Antidepressants and the adolescent brain: Changing the course of neurodevelopment?
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CHAPTER 1

GENERAL INTRODUCTION AND THESIS OUTLINE
General introduction

‘A child is not just a miniature adult’  (J.J. Rousseau, 1712-1778)

Since the time of Rousseau, developmental biology and child psychology have become an indisputable part of present-day science and it is nowadays undebated that a child (or adolescent) is not to be considered equal to an adult, not only in terms of its behavior and cognitive abilities, but also in terms of medicine. It is therefore striking that the largest part of the available medications are still primarily being tested in adults. The reason for this is that ‘pediatric studies are not only difficult to carry out from a practical point of view, they were also considered, until recently, as ethically difficult to defend’ (Wohlfarth et al., 2009). As a result, a considerable number of medicines are being prescribed in general practice that are not licensed for use in children or adolescents under 18 years of age (‘off-label’), or not even licensed at all (‘unlicensed’). Also in the Netherlands the prescription of this type of medication to children and adolescents is common practice, with about half of all pediatric drug prescriptions being either ‘off-label’ or ‘unlicensed’ (‘t Jong et al., 2002; Schirm et al., 2002). Also the prescription of psychotropic drugs (medicines that act primarily upon the central nervous system) like methylphenidate and antidepressants, and even antipsychotics, to children and adolescents is becoming the rule rather than the exception (Andersen and Navalta, 2011; Hammad et al., 2006), and is in most cases unlicensed (except for methylphenidate (Ritalin®), which is ‘off-label’ for adult use). Considering the fact that human brain development is ongoing until at least the early twenties, this is worrisome at the least (Paus et al., 1999; Sowell et al., 2003). In view of the many scientific publications regarding this topic, awareness of this important matter inside and outside the scientific world is evident, although everyday practice is still lagging behind (Jureidini et al., 2004; Lancet Editorial Note, 2006; Vitiello, 2003; Wohlfarth et al., 2004).

Childhood depression is considered relatively rare under the age of 12, but its prevalence increases from 1-2% to 8-14% by the end of adolescence (Kapornai and Vetro, 2008; Kessler et al., 2001). Also, adolescent onset of major depression is associated with a more chronic, severe and disabling pattern, including higher rates of family history and more suicide attempts than adult onset depression (Zisook et al., 2007). In 2006, the antidepressant fluoxetine (Prozac®) was approved for the treatment of moderate-to-severe depression in children aged 8 years and older. The efficacy of antidepressants in either prepubertal children or adolescents remains heavily debated, however (Bridge et al., 2007; Hetrick et al., 2007; Jureidini et al., 2004; Wohlfarth et al., 2004). More strikingly, both the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) initially stated, in 2004, that antidepressants were contraindicated for treating depression in children and
adolescents, after concerns about increased suicide risk in these specific age categories and concerns about developmental and reproductive toxicity of fluoxetine in particular. Meanwhile, although the steep increase in the rate of juvenile antidepressant prescriptions in the past 20 years did initially decline due to the warnings, prescription rates are now rising again (Wijlaars et al., 2012), also in the Netherlands (Volkers et al., 2007). In 2007, approximately 8,500 children and adolescents under age 21 were being treated with antidepressants in the Netherlands alone (Stichting Farmaceutische Kerngetallen 2007; www.sfk.nl).

**Rationale and aim of the thesis**

Fluoxetine (FLX) is a selective serotonin reuptake inhibitor (SSRI). Although numerous trials have shown robust safety of this type of antidepressant in adults, there are justified concerns about the long-term effects of these psychotropic medications on brain development in children and adolescents (Andersen and Navalta, 2004). Clinical safety data on their long-term effects are scarce and if available, studies have been conducted mainly in adults. The available preclinical data on the effects of FLX on brain development is not only limited, but also primarily aimed at perinatal exposure. The few studies that exist on juvenile exposure indeed suggest age-related differences, although focus lies heavily on behavioral aspects instead of the underlying biochemical alterations. These age-related findings lay beside the still not dispelled concerns about the heightened suicide risk after SSRI treatment in youngsters and together all of these concerns emphasize the need and urgency for intensive investigation. This clear need for further investigation on the short- and long-term developmental effects of FLX in depressed children and adolescents is supported by EMA, who have placed FLX in this context on their priority list (http://www.emea.europa.eu/htms/human/paediatrics/prioritylist.htm). The aim of this thesis was therefore to gain more insight in the effects of SSRIs, and FLX in particular, on neurodevelopmental processes in the adolescent brain, with an emphasis on serotonergic functioning.

**SSRIs and the serotonin system**

SSRIs like FLX mainly act on the serotonin system. Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine neurotransmitter that plays a role in many important brain functions, ranging from regulation of circadian rhythms to complex cognitive processes linked to learning, memory and emotion. Changes in 5-HT function are known to be involved the pathophysiology of many psychiatric disorders, with depression and anxiety being the most well-known (Sharp, 2010). The cell bodies of 5-HT neurons are restricted to clusters of cells located in the brain stem, the raphe nuclei, with the dorsal raphe nucleus (DRN)
being the largest of the brainstem’s serotonergic nuclei and containing about 50% of 5-HT neurons. Their axons however innervate nearly every area of the brain. And although 5-HT neurons make up for only one in a million (1 x 10^-6) of all neurons in the entire mammalian central nervous system, serotonergic terminals may account for as many as 0.2% of all axon terminals in rat cortex, making 5-HT one of the most widely distributed neurotransmitters within the brain (Jacobs and Azmitia, 1992; Pineyro and Blier, 1999).

Working mechanisms

5-HT is synthesized from the essential amino acid tryptophan in the presence of its rate-limiting enzyme tryptophan hydroxylase (TPH). After release into the synaptic cleft, it diffuses to activate post-synaptic 5-HT receptors located on the dendrites, cell bodies and synaptic terminals of adjacent neurons. 5-HT action is regulated primarily via reuptake of 5-HT from the extracellular space and back into the pre-synaptic neuron by the 5-HT transporter (SERT) (Torres et al., 2003). This controlled and high affinity reuptake via SERT accounts for the removal of about 90% of released 5-HT (Benninghoff et al., 2012). SSRIs selectively inhibit this reuptake of 5-HT by blocking the SERTs, this way elevating the extracellular levels of 5-HT. After reuptake in the pre-synaptic cell by SERT, the 5-HT gets either stored in pre-synaptic vesicles for reuse or it is metabolized into 5-hydroxyindoleacetic acid (5-HIAA). Another key mechanism involved in the control of 5-HT homeostasis is negative feedback inhibition by pre-synaptic 5-HT auto-receptors, of which the 5-HT1a and 5-HT1b are best characterized (Best et al., 2010; Sharp, 2010). 5-HT1b auto-receptors, located at the nerve terminals themselves, decrease the synthesis and release of 5-HT when extracellular concentrations rise, whereas 5-HT1a auto-receptors affect firing rates in the DRN itself. Subsequently, these negative feedback mechanisms are themselves regulated by dynamic changes in auto-receptor expression levels, which is demonstrated by the quick internalization of these auto-receptors by for example SSRI exposure (Descarries and Riad, 2012; Newman et al., 2004). See Figure 1 for a schematic overview of 5-HT synthesis, release and uptake.

While most of these direct effects of SSRIs on the 5-HT system are now unraveled, their exact mechanism of action is still unknown. This status quo is nicely stated by Benninghoff et al.: “while SSRIs have a proven effect on the patients’ mood, the underlying mechanisms for their beneficial impact still remain partially unclear mostly because the known effects of altered 5-HT levels on surrounding brain tissue do not suffice as satisfactory explanation of the therapeutic effects observed in patients receiving chronic SSRI-treatment” (Benninghoff et al., 2012). One of the main issues is that antidepressants such as SSRIs are only effective after long-term use, indicating that slowly proceeding neurophysiological adaptations of the brain are needed for the antidepressant effect to kick in. Several theories on the
underlying working mechanisms however exist, ranging from the requirement of complete desensitization of the negative feedback mechanisms to regulation of gene expression and adult neuroplasticity (Duman et al., 2001; Tardito et al., 2006). Either way, it is safe to say that the ways in which SSRIs exert their therapeutic effect are extremely complex and yet far from unraveled. Also, it is clear that these working mechanisms are by no means restricted to the 5-HT system, but that other neurotransmitters such as noradrenaline (NA), dopamine (DA), glutamate and GABA are involved as well (Amargos-Bosch et al., 2005).

**Developmental role of 5-HT**

So, even though it is still unresolved how SSRIs precisely work, we do prescribe them to children and youngsters with developing brains. Why is there reason to believe that changing 5-HT function in a developing brain can be potentially harmful? 5-HT is already present in the brain during early prenatal development, even prior to the onset of neurotransmission or the formation of conventional synapses, and has therefore been suggested as early as 1978 to play a role as ‘developmental signal’ in the construction and plasticity of brain circuits (Lauder and Krebs, 1978).

![Figure 1. Schematic view of a serotonergic synaptic terminal. Adapted from (Fernandez and Gaspar, 2012). Abbreviations: 5-HT: serotonin, 5-HTP: 5-hydroxy-tryptophan, Tph2: tryptophan hydroxylase 2, Aadc: aromatic amino acid decarboxylase, SERT: 5-HT transporter, MAO: monoamine oxidase, 5-HIAA: 5-hydroxyindoleacetic acid, Vmat2: vesicular monoamine transporter 2.](image)

Indeed, 5-HT was recognized to play a role in numerous developmental events, including cell proliferation, migration, and differentiation, cell death and synaptogenesis (Gaspar et al., 2003; Whitaker-Azmitia et al., 1996). Most of the 5-HT system is already fully functional around birth (Murrin et al., 2007) and disruptions in 5-HT function will therefore
have more profound effects during early (prenatal) development. Still, development of 5-HT transmission is ongoing until adulthood. Animal studies have shown that 5-HT neurotransmission undergoes widespread remodeling from youth through adolescence into adulthood, which is during adolescence most pronounced in the frontal and limbic regions (Crews et al., 2007; Olivier et al., 2011). During this period the number of 5-HT synapses is known to fluctuate, there is a steady increase of SERTs in mainly the frontal cortex, and a clear reorganization of 5-HT receptor expression (Crews et al., 2007; Moll et al., 2000). Also, several brain regions, as for instance the prefrontal cortex (PFC) and the dentate gyrus (DG) of the hippocampus, still undergo structural maturational processes during youth in which 5-HT plays a role, including synaptogenesis, synaptic pruning and white matter maturation (Brenhouse and Andersen, 2011). So, possible long-lasting changes can occur when altering neurotransmitter function during these late developmental processes; a process which is termed ‘neuronal imprinting’ (Andersen and Navalta, 2004).

**Imprinting effects of juvenile drug exposure**

Brain development depends on the emergence of critical developmental processes during which widespread reorganizations of brain morphology and function take place and is therefore sensitive to pharmacological interventions that occur during these processes (Swaab and Boer, 2001). Clearly, adolescence is one of these so-called ‘windows of vulnerability’. Several neuroimaging studies have helped to visualize these intense levels of neuronal plasticity in puberty (Toga et al., 2006). After an initial period of axonal overgrowth, most circuits in the brain are refined and rewired by a gradual but extensive loss of synapses (as many as 40%) together with strengthening and maturation of remaining synaptic connections (Blakemore, 2008; Casey et al., 2008; Paus et al., 1999). This loss of earlier formed synaptic connections, called pruning, peaks during adolescence and accounts for substantial region-specific decreases in gray matter density in puberty (Giedd, 2008), while maturation of remaining connections leads to increases in white matter (Paus et al., 1999). This vulnerability of the adolescent brain becomes evident with the onset of some major neuropsychiatric disorders, including ADHD, depression, OCD, eating disorders and schizophrenia, and a severe liability to drug abuse (Adriani and Laviola, 2004; Andersen, 2003). Furthermore, there is an increased likelihood that addiction will develop when psychoactive drug use starts early during adolescence, indicative of age-dependent differences in pharmacological sensitivity to drugs (Clark et al., 1998).

Imprinting describes the long-term effects of a drug or event that last well after the removal of the originating cause. These effects might even be not fully noticeable until well after the time of exposure and may thus arise only during adulthood (Andersen, 2003). In this
view, Andersen and Navalta have put forward the ‘equal, but opposite’ hypothesis; stating that, although the initial mechanisms of action of psychotropic drugs are most probably similar in both the immature and mature brain, chronic exposure in adult subjects will result mainly in transient compensatory reactions, while in juveniles these same compensatory reactions might permanently affect the development of the involved neurotransmitter systems and subsequently lead to decreased sensitivity to the drug or even to effects that are opposite to the original drug effect (Andersen and Navalta, 2004). As said before: much of the 5-HT system is already fully functional around birth (Murrin et al., 2007). This argument along with the notion that targeted deletions of 5-HT receptors or of genes involved in 5-HT metabolism cause no gross abnormalities of brain development (Gaspar et al., 2003) suggests that the effects of SSRIs during childhood and adolescence will be minimal. The reason that targeted disruption of 5-HT transmission early in life has so little devastating effects is probably due to the large variety of 5-HT receptors and other 5-HT modulators and their limited set of actions during specific periods in development in specific brain areas, so that many ways of compensation exist (Gaspar et al., 2003). Still, the 5-HT system is not at ‘adult’ level during adolescence. And effects of early changes in 5-HT homeostasis are in fact present, especially resulting in deviant adult behavior (and physiopathology of psychiatric diseases), although not as striking as one may expect.

Preclinical studies on the lasting effects of juvenile SSRI exposure have focused on behavioral aspects, while studies on the effects of neurochemical outcome measures and especially on neurotransmitter function still are scarce. Considering behavior, the general consensus is that exposure to SSRIs very early in rodent development can lead to depression- and anxiety-like behaviors in adulthood, which is in line with the earlier mentioned ‘equal-but-opposite’ hypothesis (Olivier et al., 2011). However, the behavioral effects of adolescent exposure are less clear. Available studies report conflicting findings, both for the acute as well as for the long-term behavioral effects. Chronic SSRI treatment during adolescence has been reported to result in both anxiolytic as well as anxiogenic responses and both depressogenic as well as antidepressant behavior in adulthood, while other studies failed to demonstrate lasting effects (Iñiguez et al., 2010; Norcross et al., 2008; Oh et al., 2009; Vorhees et al., 2011). Acutely, SSRIs have been found to have either antidepressant effects (Bhansali et al., 2007; Homberg et al., 2011), or anxiogenic effects (Oh et al., 2009) on adolescent behavior. It must be noted that in all above-described studies the used age ranges, dosages, routes and number of administrations and washout periods differ considerably, in this way complicating direct comparison. As said, studies regarding the effects of adolescent SSRI exposure on neurotransmitter function or related outcome measures are limited in number. However, there are reports of age-dependent effects of chronic FLX on SERT expression
(Bock et al., 2005; Wegerer et al., 1999) on markers of both 5-HT and DA function (Karanges et al., 2011) and on dendritic spine proliferation (Norrholm and Ouimet, 2000). Also, a small number of studies focused on the effects of juvenile exposure on adult neurogenesis, but again findings are mixed (Cowen et al., 2008; Hodes et al., 2009; Navailles et al., 2008).

Pharmacological MRI as a research tool

Neuroimaging techniques that allow us to study both the anatomy and functional activity of the living brain have helped greatly improving our understanding of the underlying mechanisms of action of psychiatric diseases and of the effects of psychotropic medication thereupon. Especially functional magnetic resonance imaging (fMRI) has become one of the key research tools in neuroscience today, accompanied with an exponential growth in the application of MRI tools (Martin and Sibson, 2008). A powerful feature of MRI methodologies is their non-invasive nature and consequent widespread application to studies involving both animals and humans. The most commonly used fMRI technique is blood-oxygen-level-dependent (BOLD) fMRI. This technique is based upon the oxygenation state of the blood. Deoxygenated blood has paramagnetic properties which can influence the MR signal; its presence leads to signal decrease. Neuronal activation causes a local increase in blood flow which ‘overshoots’ the metabolic requirement of the nerve cells. The resultant increase in perfusion rate and in the amount of oxygenated blood in the region of activation can now be visualized as a change in raw image intensity when using appropriate MR acquisition sequences. These signal changes can be subsequently mapped onto ‘anatomical’ MR images of the same brain (Anderson et al., 2008; Leslie and James, 2000; Tracey, 2001). Perfusion, or cerebral blood flow (CBF), can also be measured with other MRI techniques such as arterial spin labelling (ASL). With ASL, arterial blood water is magnetically labeled using radiofrequency (RF) pulses and used as a diffusible flow tracer. By subtraction of labelled and non-labelled (control) images, a perfusion contrast can be obtained giving absolute CBF values in a specific region. CBF is expressed in well characterized physiological units of mL/100 g/min, reflecting the volume of flow per unit brain mass per unit time (Wolf and Detre, 2007). This is a more absolute measure than BOLD signal change.

By combining fMRI with pharmacological intervention, the actions of psychotropic drugs on the central nervous system can be visualized, an application that is referred to as pharmacological MRI or phMRI. This is a relatively non-invasive method, which offers a good spatial and temporal resolution allowing longitudinal studies to follow disease progression and/or treatment efficacy. Until recently, pharmacological action was typically visualized using positron emission tomography (PET) (Aznavour et al., 2006) or single photon emission computed tomography (SPECT) (Hwang et al., 2007). These techniques allow the localization
of radioligands, for example targeted at a specific receptor or transporter, in the living body. This makes PET and SPECT more direct measures than fMRI, but these techniques also have important shortcomings. First, the radiation exposure that is involved leads to safety concerns regarding the radioactive load and repeatability issues. Second, PET and SPECT suffer from a relatively low spatial resolution, and there is still a relative lack of useful radioligands currently available for receptor studies (Paterson et al., 2013; Saulin et al., 2012; Smith et al., 2002). Last, this type of imaging is relatively costly compared to MRI.

Considering the amount of psychotropic substances available, phMRI generates endless possibilities. It can for example be used for the in vivo assessment of neurotransmitter function, by applying a drug that specifically targets that neurotransmitter system. In this way, it is possible to use phMRI to assess 5-HT function by visualizing the effects of for example an SSRI or 5-HT receptor (ant)agonist. Numerous studies in both animals and humans have already validated this specific use of phMRI (Anderson et al., 2008; Martin and Sibson, 2008). Although precise underlying mechanisms are yet unknown, animal studies have shown that alterations in extracellular 5-HT concentrations and/or the blockade or stimulation of 5-HT receptors alter the MR signal in a region-specific manner (Martin and Sibson, 2008). An additional advantage of phMRI is its possibility to either determine direct modulation of brain function, for example during a long resting-state or baseline fMRI sequence following drug infusion, or indirect modulation of brain activation, for example caused by an activation paradigm to specifically highlight brain areas related to a specific cognitive, sensory, or motor task (Anderson et al., 2008; Tracey, 2001). In this way, 5-HT phMRI has shown the involvement of 5-HT in a broad range of neural processes ranging from motor function through ‘cold’ cognition, such as memory and response inhibition, to emotional processing (Anderson et al., 2008).

Besides these advantages, there are also some practical limitations to phMRI to consider. Firstly, although less invasive than PET or SPECT, phMRI still requires the administration of a drug and often this is administered intravenously to ensure rapid uptake in the brain visible within one scan. This makes the technique less suitable for vulnerable patient populations such as young children. Although oral administration of the challenge drug is possible and has been used in numerous studies (Anderson et al., 2008), individual differences in drug response and drug clearance together with the inability to scan on-and off the drug within one scan session will account for additional sources of variation. Secondly, the possible systemic and global cerebrovascular effects of the drug challenge should be taken into consideration, since these are able to affect the MR signal (Martin and Sibson, 2008), although the same accounts for PET/SPECT studies. Additionally, one should keep physiological variables such as heart rate, temperature and O$_2$ saturation as constant as
possible, as these also influence the hemodynamic response. This is especially of relevance in pre-clinical phMRI, where a certain level of anesthesia is often required. Last but not least, since phMRI is a relatively new imaging approach, for most of the phMRI applications, the reliability of the specific technique and thus its usefulness as a research tool needs to be further established.

Outline of this thesis

The aim of this thesis was to gain more insight in the effects of SSRIs, and FLX in particular, on neurochemical processes in the adolescent brain, and on how these differ from the effects on the adult brain, with a strong focus on the serotonergic neurotransmitter system. We first illustrate the concept of chemical imprinting after 5-HT manipulation in Part I. In Part II, we test this principle for FLX in a number of animal studies (the main focus of this thesis) and in Part III, methodological issues are discussed regarding imaging of the 5-HT system.

General Introduction

Chapter 1 offers a short introduction on the significance and relevance of studying the effects of antidepressants on the developing brain. The prevalence of depression and prescription rates of antidepressant drugs to children and teenagers force us to look into possible imprinting effects of these drugs on key neurotransmitter systems such as serotonin. Neuroimaging techniques such as pharmacological MRI can have a very useful role herein.

Part I: Chemical imprinting after 5-HT manipulation

Possible long-lasting changes in neurotransmission can occur when neurotransmitter function is altered during brain development. This is called neuronal imprinting, or, when a chemical substance is used to alter neurotransmitter function, chemical imprinting. In chapter 2, we describe a study in which the age-at-first-exposure to MDMA, a potent 5-HT releaser, is related to adult SERT expression in both the human and rat brain. Chapter 3 presents the results of several early pilot experiments that were performed in order to assess the effects of FLX on the late developing rat brain, including behavior, SERT expression and phMRI results.

Part II: Age-related effects of chronic fluoxetine in the rat brain

The aim of the animal studies described in this part of the thesis was to identify age-related effects of chronic FLX treatment in the rat’s brain. All studies use the same study design
and treatment protocol combined with different read-out systems to assess 5-HT-related neurochemical processes. Chapter 4 describes a pharmacological MRI study in which we compared the effects of FLX treatment on the brain’s responsiveness to an acute 5-HT challenge between adolescent- and adult-treated animals. Chapter 5 focuses on the effects of preceding chronic FLX treatment on extracellular monoamine levels before and after an acute 5-HT challenge. In chapter 6, three explorative studies on the age-related effects of chronic treatment on gene expression, SERT availability and TPH expression are discussed. Chapter 7 concludes with a study on the effects of chronic treatment on adult neurogenesis and how this again differs between adolescent-treated and adult-treated animals.

**Part III: Methodological issues of 5-HT phMRI**

This part of the thesis focuses on the importance, but also on the difficulties of 5-HT imaging. In chapter 8 we present a technical overview of how to perform 5-HT phMRI in rats. Chapter 9 and 10 describe an extensive pilot experiment in which the test-retest reliability of phMRI with an oral challenge was assessed in healthy female volunteers. In chapter 9, we used a perfusion-based method (arterial spin labeling) to overcome the potential problems with signal variation due to the requirement of repeated scan sessions. Chapter 10 describes the effects of an oral 5-HT challenge on task-related brain activity in a BOLD-based fMRI study. Finally, in chapter 11 we introduce the methodology of the ongoing clinical medication trial used to assess the effect of FLX treatment in adolescent and adult MDD patients.

**Summary, general discussion and conclusion**

The most important findings of the studies described in this thesis are summarized and discussed in chapter 12. A Dutch summary of the main findings and their implications can be found in the appendix.