The role of the serotonin 5-HT3 receptor in cortical development
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Prenatal fluoxetine exposure induces life-long 5-HT₃ receptor-dependent cortical abnormalities and anxiety

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Submitted
Abstract

There is accumulating evidence that serotonin is a key factor in the development of the central nervous system, and that alterations in serotonergic signaling during development play an important role in the etiology of several neurodevelopmental disorders. Here we show that in utero exposure to the widely used antidepressant and selective serotonin reuptake inhibitor, fluoxetine, results in life-long abnormalities of cortical cytoarchitecture and in behavioral disturbances resembling anxiety. These developmental abnormalities critically depend on the 5-HT$_3$ receptor since they can be rescued in vitro through pharmacological block of the 5-HT$_3$ receptor. Moreover, the adverse behavioral phenotype after in utero exposure to fluoxetine is absent in 5-HT$_{3a}$ receptor knockout mice. These findings show for the first time that serotonergic signaling mediated by 5-HT$_3$ receptors is critical for cortical morphology during development and the behavioral repertoire during later life. In addition, these data may have clinical implications for the use of fluoxetine during pregnancy.
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Introduction

During the development of the brain, a series of events take place in which the classical neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) plays an important role (Gaspar et al. 2003; Daubert and Condron 2010). The importance of serotonergic signaling is highlighted by studies in which disturbances in serotonergic homeostasis resulted in structural changes of the brain and which impact the behavior in adult life. For instance, lesions of serotonergic innervation to the cortex results in abnormal formation of cortical columns (Janusonis et al. 2004), increase in cortical width (Boylan et al. 2007; Hohmann et al. 2007) and maturation of dendrites (Vitalis et al. 2007). In addition, genetic changes in either the synthesis or reuptake of serotonin result in similar cytoarchitectural abnormalities and are associated with an altered behavioral repertoire in later life (Alvarez et al. 2002; Holmes et al. 2003). Changes in brain development as a result of block of reuptake of serotonin by in utero exposure to antidepressants like fluoxetine have received quite some attention given the fact that SSRIs are the first choice of antidepressant treatment in pregnant women (Nonacs et al. 2003; Payne et al. 2009).

It has been shown that in utero exposure to fluoxetine induces a paradoxical effect in later life, i.e. the behavioral symptoms are opposite to those induced by adult fluoxetine exposure, and include increased emotional and anxiety behavior (Ansorge et al. 2004; Noorlander et al. 2008; Homberg et al. 2009). However, the mechanisms underlying the serotonin-induced changes in brain development and behavior are largely unknown. Recently, we showed that serotonin controls the maturation and complexity of dendrites of cortical neurons, and that this effect is mediated by the 5-HT₃ receptor, the only ligand-gated ion channel in the family of serotonin receptors (Chameau et al. 2009). Decrease of the serotonergic tone by either pharmacological block of the 5-HT₃ receptor, or by using the 5-HT₃A receptor knockout mouse (Zeitz et al. 2002), results in an aberrant hypercomplexity of cortical layer 2/3 pyramidal neurons (Chameau et al. 2009). (Supplemental Figure S1). We hypothesized that fluoxetine, by increasing the serotonergic tone, would induce opposite effects on the cortical cytoarchitecture.
Material and Methods

Animals and drug administration

Pregnant C57/BL6J mice were maintained in a facility with a 12h dark/light cycle and with ad libitum access of food and water. Successful mating was determined by the detection of a vaginal plug. The plug date is considered to be day 0 of gestation (embryonic day 0 (E0)). From E8 until E18 pregnant dams were injected intraperitonally (i.p) with either fluoxetine (0.6 or 0.8 mg/kg/day) or with equal volumes of sterile saline. Offspring was studied between P0 and P450. Animals were weaned at P21, group housed with 2-4 same sex littermates until experimentation. All experiments were approved by the ethical committees of the participating institutes.

Organotypic slice culture

Pups were sacrificed at P0 and 400 μm thick coronal brain slices were cut on a vibroslicer (Leica VT1000S) and subsequently cultured on culture inserts (Falcon, 1 μm pore size) for 5-7 days at 37˚C in a humidified atmosphere containing 5% CO₂. Slices were maintained in Neurobasal medium (Invitrogen) supplemented with B27 (1:50), 2 mM L-glutamine and 100 μg/ml penicillin/streptomycin. 100 nM of the selective 5-HT₃ receptor antagonists (tropisetron, bemesetron, granisetron and ondansetron, all from Sigma) or 10 μM fluoxetine was added to the culture medium starting at the day of culture, and half of the culture medium was refreshed every 2 days.

Electroporation

For electroporation of fresh slices, offspring was sacrificed between P6 and P9 and 300μm thick coronal slices were cut on a vibroslicer (Leica VT1000S). All slices including cultured slices were kept submerged at room temperature and continuously superfuged with ACSF containing in mM NaCl (120), KCl (3.5), CaCl₂ (2.5), MgSO₄ (1.3), NaH₂PO₄ (1.25), NaHCO₃ (25) and glucose (25), continuously bubbled with carbogen (95% O₂ and 5% CO₂) pH 7.4. Layer II/III cortical neurons were visualized using infrared differential interference contrast videomicroscopy on a Zeiss FS2 microscope. Pipettes were pulled from boroscillate glass and had a resistance of 5-12 MΩ when filled with biocytin hydrochloride (6mg/ml; Sigma) dissolved in internal solution containing (in mM): K-gluconate (110), KCl (30), CaCl₂ (0.5), EGTA (5), HEPES (10), Mg-ATP (2), pH 7.3 with KOH.
To electroporate the solution into the neurons, the micropipette was placed against the cell wall and electrical pulses (three trains of 300 square pulses of 1 ms duration and 10 ms interval at 100 Hz) were delivered across a silver wire placed inside a glass micropipette and a silver wire ground electrode placed in the culture medium. After electroporation, slices were fixed overnight in 4% paraformaldehyde.

**Immunohistochemistry**

For the visualization of the electroporated cells slices were rinsed in phosphate buffered saline pH 7.4, endogenous peroxidases were removed with 3% H$_2$O$_2$ in PBS for 30 min, rinsed again and permeabilized with 2% triton X-100 (Sigma Germany) in PBS for 1 hour. Subsequently, slices were incubated with ABC (Vector labs UK) for 2 hours and staining was visualized with a DAB (invitrogen USA) reaction. After 8-20 min reaction was stopped and slices were mounted with moviol.

**Golgi-Cox impregnation**

In adult animals, neurons were visualized using the Golgi-Cox method according to Smit-Rigter et al. (2009). Briefly, at P60 or P>365 animals were decapitated and after dissection brains were placed in vials containing Golgi-Cox solution and stored in the dark for 28 days. Brains were rinsed in milli-Q water, dehydrated in ethanol and embedded in celloidine and immersed in chloroform for a maximum of 16 hours. Chloroform was discarded and brains were subsequently immersed in 70% ethanol. Brains were sliced on a vibratome at a thickness of 200 µm. Sections were stained in 16% ammonia, rinsed in milli-Q water, fixed in 1% sodiumthiosulphate, rinsed in milli-Q, dehydrated in ethanol and placed in histoclear before mounting the slices on glass slides. Slides were allowed to dry for one week before initiating the image analysis.

**5-HT transporter in situ hybridization (ISH)**

Brains of adult P60 animals were collected and immediately frozen on dry ice. Sections (20 µm) were cut and collected on SuperFrost Plus slides (Menzel Gläser). Brain sections were stored at – 80°C until use. ISH procedures were as described (Jacobs et al. 2009). The SERT probe spanned bp 1827-2326 of the Sert mRNA (NM_010484.1).
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Image analysis

Sections containing filled or Golgi-Cox impregnated neurons were scanned on a confocal microscope (Zeiss LSM 510). Objective: dry Plan Neoluor 20x/0.75; filter: LP 1; beam splitter & HFT 80/20; laser HeNe 543 nm; stack 2 μm and 1 μm for golgi-cox impregnated sections. Neurons were reconstructed in ImageJ using the Neuron Morpho plug-in. Dendritic complexity index was calculated according to (Lom and Cohen-Cory 1999) using the LMeasure plugin:

\[
DCI = \frac{\sum \text{branch tip orders} + \# \text{of branch tips}}{\# \text{of primary dendrites}} \times \text{total arbor length}
\]

The neurons selected from two to four animals per group were randomly chosen alternating between right and left hemisphere and located in the somatosensory cortex. Neurons had to be well filled and free of neighboring precipitate and damage. Autoradiographic BAS-TR2040 imaging plates were scanned using the FLA-5000 imaging system (Fuji) and quantitative analysis was performed using the AIDA Image Analyzer Software (Raytest). Serotonin transporter binding was analyzed in the dorsal raphe nucleus.

Novelty Suppressed Feeding test

The procedure was adapted from Gross et al. (2002). One day before testing mice were housed individually. Fifteen hours before the onset of the test mice were weighed and food was removed from the cage, while water remained available ad libitum. At the day of testing, mice were transferred to the testing room and at the onset of the test mice were placed in an open field (70 x 70 cm) with a pre-weighed food pellet in the center. For this experiment latency to begin chewing food was measured. Immediately after beginning to eat or 5 minutes after being placed in the open field, mice were returned to their homecage where they could continue to consume the pellet for 5 minutes. The amount of food consumed was measured by weighing the food pellet afterwards. At the end of the test animals were weighed again.
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Statistical analysis

Results are expressed as mean ± SEM and compared using either a Mann-Whitney U test or a Student's t-test. Values are considered significant at $p < 0.05$. 
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Results and Discussion

Pregnant mice were treated from E8 onwards until confinement with daily i.p. injections of fluoxetine (0.6 mg/kg or 0.8 mg/kg) or saline as described previously (Noorlander et al. 2008). The brains from the offspring were used to determine the complexity of the dendritic tree of layer 2/3 pyramidal neurons in the somatosensory cortex at three ages: around one week after birth (postnatal day (P) 6-9), at adulthood (P60), and in mice older than one year (P>365). Layer 2/3 pyramidal neurons were visualized by either electroporation with biocytin in acute cortical slices, or by Golgi impregnation.

The complexity of the dendritic tree was quantified into a single parameter, the Dendritic Complexity Index (DCI), which takes into account the extent of branching and the total length of the dendrites (Figure 1A). In agreement to our hypothesis, the complexity of the layer 2/3 pyramidal neurons was significantly reduced to ~50% in prenatal fluoxetine-exposed animals as compared to controls (Figure 1B,C). The reduction in complexity was already evident in young animals, and lasted lifelong, even in mice up to 16 months of age (Figure 1C). These results strongly suggest that an increase in serotonergic tone, induced by fluoxetine, results in an aberrant development of the dendritic tree by a mechanism which is similar, but opposite, to our previous findings in which the dendritic complexity was increased after reduction of the serotonergic activity (Chameau et al. 2009). Therefore, we hypothesized that treatment with selective 5-HT₃ receptor antagonists should result in a reversal of the initial decreased DCI phenotype. We tested this hypothesis by making organotypic slice cultures of mice exposed in utero to fluoxetine or saline, and treating these slice cultures with the selective 5-HT₃ receptor antagonist tropisetron. The complexity of layer 2/3 pyramidal neurons from saline-treated mice was indeed increased by tropisetron (Figure 1D). In agreement with our earlier findings (Figure 1C), the complexity of neurons from fluoxetine-treated mice was initially reduced and importantly, remained reduced under the slice culture conditions (Figure 1D). However, when slice cultures from fluoxetine-treated mice were cultured in the presence of tropisetron, the complexity was reverted (Figure 1D). This strongly suggests that fluoxetine exerts its effect on dendritic complexity through a mechanism which involves 5-HT₃ receptors.
Figure 1. Prenatal exposure to fluoxetine leads to hypotrophic dendrites of cortical layer 2/3 pyramidal neurons. (A) Example of the quantification of dendritic complexity of layer 2/3 pyramidal neurons. Branch tip orders are assigned after each bifurcation of the dendritic tree, and the number of branch tips are counted. (B) Examples of layer 2/3 pyramidal neurons, stained with biocytin, from young mice (P6-9) which were exposed in utero to either saline or 0.6 mg/kg fluoxetine. The arrow head indicates the primary dendrite originating from the soma. Note that the dendrite of the fluoxetine-treated neuron has less branches (indicated by asterisks) as compared to the saline-treated neuron. Scale bars: 10 µm. (C) DCI, as percentage of saline-treatment, in mice exposed in utero to 0.6 mg/kg (black bars) or 0.8 mg/kg (open bars) fluoxetine at the age of 6-9 days after birth, at adulthood (P60) and in mice over 1 year old (P>365). (D) DCI of layer 2/3 pyramidal neurons in organotypic slice cultures prepared from mice which were exposed to either saline or 0.6 mg/kg fluoxetine, and in culture treated with either vehicle or 100 nM tropisetron. Number of cells is indicated in the bars. Asterisks indicate levels of significance (*p<0.05,** p<0.01, *** p<0.001).

To rigorously test the idea that signaling via 5-HT_3 receptors is responsible for the fluoxetine-induced changes in cortical cytoarchitecture, we applied fluoxetine to pregnant mice which lack the gene for the obligatory 5-HT_3A subunit of the 5-HT_3 receptor (Zeitz et al. 2002). As we have shown previously, the dendritic complexity of layer 2/3 pyramidal neurons in the 5-HT_3A knockout is increased as compared to wilttype (Chameau et al. 2009) (Figure 2B). In contrast to wildtype mice, however, fluoxetine does not decrease the complexity in the 5-HT_3A knockout animals (Figure 2B).
The lack of effect of fluoxetine in the 5-HT$_{3A}$ knockout is not due to the lack of its substrate, the serotonin transporter, as the expression of the serotonin transporter in the 5-HT$_{3A}$ knockout is indistinguishable from that in wildtype (Supplemental Figure S2).

**Figure 2.** Prenatal fluoxetine does not affect dendritic morphology of layer 2/3 pyramidal neurons in 5-HT$_{3A}$ receptor knockout mice (A) Examples of reconstructed layer 2/3 pyramidal neurons from wildtype mice and their transgenic littermates which were exposed *in utero* to either saline or 0.6 mg/kg fluoxetine. Scale bar: 10 µm. (B) DCI, expressed as percentage of wildtype saline, in young and adult mice exposed *in utero* to either saline or 0.6 mg/kg fluoxetine. Note that the DCI of knockout saline is already increased as compared to wildtype saline. (C) DCI of layer 2/3 pyramidal neurons from the mothers. Number of cells is indicated in the bars. Asterisks indicate levels of significance (* p<0.05, ** p<0.01, *** p<0.001).
It is of interest to note that fluoxetine does not affect the cortical organisation in the mothers (Figure 2C), coinciding with a lack of effect of fluoxetine on the dendritic complexity when applied acutely to organotypic slice cultures (Supplemental Figure S1). This illustrates that the timely activity of both serotonin and the 5-HT$_3$ receptor during development determines the cortical cytoarchitecture.

The long-term change in cortical cytoarchitecture as a consequence of in utero fluoxetine exposure pointed our attention to the previously described behavioral abnormalities in fluoxetine-exposed animals (Noorlander et al 2008). Fluoxetine-exposed animals score significantly higher in anxiety-related tests such as the novelty suppressed feeding test. To test this hypothesis, we set up an experiment where we treated wildtype and 5-HT$_{3A}$ knockout animals with 0.6 mg/kg fluoxetine. In agreement to our initial idea, the development of an enhanced latency to feed in a novelty suppressed feeding test was absent in the fluoxetine-exposed 5-HT$_{3A}$ knockout animals (Figure 3A), whereas other related behavioral parameters were unchanged between wildtype and knockout (Figure 3B-D). Taken together, the absence of 5-HT$_3$ receptor-mediated signaling prevents the adverse effects of prenatal fluoxetine exposure, suggesting that 5-HT$_3$ receptor signaling is critically involved in the development and regulation of these behaviors.

In conclusion, our data suggest that the prenatal exposure to fluoxetine interferes with the serotonergic tone during prenatal brain development, which is essential for the correct cortical cytoarchitecture and the development of adult behavioral repertoire in a 5-HT$_3$ receptor-dependent mechanism. There is accumulating evidence that serotonin plays an important role in the etiology of several neurodevelopmental disorders (Holmes et al. 2003; Boylan et al. 2007; Hohmann et al. 2007; Daubert and Condron 2010), but the molecular mechanisms underlying the effects of serotonin are largely unknown. Here, the 5-HT$_3$ receptor presents itself as a molecular target for understanding the intricate process of the development of the cortical cytoarchitecture. In addition, the 5-HT$_3$ receptor may be a target to interfere with cortical developmental disorders. In view of the above, it would be worthwhile to contemplate about a re-evaluation of the use of fluoxetine as antidepressant treatment in pregnant women.
Figure 3. Increased latency in a novelty-suppressed feeding task induced by prenatal exposure to fluoxetine is absent in 5-HT₃A knockout mice. (A) Fluoxetine induces an increase in latency to feed in the novelty-suppressed feeding test in wildtype mice, but not in 5-HT₃A knockout mice. Number of animals is indicated in the bars. Asterisks indicated p<0.05. Other behavioral parameters such as weight loss after food deprivation (B), time spent feeding upon completion of the test (C) and total food consumption (D) were neither dependent on the genotype of the animal, nor on the in utero treatment.
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Supplemental Figure S1. DCI, expressed as percentage of control, of layer 2/3 pyramidal neurons in organotypic slice cultures, treated with 100 nM of the selective 5-HT$_3$ receptor antagonists tropisetron (ICS205-930), bemesetron (MDL72222), granisetron (Kytril®), ondansetron (Zofran®) or with 10 µM fluoxetine. Organotypic slice cultures were prepared from mice at P0 - P1, and neurons were electroporated with biocytin at day 6-7 in culture.

Supplemental Figure S2. Expression of the 5-HT transporter is not altered in the 5-HT$_{3A}$ knockout mouse. ISH for the 5-HT transporter is shown in coronal sections through the raphe nuclei from rostral (A, A’) to caudal (G, G’) of adult wildtype (WT) and knockout (KO) mice.