was obtained to follow the development of these children. All mothers, regardless of their mood or medication status, were physician-referred or self-referred from the Reproductive Mental Health Clinic at British Columbia Women's Hospital and Health Centre (a tertiary-care service), community midwife clinics or family physician practices in the greater Vancouver metropolitan area. All SRI-treated mothers had started taking medications based on clinical need, had a diagnosis of a mood disorder, and were already taking antidepressant medications at the time of conception. Women in the non-SRI group had a range of mood symptoms at the time of recruitment as assessed by the Hamilton Rating Scale for Depression (HAM-D; see **Table 1**). Of the original 98 mothers, 4 withdrew before the baby was born and another 4 withdrew before the end of the child's first year. At 6 years, an additional 26 children were unavailable for study (22 families had moved and 4 mothers had withdrawn by 3 years). At the time of this study, 64 children (26 prenatally SRI-exposed and 38 non-exposed) were seen at mean age 6.3 years ($SD = 0.51$ years). From this sample, 25 exposed and 32 non-exposed had both prenatal maternal mood scores and samples of the child's blood available for genotyping.

Table 1 | Maternal characteristics.

	Non-exposed ($n = 38$)	SRI-exposed ($n = 26$)	*T*	*p*-value
Prenatal HamA (mean) (*SD*) ($n = 35$ non-exposed and 25 SRI-exposed)	5.5 (4.75)	9.58 (6.75)	−2.8	0.007
Prenatal HamD (mean) (*SD*) ($n = 34$ non-exposed and 25 SRI-exposed)	3.64 (4.26)	8.54 (6.28)	−3.66	0.001
Maternal smoking during pregnancy	0	0		0
Maternal alcohol consumption (drinks in pregnancy) ($n = 37$ non-exposed)	2.49 (4.49)	3.69 (8.35)	−0.74	0.462
Maternal age at birth (years)	33.21 (4.93)	31.42 (4.6)	1.45	0.151
Maternal education (years)	17.8 (2.71)	15.27 (2.39)	3.83	0.001
MATERNAL *SLC6A4* GENOTYPE (n's)				
LL	10	7		0.704
1S	27	18		

CHILD MENTAL HEALTH SYMPTOMATOLOGY

Measures of child mood and behavior were obtained from the mental health symptomatology section of the MacArthur Health and Behavior Questionnaire (HBQ; Boyce et al., 2002; Essex et al., 2002) that was completed by maternal report (HBQ-P) and yielded measures of internalizing, externalizing and Attention-Deficit and Hyperactivity Disorder (ADHD) behaviors for each child. The HBQ was derived from the Ontario Child Health Study measure designed to map onto DSM-III-R symptom criteria (Boyle et al., 1993). The HBQ-P has strong psychometric properties and has been used to assess child mental health across multiple ages from 4.5 years into adolescence (Ablow et al., 1999; Essex et al., 2006; Shirtcliff and Essex, 2008) The mental health scales have been shown to discriminate groups of children with and without signs of early psychopathology (Luby et al., 2002).

The HBQ-P, administered in questionnaire format, assesses symptoms ranging from "never or not true" to "often or very true." Symptoms in three domains were analyzed: (1) ADHD symptoms consist of items indexing inattention, impulsivity, and hyperactivity. (2) Externalizing symptoms consist of items indexing oppositional defiant behaviors and conduct problems. (3) Internalizing symptoms consist of items indexing symptoms of depression, separation anxiety, and generalized anxiety. In addition to mean symptom level, the percentage of children above clinical cutoffs was examined. Clinical cutoffs for parent reported ADHD, externalizing, and internalizing symptoms (1.2, 0.68, 0.71, respectively) were set based on previous analysis of the HBQ-P (Lemery-Chalfant et al., 2007) with children of approximately the same age as in the present study.

EF TASKS

EFs were assessed using the H&F task, a computerized measure that has been validated with children 4–13 years of age and with adults (Davidson et al., 2006; Diamond et al., 2007). This task assesses inhibition, working memory and cognitive flexibility. A stimulus appears to the right or left of a computer screen

on every trial. On Block 1 of the task (the congruent block), participants have only to do what comes naturally (i.e., pressing on the same side as the stimulus); no EFs are taxed. On Block 2 (the incongruent block), participants had to resist that prepotent response and instead press on the side opposite the stimulus. On Block 3 (the mixed block), the two types of trials are randomly intermixed, requiring remembering both rules and mentally translating "same [or opposite] side" into "right [or left] hand," and flexibly switching between the two rules, inhibiting one to apply the other.

The children came to the study center mid-morning and first performed a warm up task (about 5 min). During the task, children were told to respond to a stimulus as fast as they were able and this gave them practice with the computerized set-up. The children then performed the H&F task (about 10 min). Practice trials were given before both the congruent and incongruent blocks (see Davidson et al., 2006; Diamond et al., 2007). In both blocks, children were given up to 6 s to respond, and 10 s in the mixed block. Responses $>$ 2000 ms were considered incorrect (inattentive) and those $<$ 250 ms, impulsive. Both responses were excluded. Five trials out of 1860 trials were $>$ 2000 ms (0.26%), and there were no trials $<$ 250 ms (out of 1860 trials in total). Outlier trials were removed by using a lower and upper threshold of 2 standard deviations from the mean RT per trial type per block and per subject.

Two dependent measures were tabulated for each block (i.e., Congruent, Incongruent, etc): (1) correct responses or *accuracy* (% correct = #correct/[# trials]) and (2) Reaction time or *speed of response* (reaction time, RT $>$ 250 msec for correct trials only). Reaction time was a *Choice RT* tabulated every time a stimulus appeared during the three-block task and the stimulus appeared at random intervals (one button used) (Kail and Salthouse, 1994).

MATERNAL MOOD

Maternal mood was assessed during the third trimester of pregnancy (mean 33.8 week; *SD* 1.25 weeks), and again at the 6-year timepoint using the HAM-D; (Hamilton, 1960), a 21-item clinician-rated measure of depressive symptoms with a score ranging from 0 to 63.

SLC6A4 GENOTYPING

Genomic DNA was extracted from neonatal whole blood samples using the Flexigene DNA Blood Kit (Qiagen, Valencia, California). The S and L alleles of *SLC6A4* were identified as previously described in (Lesch et al., 1993). Polymerase chain reaction was performed with oligonucleotide primers flanking the polymorphism (corresponding to nucleotide positions -1416 to -1397 [stpr5, 5_- GGCGTTGCCGCTCTGAATGC] and -910 to -888 [stpr3, 5_-GAGGGACTGAGCTGGACAACCAC]) of the 5_-flanking regulatory region of *SLC6A4* to generate a 484-bp (S short allele) or a 528-bp (L long allele) polymerase chain reaction product. Polymerase chain reaction amplification was performed in a final volume of 30 μL with 50 ng of genomic DNA, 2.5mM deoxyribonucleotides (dGTP/7-deaza-2_-dGTP = l/l), 0.1 μg of sense and antisense primers, 10 mM Tris hydrochloride (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, and 1 U of Taq DNA polymerase. For quality control, 5% of the samples were randomly chosen to be retested and their genotypes were consistent with previous results.

STATISTICAL ANALYSES

Two separate analytic approaches were used to study behavioral outcomes. To analyze child behavioral differences using maternal report, a multivariate analysis of covariance (MANCOVA) was used to examine group (SRI exposed vs. non exposed) differences in child behavior, with child age (at the time of the 6 year study, prenatal (3rd trimester) and postnatal (6 year) maternal mood as covariates. Maternal mood was used as a continuous measure to allow us to account for the wide range of depressive symptoms observed among both SRI-exposed and non-exposed groups. Across time, some mothers in our untreated group became depressed and some crossed over to the SRI treated group. Maternal mood measures at both time points (prenatally and at the 6 year study) helped to account for these changes.

General Estimating Equation (GEE) modeling was used to examine group (SRI exposed vs. non-exposed) and genotype (LL vs. at least one S allele) in relation to each of the three computerized H&F conditions. Due to a limited number of children with SRI exposure and two short alleles ($n = 7$), children with at least one short allele (LS and SS) were grouped together to yield the $\geq$1 S allele group. With the GEE approach we were able to examine main effects (SRI exposure and genotype) and interactions simultaneously. The role of genotype was examined as a possible moderator of the effects of prenatal exposure on EFs by comparing performance for each EF task block and interactions between exposure group and *SLC6A4* genotype (LL vs. at least one S allele [SS or LS]), accounting for pre- and post-natal maternal mood. GEE extends the generalized linear modeling to allow for analysis of repeated measurement of accuracy (a binomial dependent variable). GEE analyses were performed using SPSS Statistics 18. All *p*-values less than 0.05 were considered significant.

RESULTS

Demographic and behavioral outcomes for the mothers and their children are presented in **Tables 1**, **2**. With the exception of maternal education, maternal mood prenatally and at the 6 year study, child age at the time of the study ($p = 0.03$), and the children's 5-min APGAR scores ($p = 0.026$), no significant group differences (SRI exposed vs. non-exposed) were observed. As SRI-exposed children were older than the non-exposed children at the time of the study, child age was included as a covariate in the analyses. While the 5-min APGAR scores were statistically different between groups, the clinical impact of these differences (i.e., between scores of 9.13 vs. 8.73, **Table 1**) would not reflect a significant difference in outcome and thus were not included in further analyses. We did not include maternal education as a covariate as all mothers had high levels of education.

CHILD MENTAL HEALTH

In SRI-exposed children, significantly fewer ADHD ($p = 0.03$) and disruptive externalizing symptoms ($p = 0.019$) were reported by parents, after adjusting for child age, 3rd-trimester

maternal mood and maternal mood at the time of the study. No differences in internalizing behaviors were found between exposure groups (**Table 2**). Maternal depression symptoms were associated with increased report of externalizing ($r = 0.314$; $p = 0.01$) and ADHD behaviors ($r = 0.251$; $p = 0.039$). In separate GEE models, internalizing and externalizing behaviors, respectively, were not predictive of EF performance, regardless of concurrent mother's mood.

EF TASK (HEARTS AND FLOWERS)

To examine the effect of SRI exposure, *SLC6A4* variant and maternal mood on EF performance (accuracy and reaction time), a GEE model was run separately on each of the three blocks of the EF task. SRI exposure (yes/no) and *SLC6A4* variant (LL vs. at least one S) were factors. Trials was a repeated within-subject variable, and maternal mood measures (prenatal and at the 6 year study) and child age at test day were covariates. The outcome was either accuracy (% correct response) or RT (in milliseconds) on the EF task.

Table 2 | Child characteristics.

Child characteristics	Non-exposed ($n = 38$)	SRI-exposed ($n = 26$)	*T*	*p*-value
Gestation age at birth (mean weeks) (*SD*)	40.0 (1.35)	39.4 (1.63)	1.54	0.128
Birth weight (grams) (*SD*)	3531 (470)	3317 (480)	1.77	0.081
Birth length (cm) (*SD*)	51.7 (2.8)	50.71 (2.54)	1.48	0.143
Head circumference (cm) (*SD*)	35.0 (1.36)	34.42 (1.27)	1.69	0.095
Apgars score (1 min)	8.03 (1.6)	7.65 (1.55)	0.93	0.358
Apgars score (5 min)	9.13 (0.53)	8.73 (0.87)	2.28	0.026
Sex (m:f)	18:20	10:16	−0.7	0.488
SLC6A4 genotype (n's)				0.946
LL	11	9		
1S	23	16		
Age at study (yr) (*SD*)	6.22 (0.55)	6.51 (0.45)	−2.28	0.03
Child mental health symptomatology			***F***	***p*-value**
Internalizing symptoms^ *Composite of depression, separation anxiety, and generalized anxiety scores*	0.33 (0.25)	0.33 (0.22)	0.253	0.617
% above clinical threshold	6.3	8		
Externalizing symptoms^ *Composite of oppositional defiant behaviors and conduct problem scores*	0.29 (0.22)	0.23 (0.17)	5.831	0.019
% above clinical threshold	6.3	0		
ADHD Symptoms^ *Composite of inattention, impulsivity, and hyperactivity scores*	0.67 (0.39)	0.47(0.37)	4.954	0.03
% above clinical threshold	9.4	0		

^Controlling for maternal mood (prenatal and 6 year) and child age.

REACTION TIME

Overall, no SRI exposure group differences in RT (**Figure 1**) were found in any of the 3 blocks. RT increased with increasing task difficulty, but that did not differ by SRI exposure or genotype (LL vs. ≥1 S allele). In a separate analysis of Choice RT task, older children were faster ($B = -35.5$, $p = 0.048$). To control for speed of responding faster, Choice RT was added to an overall GEE RT model as a covariate. There was still no significant effect for SRI exposure or SLC6A4 genotype (LL vs. ≥1 S allele) in the GEE RT model.

ACCURACY

Overall, no SRI exposure group differences in accuracy (**Figure 2**) were found on block 1 or 2. Differences emerged in the most difficult third block where exposed children showed higher accuracy (suggesting better cognitive flexibility). However, the results were not significant when controlling for child age. Not surprisingly, older children performed better on the EF test ($p = 0.004$); the results of the models were adjusted for child age. Maternal depressed mood at 3rd trimester contributed, but not statistically significantly ($p = 0.064$, $OR = 0.943$).

With each block, differences in accuracy between allelic variations and exposure groups began to emerge, but only in block 3, with accuracy as the dependent variable, a significant main effect for *SLC6A4* genotype, child age and maternal mood emerged, as well as a significant 3-way interaction between prenatal SRI exposure, *SLC6A4* variant, and maternal mood at age 6 years in the GEE model (**Table 3**), controlling for child age.

When the mother's current depressed mood symptoms were relatively low (measured on test-day), EF performance did not differ with the presence of prenatal SRI exposure (**Figure 2**) or by child genotype (**Figure 3A**, using maternal mood grouped by quartiles to illustrate the GEE results). However, the more depressed the mother was currently, the more performance

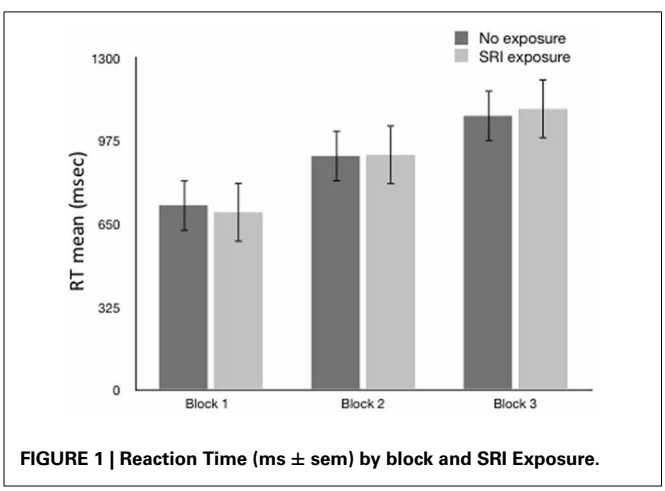

FIGURE 1 | Reaction Time (ms ± sem) by block and SRI Exposure.

between the groups began to diverge. In the face of higher depressive maternal symptoms (4th quartile), EF performance of children with no prenatal SRI exposure was poor. Accuracy was significantly and inversely related to how depressed their mother was currently ($B = 0.099$; 95% CI [0.035–0.163]; $\chi^2 = 9.295$; $p = 0.002$) and this was particularly true for children with the LL variant ($B = -0.092$; 95% CI [−0.171–−0.014]; $\chi^2 = 5.29$; $p = 0.021$). Namely, children prenatally exposed to SRIs and with at least 1 S allele and high concurrent maternal depressive symptoms showed no decrement in accuracy. However, among children with LL variant of SLC6A4, accuracy was worse in children with symptomatic mothers (3rd and 4th quartiles) compared with those with less symptomatic mothers. In contrast, children with =1 S allele had relatively stable performance regardless of mothers' depressive mood states (**Figure 3B**).

DISCUSSION

On a test of EFs (H&F; requiring inhibition, working memory, and cognitive flexibility), the effect of prenatal SRI exposure was markedly different in 6-year-old children depending on the child's *SLC6A4* genotype and mother's concurrent mood. SRI exposed children with an LL genotype showed pronounced differences in their EFs depending on their mother's current mood. SRI-exposed children with at least one short *SLC6A4* allele showed resilience (no impairment in inhibition and attention). Even in the face of more symptomatic mothers, the accuracy of the ≥1 S children on the difficult mixed Block 3 of the H&F test did not differ. In contrast, children with two L alleles, were far more sensitive to the context of life with a depressed mother. When their mother had few or no depression symptoms, LL children did

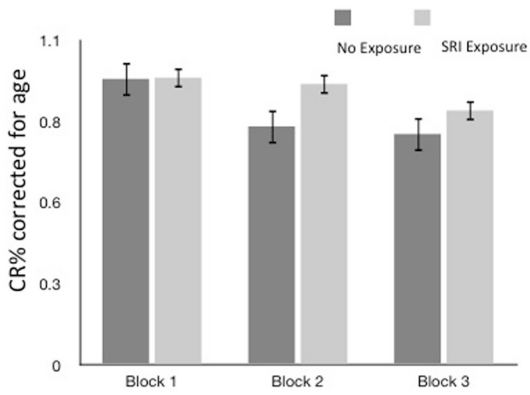

FIGURE 2 | Accuracy (CR % ± SEM) by block and SRI exposure (CR% corrected for age).

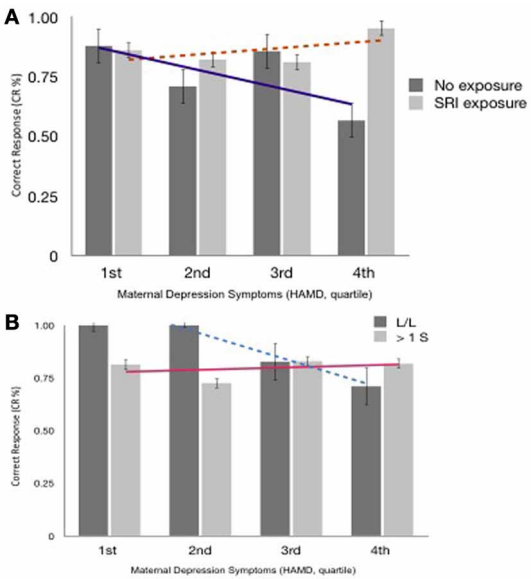

FIGURE 3 | (A) Accuracy (% Correct Response, Block 3 ± SEM), SRI exposure & Maternal Depressed Mood at 6 years postpartum (quartile). Trend lines reflect differences in CR% across maternal mood between exposure groups. **(B)** Accuracy (CR%, Correct Response, Block 3 ± SEM), *SLC6A4* Allelic variations & Maternal Depressed Mood at 6 years postpartum (quartile). Trend lines reflect differences in CR% across maternal mood between allele groups.

Table 3 | GEE model results (reflecting slope) showing the effect of SRI exposure, *SLC6A4* variant, and maternal mood on EF task accuracy.

Parameter	*B**	95% Wald confidence interval		Wald χ2	*p*-value
		Lower	Upper		
SRI exposure	0.516	−0.236	1.268	1.807	0.179
SLC6A4 LL	1.051	0.263	1.838	6.838	**0.009**
Third trimester mood (HAMD)	−0.059	−0.122	0.003	3.429	0.064
Maternal Mood Study Day (HAMD)	0.099	0.035	0.163	9.295	**0.002**
Child age at study day	0.682	0.221	1.143	8.398	**0.004**
Prenatal mood * SRI non-exposed * *SLC6A4* LL	−0.047	−0.162	0.068	0.632	0.427
Prenatal mood * SRI non-exposed * *SLC6A4* ≥ 1 s	0.056	−0.024	0.136	1.884	0.17
Prenatal Mood * SRI exposed * *SLC6A4* LL	−0.02	−0.094	0.055	0.271	0.602
Six year maternal mood * SRI non-exposed * *SLC6A4* LL	−0.171	−0.261	−0.082	14.098	**<0.001**
Six year maternal mood * SRI non-exposed * *SLC6A4* ≥ 1 s	−0.139	−0.216	−0.061	12.237	**<0.001**
Six year maternal mood * SRI exposed * *SLC6A4* LL	−0.092	−0.171	−0.014	5.29	**0.021**

*B** *is the non-standardized regression coefficient. Bold value indicates p < 0.001.*

extremely well—no other group regardless of *SLC6A4* genotype or mother's mood had mean scores that were as high. However, when their mothers were highly symptomatic, they performed worse than any other group including ≥1 S children with equally symptomatic mothers and LL children with less symptomatic mothers.

Differences in accuracy were most evident on the most-demanding EF Block. In general EF differences between groups often emerge only when cognitive skills are pushed to their limit. Children's EFs were not significantly affected by the child's mood (anxiety or depressive symptoms), though in this cohort there was little variation in the children's subclinical mood symptoms. Parents reported fewer inattentive and externalizing behaviors in children with prenatal SRI exposure regardless of the child's genotype. That might be because the SRI improved the child's postnatal environment (by improving the mother's mood) or because the effect on the 5HT signaling in the child secondary to prenatal SRI exposure. The benefit of one S allele of *SLC6A4* to EFs (cognitive control, self-regulation, inhibitory control) in children prenatally exposed to an SRI antidepressant became most apparent when children were in a particular environment (i.e., when their mothers were relatively more depressed). In that environment, the effects of the LL exposed children suffered but the effects of the exposed with ≥1 S allele did not. In this way maternal depression might act as a "prism," dramatically increasing variability in EFs, according to prenatal SRI exposure and allelic variation.

Critical to identifying the impact of prenatal SRI exposure, is distinguishing the effects of the antidepressant from the maternal mood disturbance (pre and postnatal) that resulted in antidepressant medication use. Sensitivity to maternal depressed mood and its impact on cognitive development has been widely reported across childhood (Gelfand and Teti, 1990; Goodman and Gotlib, 1999; Elgar et al., 2004; Gross et al., 2008). Long before birth, early life influences are already shaping core cognitive capacities that go on to become critical for learning and mental health during childhood (Kolb et al., 2003; Fox et al., 2010). Preterm birth (Davis et al., 2011), prenatal psychological distress (Buss et al., 2011) and maternal behavioral risks (smoking, alcohol use, drug use) (Espy et al., 1999; Schonfeld et al., 2006; Blood-Siegfried and Rende, 2010) exert an influence on early EFs. Early and chronic exposure to maternal symptoms adversely affects early development of EFs (Hughes et al., 2013). Yet, not all outcomes on cognitive developmental pathways are necessarily negative in this setting (DiPietro et al., 2006), raising critical questions of how maternal mood affects cognitive development and who remains at risk, even in the presence of maternal pharmacotherapy. In the present study, prenatal SRI exposed children with at least one S allele showed stable EF functioning regardless of whether their mother was more or less depressed. Moreover, maternal mood in the present study, when their children were 6 years old, was mainly at a subthreshold level, well-below a typical DSM-IV criteria for Major Depressive Diorder (MDD; American Psychiatric Association, 2000), thus highlighting the importance of a spectrum of maternal mood symptoms on child development, rather than a clinical cutoff score.

Converging evidence also points to links between changes in 5-HT signaling and cognition in both animal models and humans (Munafò et al., 2009; Homberg et al., 2010), though not all studies have been consistent (Schmitt et al., 2006; Homberg and Lesch, 2011). Increased 5-HT signaling, secondary to SRI treatment and genetic variations, has been associated with improved cognitive functions. 5-HT transporter knockout rodent models, analogous to an extremely low activity (short allele) variant, have been associated with improved cognitive flexibility during reversal learning tasks (Brigman et al., 2010; Nonkes et al., 2012), as well as morphological frontal cortex changes reflecting an increase in central 5-HT levels (Jedema et al., 2009; Kalueff et al., 2010; Nonkes et al., 2010). Consistent with these findings, early developmental exposure to fluoxetine has been associated with improved spatial learning in rats (Bairy et al., 2006). In humans, carriers of the S allele showed improved performance on an attentional inhibition task (Roiser et al., 2007). Adults homozygous for SS alleles outperform LL carriers on cognitive tasks requiring inhibitory control, including episodic memory and attention (Roiser, 2011), reaction time (Enge et al., 2011) and executive attention (Strobel et al., 2007). Among S carriers, better performance on the Wisconsin Card Sorting test has been reported (Borg et al., 2009), reflecting the impact of increased 5-HT signaling on cognitive flexibility. In contrast, lower 5-HT levels also appear to impair reversal learning (Clarke et al., 2007). Cognitive consequences of increased 5-HT signaling associated with SRI antidepressant exposure have shown mixed results as well. In animal models, not all findings reflect the same impact on cognitive flexibility (Homberg et al., 2007b).

The neuroanatomical, and functional consequences of changing 5-HT levels depend on the timing (critical periods) and direction (increased or decreased) of the developmental exposure to changes in 5-HT signaling and may differ from the impact of an acute exposure in a mature organism. (Ansorge et al., 2007; Kalueff et al., 2010). In a rodent model, SRI exposure during a very specific postnatal period (postnatal days 4–21) of development is also associated, paradoxically, with reduced exploratory behavior, and depressive and anxiety-related behaviors in adulthood. These effects mimic the very effects of genetic 5-HTT inactivation (i.e., gene knockout models leading to the absence of the transporter); suggesting that increased serotonergic signaling during a developmentally critical period predisposes to subsequent affective disturbances (Lira et al., 2003; Ansorge et al., 2004, 2008). This central serotonergic autofeedback hypothesis suggests that increased feedback signaling in the presence of high serotonergic tone blunts maturation of the 5-HT system via long-term developmental activation of inhibitory receptors (i.e., 5-HT_{1a}), paradoxically leading to psychopathology later in life (Hensler, 2006; Ansorge et al., 2007; Simpson et al., 2011). While one might consider that maternal SRI treatment during pregnancy could potentially confer benefit on fetal neurodevelopment—via improved maternal mood—such exposure could also have detrimental effects later in childhood, reflecting a long-term consequence of decreased serotonergic tone. SRIs may elevate fetal 5-HT levels, but then ultimately lead to decreased 5-HT signaling later in life and restricted serotonergic system development. The serotonergic auto-feedback hypothesis, however, is not a unitary construct and further work

is needed to understand how developmental changes in 5-HT signaling influences the downstream interaction with the social environment inherent to life with a depressed mother that together contributes to childhood behavior in this setting (Oberlander et al., 2009).

Importantly, not all factors that affect 5-HT signaling confer the same risk. Early life experiential variables influence susceptibility to environmental factors (Moffitt et al., 2005; Caspi and Moffitt, 2006) and not all outcomes associated with the short allele are necessarily negative (Risch et al., 2009). While adults with two short alleles may be at increased risk for depression (Caspi et al., 2003) following early life adversity, those raised in a nurturing environment may ultimately have a lower risk for depressive symptoms (Taylor et al., 2006). Increased central 5-HT associated with the *SLC6A4* short allele may therefore contribute to an increased sensitivity to environmental stimuli or hyper vigilance, leading to adaptation in one setting or an increased risk for poor mental health in another. In other words, in a low reward or low adversity setting, such hyper vigilance may confer an actual benefit that increases processing of relevant stimuli improving learning and social cognition (Homberg and Lesch, 2011). In the current study, serotonergic tone, via either prenatal SRI exposure or *SLC6A4* allelic variations, appeared to affect a self-regulatory capacity that might heighten sensitivity to a world with a depressed mother. Highly vigilant individuals may therefore either become vulnerable or resilient, depending on the demands of that social environment.

Our findings may also illustrate the influence of how allelic variations in the context of both early (i.e., fetal) and ongoing (i.e., postnatal/childhood) life experience shape a "biological sensitivity to context" (Boyce et al., 1995; Ellis et al., 2011) influencing adaptation and the diversity of child developmental outcomes following early changes in 5-HT signaling. This model proposes that phenotypic plasticity might enable a child to match their biological and behavioral capacities to the demands of their developmental environment. In this context, genetic variations may confer advantages for some children in supportive environments, but disadvantages for others who face social adversity in the context of maternal depression (Boyce and Ellis, 2005). Our findings showing higher accuracy in the non-exposed, LL children in the context of a minimally depressed mother, supports this claim.

Our findings point to a broader understanding of the impact of serotonin developmental neurobiology. While the "S" allele has been widely considered the "risk" or "sensitive" allele whereby the effect varies with context (Barr et al., 2004; Belsky and Pluess, 2009; Homberg and Lesch, 2011; van Ijzendoorn et al., 2012) our findings suggest that under certain circumstances carriers of the L allele may also be equally or even more sensitive to context. How this reflects the underlying changes in serotonin signaling (i.e., increased or decreased serotonin at developmentally sensitive times) remains a matter of speculation (Oberlander et al., 2009). Under some circumstances the L allele may confer vulnerability such as fear in adults exposed to carbon dioxide (Schruers et al., 2011) or aggression in 3-year old children of prenatally anxious mothers (Oberlander et al., 2010) when compared with LS or S allele carriers. Our findings take this observation one step further. Even with similar prenatal exposures, two children with different genetic inheritance show divergent developmental outcomes depending on the environmental circumstances they find themselves in at 6 years of age. Namely, while the impact of allelic variations may be environmentally dependent and the influence can, depending on the childhood context they grow into, go in both directions, thereby reflecting both developmental risk in some settings *and* resiliency in others. In this way, gene by environment outcomes may reflect a "conditional adaptation" (Boyce and Ellis, 2005) whereby allelic variations can be susceptible to both stressful and supportive contexts—for better and for worse (Belsky et al., 2007).

Conceivably there could be both advantages and disadvantages to improved EF performance. On one hand heightened vigilance may reflect an increased sensitivity in the social world of early childhood. However, it may also reflect a relative deficit in self-regulatory capacity which might illustrate a "leading edge" or susceptibility for a mood disorder that may emerge later in childhood (Taghavi et al., 1999). Interestingly, in a rodent model, an early increase in 5-HT signaling was associated with early fluoxetine exposure and paradoxically leads to increased anxiety and depression behaviors in adulthood (Ansorge et al., 2004). Earlier we reported that increased anxiety and depressive symptoms were observed by parents in 3 year olds with prenatal SRI exposure, though current increased maternal depression symptoms also contributed to child behavior (Oberlander et al., 2010). Now by 6 years of age, in the same cohort, levels of anxious behaviors did not differ between non-exposed children and fewer externalizing and attentional behaviors were observed in the exposed children. The long term implications of this unfolding longitudinal pattern remains unknown, however, improved EFs may reflect an endophenotype that includes increased vigilance that may evolve into a clinically apparent mood disorder in later childhood. Although increased vigilance may confer benefits for short-term tasks in one context (e.g., during a laboratory EF testing), it may be disadvantageous in the long run under other typical childhood circumstances (e.g., during an entire school day). While improved cognitive control in one setting may confer a developmental advantage (such as life with a depressed mother), the long term consequences of our findings in other childhood contexts (e.g., stressful classroom) need further study.

LIMITATIONS

A number of limitations need mentioning. First, without direct measures of central changes in 5-HT signaling in utero and again at 6 years, we can only infer that prenatal SRI exposure and genetic variations did indeed alter 5-HT function accounting for our findings. Further, serotonergic system function is dependent on multiple neurochemicals, receptors and related genes, and a focus on prenatal SRI exposure and genetic variations for 5-HT transporters offer only a limited insight into a complex developmental system underlying early human cognitive development. Additionally, study of parent-child relationships which have been noted as key influences on individual differences in a developing child's executive capacities (Carlson, 2003; Hughes and Ensor, 2009; Bernier et al., 2011) are needed.

SUMMARY

This study sought to examine the long-term effects of prenatal SRI exposure on EFs at 6 years of age and to determine whether effects are moderated by maternal mood and/or genetic variations. For prenatally SRI-exposed children, regardless of maternal mood, accuracy of children with reduced 5HTT expression (at least one short [S] allele) remained stable regardless of maternal depressive symptoms. In particular, even with somewhat depressed mothers (though all symptoms were below clinical threshold), these children's EFs were comparable to children with the same genotype whose mothers showed few if any depressive symptoms—in this sense, they showed resilience. In contrast, children with two long (L) alleles appeared sensitive to context. When their mothers reported relatively fewer depressed symptoms, LL children showed extremely good EF performance—better than any other group. When mothers reported more depressive symptoms, LL children's EF performance was worse than that of any other group. Further, parents reported fewer inattentive behaviors in their SRI exposed children.

In the face of a mother with a relatively more depressed mood (albeit not at clinical levels), EFs were best preserved in children prenatally exposed to SRIs and with at least one short *SLC6A4* allele. Yet, prenatally-exposed LL children hold out promise of possibly superior EF if their mother's mood remains euthymic or improves. Together these findings may reflect effects of both increased and decreased early serotonergic signaling associated with an increased sensitivity to social and relational contexts. While improved EFs might reflect an apparent resiliency to an "at risk" social environment, the long term and clinical implications of these findings remain to be determined.

ACKNOWLEDGMENTS

We thank the mothers and their children who participated and contributed to this work. We are grateful to Joannie Maynard and Deborah Heard for their assistance with data collection. This research was funded by March of Dimes Foundation (USA), the Child and Family Research Institute (UBC) and the Canadian Institutes of Health Research [Tim F. Oberlander (PI), CIHR #MOP 57837] and 6 year grant (MOP-86296). Tim F. Oberlander is the R. Howard Webster Professor in Brain Imaging and Early Child Development (UBC). Whitney M. Weikum was supported by postdoctoral fellowships from CIHR, Michael Smith Foundation for Health Research and funding from the Sunny Hill Foundation. Funding sources had no role in the study.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 19 July 2013; accepted: 24 September 2013; published online: 11 October 2013.

Citation: Weikum WM, Brain U, Chau CMY, Grunau RE, Boyce WT, Diamond A and Oberlander TF (2013) Prenatal serotonin reuptake inhibitor (SRI) antidepressant exposure and serotonin transporter promoter genotype (SLC6A4) influence executive functions at 6 years of age. Front. Cell. Neurosci. 7:180. doi: 10.3389/fncel.2013.00180

This article was submitted to the journal Frontiers in Cellular Neuroscience.