YVES AUBERT

SEX, AGGRESSION AND PAIR-BOND

A STUDY ON THE SEROTONERGIC REGULATION OF FEMALE SEXUAL FUNCTION IN THE MARMOSET MONKEY

Sex, Aggression and Pair-bond:
a study on the serotonergic regulation of
female sexual function in the marmoset monkey

Yves Aubert

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TABLE OF CONTENTS

CHAPTER 1

General Introduction

TABLE OF CONTENTS

1. FEMALE SEXUALITY AND FEMALE SEXUAL DYSFUNCTION

Sexual reproduction is one of the most universal, fundamental and essential processes that all species have in common. Sexuality in humans, however, goes far beyond the basic principles of reproduction and entails complex interactions of biological, psychological and sociocultural variables. Few other biological systems are as multifaceted as sexuality. Beyond neurobiological factors that influence sexual function, several studies, including the National Health and Social Life Survey (NHSLS) that was conducted in the United States [1], point out that sexuality in women is closely linked to psychological factors, quality of their relationship with a partner and lifestyle [2-7], and that all are influenced by cultural, family and personal expectations [2, 3; 8-10]. Ultimately, sexuality serves a basic emotional health need for all individuals. Despite this understanding, sexual function in general, and female sexual function in particular, remains a relatively understudied area of scientific and clinical investigation, although pharmacotherapeutic interest has intensified research in recent years [11].

Masters and Johnson (1966) were among the first to describe and characterize female sexual function by proposing a linear, four stage model of the human sexual response. This traditional view divides a woman's sexual response into four sequential stages, starting with spontaneous sexual desire that leads to sexual arousal, and that is then followed by orgasm and resolution [12]. Later observations made it evident that this view of the female sexual response is just one of several normal variants in sexual responses in women [13]. For example, studies of women in established relationships show that desire by itself is not a frequent reason to engage in sexual activity [14], while other reasons such as the nurture of emotional closeness with a partner, the increase of their own sense of well-being, or the avoidance of negative consequences of sexual inactivity are commonly reported to also play a role [2, 3, 9, 10]. Other reports suggest that that female sexual desire and sexual arousal are overlapping concepts that women find difficult to separate [13]. At the same time, functional imaging studies in women reveal that subjectively perceived sexual arousal is often unrelated to objectively measured sexual arousal (reviewed in [6, 7, 15]). These and other observations led to novel views on women's sexuality and spurred the development of alternative models of the female sexual response cycle, centering around sociocultural and relationship factors [2, 8]. The model proposed by Basson (Figure 1, [8]) may be the most accurate for women with a history of sexual problems [16].

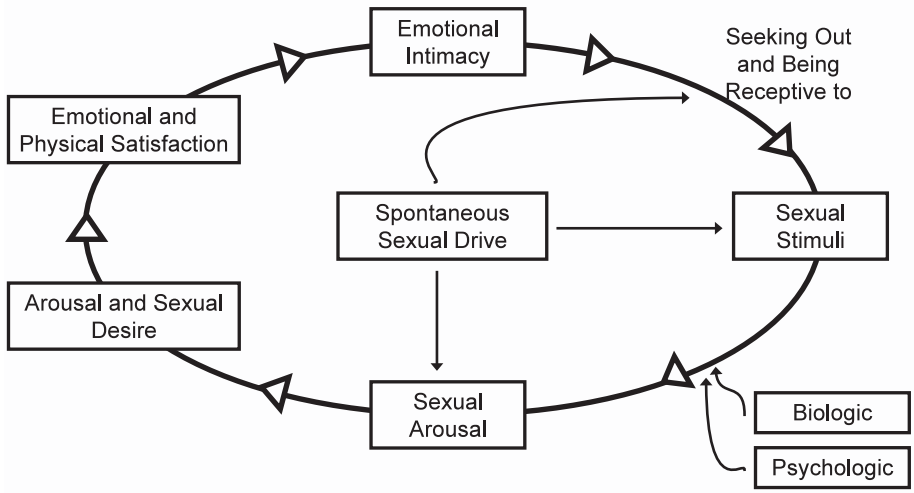


Figure 1. Female sexual response cycle. Adapted from [8].

Following the groundbreaking work by Masters and Johnson, the Diagnostic and Statistical Manual of Mental Disorders (DSM) implemented a classification system for female sexual dysfunctions (FSDs) that is based on the female sexual response stages. Sexual problems are divided into disorders of desire (hypoactive sexual desire disorder (HSDD), sexual aversion), arousal, orgasm and pain (dyspareunia and vaginismus). In the 4th edition (DSM-IV) and text-revised 4th edition (DSM-IV-TR), attention is paid to psychological factors such as interpersonal distress, which must be subjectively perceived by the female patient for the diagnosis of certain sexual dysfunctions, including HSDD. The NHSLS reveals that sexual dysfunction is prevalent across the entire demographic population and found in 43% of the women who participated in the NHSLS study at some point in their lives [1]. Among female sexual desire subgroups, low sexual desire is the most frequently reported complaint in women (32%; Figure 2). Clinically relevant, personally distressing occurrence of HSDD is reported in approximately 1 in 10 women [6, 7, 17]. With the exception of estrogen therapy for impaired genital vasocongestion in response to sexual stimulation and, in some countries, testosterone formulations for reduced desire, there are currently no approved drugs for the treatment of sexual dysfunction in women despite their high prevalence [18].

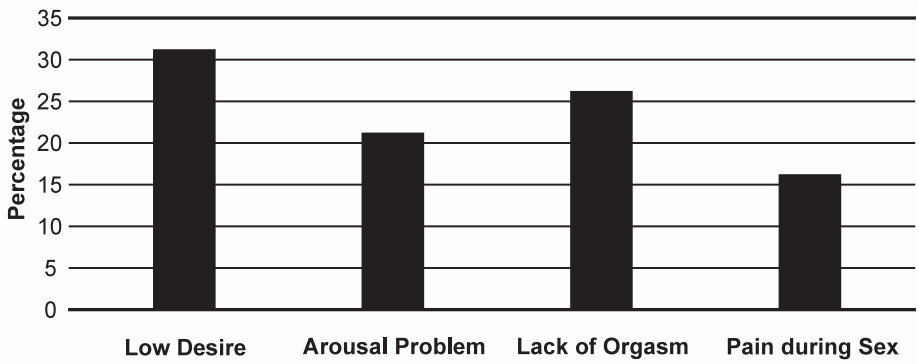


Figure 2. Prevalence of sexual dysfunction in women. Adapted from the National Health and Social Life Survey (NHSLS) [1].

Sexual desire and sexual arousal have traditionally been regarded as two distinct though related phenomena, a concept which seems to fit the sexual experience in men, but less in women [19]. In fact, some women find it difficult to differentiate arousal from desire [13]. Bancroft and Graham [13] describe sexual desire and sexual arousal as overlapping concepts. They suggest that sexual arousal involves (i) information processing of sexual stimuli involving unconscious and conscious cognitive mechanisms, (ii) incentive motivation that includes activation of the dopaminergic incentive motivational system, (iii) induction of generalized central nervous system (CNS) arousal, and (iv) genital response. The state of sexual desire, in contrast, involves only some of these components (usually components (i) and (ii)). The DSM-IV-TR defines HSDD as persistent or recurrent deficiency or absence of sexual fantasies and thoughts, and/or desire for, or receptivity to, sexual activity, which causes personal distress or interpersonal difficulties and is not caused by a medical condition or drug. Distress is an integral part of HSDD diagnosis. A diagnosis of HSDD requires a clinical evaluation, taking into account factors that affect sexual functioning, such as age and the context of the woman's life [20]. Despite of its prevalence, the acronym HSDD is poorly understood by clinicians, and the current DSM-IV-TR definition of HSDD has been criticized. There is an ongoing debate for combining arousal and desire into one disorder, and to include duration, intensity, and frequency to the definition of HSDD in the upcoming DSM-5 edition [20, 21]. The debate, however, goes beyond the scope of this thesis and its outcome neither affects objectives nor results of the experimental chapters presented in this book (Chapters 2-5).

2. THE NEUROBIOLOGY OF FEMALE SEXUAL BEHAVIOR

Following the principle that animal and human behavior is based on an underlying neurobiological substrate, neuroscientists are facing the challenge of dissecting and reducing the multifaceted behavior of female sexuality into separate components that can be studied individually. Health psychologists and ethologists, on the other hand, are equally challenged with the integration of complex etiological factors to explain the behavioral expression of female sexuality. The display of female sexual behavior, as well as the lack thereof, is the net result of motivational states, sensory stimuli, hormonal and neurochemical environments, previous experience, cultural influences and many other factors.

For practical and ethical reasons, many studies attempting to unravel the neurobiology of female sexual behavior have been performed in animals, and mostly in rodents. Pfaff and colleagues succeeded in describing the complete neural circuit of a primary reproductive behavior, lordosis behavior, in the female rat [22]. Lordosis behavior is a sexual response of the female to male tactile stimulation and consists of a ventral arching of the spine, thus allowing for copulation. In short, somatosensory stimulation of the female's flanks, tailbase and perineum by the male's paws and penis activate pressure receptors that signal via ascending spinal neurons to nuclei in the brain stem (medullary reticular formation and lateral vestibular nucleus), midbrain (midbrain reticular formation and periaqueductal gray, PAG) and hypothalamus (medial preoptic area, mPOA, medial anterior and ventromedial hypothalamus, VMH), where estradiol and progesterone are necessary to facilitate the behavior. Integrated signals are projected via the brain stem nuclei to descending pathways of the lumbar spinal cord, leading to the contraction of deep back muscles to elicit lordosis behavior [22]. Further studies in rodents applying lesion, electrical stimulation, tract-tracing and Fos-immunoreactivity (Fos-IR) techniques revealed other brain structures involved in different aspects of female sexual behavior. Following mating, Fos-IR is not only activated in the mPOA, VMH, and PAG, but also in the bed nucleus of the stria terminalis (BNST) and central tegmental field (CTF) [23-27]. Chemosensory investigation of the anogenital area induces Fos-IR in the posteromedial part of the BNST only [28], while vaginocervical stimulation either by a high number of penile intromissions or manual probing induces Fos-IR in the mPOA and BNST, and also in the medial amygdala and parvocellular subparafascicular nucleus [25, 26, 28, 29]. Importantly, combined estrogen and progesterone treatment in female rodents activates Fos-IR in most of the same brain areas that are activated after mating, including the pivotal VMH and mPOA, confirming the high density of estrogen receptors in these areas [30] and suggesting that both hormonal

and environmental signals relevant to sexual function may converge in these brain structures to determine the expression of female sexual behavior.

In female cats, a similar neural circuit to rats has been found to control lordosis behavior. Neurons in the nucleus retroambiguus (NRA), located in the brain stem, send descending projections to lumbosacral motoneurons that control the lower limbs and trunk to produce the copulatory posture. The NRA receives sensory information from the midbrain central gray, which in turn is a projection area for hypothalamic, amygdaloid and cortical neurons. Estradiol regulates the strength of the NRA-lumbosacral connection, explaining why lordosis behavior only occurs when the female cat is in estrous [31].

While experiments as described above are limited in their applicability to animal studies, more recent advances in functional imaging methods provide a window into the conscious human mind and allow for the study of neural correlates of human behavior. Both functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) approaches have attempted to investigate the brain activity during different sexual states, ranging from sexual interest to arousal and orgasm. fMRI studies in women show that several cortical areas (medial prefrontal, anterior cingulate, orbitofrontal, insular, entorhinal and occipitotemporal cortices) as well as the amygdala and ventral striatum are activated in response to the visual presentation of erotic video clips [32, 33]. Interestingly, the peripheral sexual response seems not to be correlated to either the subjective sexual response or brain activation patterns [33]. In a H215O PET study, clitoral stimulation of women by their partners leads to activation of the medial areas of the somatosensory and somatomotor cortices, which represent the pelvic region. These same regions are also activated during orgasm, although in a less pronounced fashion. Orgasm is furthermore characterized by an activated rostromedial cerebellum [31, 34]. Most strikingly, while some brain areas are activated during sexual stimulation and orgasm, much larger areas are deactivated, especially during orgasm. Clitoral stimulation deactivates the amygdala and the posterior temporal lobe mainly in the left hemisphere, and these same structures, together with large areas of the ventral temporal lobe and ventral prefrontal cortex, are also deactivated during orgasm. This study, together with an fMRI study that demonstrates greater activation of the medial frontal and right inferior frontal gyri in women with HSDD when watching erotic video clips [33], suggests that the deactivation of certain neural processes involved in alertness and thought processing is important for the experience of sexual arousal and orgasm, while HSDD patients may allocate more attention to monitoring and evaluating their sexual responses.

Of equal importance as the description of anatomical structures involved in

the regulation of female sexual behavior, including female sexual desire, is the characterization of neurochemical and hormonal mediators that orchestrate the neural activity within and between these brain regions. In a recent review publication [35], Pfaus applies the concept of separate but interactive neural systems for behavioral excitation and inhibition of female sexual behavior (Figure 3) and stresses that behavior can commence either because of direct excitation, or through a process of disinhibition. Neurobiological mechanisms of this dual control model for sexual behavior include, on the excitatory side, the central dopamine (DA) and norepinephrine (NE) neurotransmitter systems, as well as the neuropeptides oxytocin (OT) and melanocortin (MC), and the steroid hormones estradiol (E2) and testosterone (T). DA facilitates sexual excitement via mesolimbic/mesocortical, nigrostriatal and incertohypothalamic pathways. The mesolimbic/mesocortical pathway sends projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc), amygdala and frontal cortex [35]. The medial prefrontal cortex (mPFC) is of particular interest as it is a key region implicated in executive control and behavioral inhibition [36, 37].

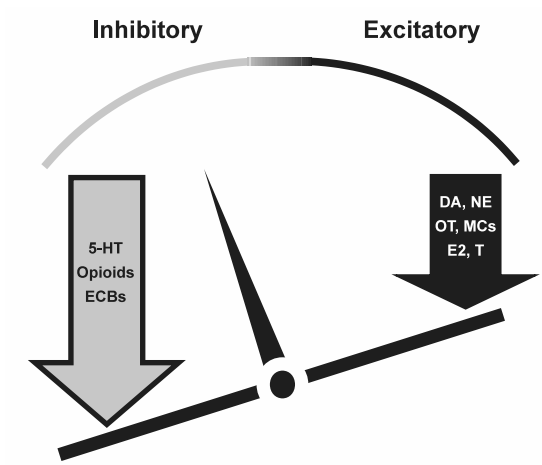


Figure 3. Excitatory-inhibitory model of female sexual behavior. Adapted from [35]. 5-HT, serotonin; ECBs, endogenous cannabinoids; DA, dopamine; NE, norepinephrine; OT, oxytocin; MCs, melanocortins; E2, estradiol; T, testosterone.

The nigrostriatal pathway sends projections from cell bodies in the substantia nigra to the striatum, while in the incertohypothalamic pathway, dopaminergic cell bodies in the zona incerta send projections to the mPOA. In estradiol and progesterone primed ovariectomized female rats, DA levels increase in the NAcc when the females are exposed to a sexually active male, and even more

during copulation. Copulation also elevates DA in the dorsal striatum [38]. DA antagonists generally inhibit proceptive, or appetitive, sexual behavior in female rats [39]. When D1 and D2 agonists are directly administered to the mPOA of estradiol primed ovariectomized rats, proceptive sexual behavior is selectively increased or decreased, depending on the specificity of the administered ligands to D1 or D2 receptors. This study suggests that it is the ratio of DA D1/D2 activity within the mPOA that influences the expression of proceptive sexual behavior [40].

The activation of the melanocortin receptors MC3R and MC4R in the brain increase female proceptive behavior in the rat, without affecting receptive (lordosis) behavior [41]. Peripheral administration of the MC3R and MC4R agonist bremelanotide induces c-fos expression in the mPOA and other hypothalamic and limbic brain regions. Direct infusion of bremelanotide into the mPOA facilitates proceptive, but not receptive, sexual behavior in the female rat, while administration to the VMH is without effect [42]. Subcutaneous administration of bremelanotide to ovariectomized and hormonally primed females also increases DA selectively in the mPOA, but not in the NAcc or VMH, suggesting that bremelanotide's effect on female sexual behavior is mediated by DA in the mPOA, a hypothesis that is supported by the observation that D1 antagonist administration to the mPOA reverses the effect of bremelanotide [Gelez H, Pfaus JG, personal communication].

The central serotonin (5-HT) neurotransmitter system is a key component in sexual inhibition, though there is evidence that activation of certain 5-HT receptor subtypes facilitates female sexual behavior [43-48]. The regulation of female sexual function by the serotonin neurotransmitter system is the main focus of this thesis, and a more detailed introduction to serotonin is presented in section 3.

Alongside the neural control of sexual function, steroid hormones play an important role in the regulation (in rodents) or modulation (in primates) of sexual behavior. Estrogens, progesterone and androgens have been recognized to be excitatory on sexual behavior in both humans and rodents [49], while glucocorticoids secreted in response to a stressful environment generally inhibit sexual behavior [49-52]. Thus, disorders of both ovarian function and the hypothalamic-pituitary-adrenal (HPA) axis, the latter responsible for the secretion of glucocorticoids from the adrenal glands, are associated with decreased sexual desire and arousal in women [53, 54]. In addition to direct endocrine effects on sexual behavior, steroid hormones modulate the central neurotransmitter systems involved in sexual function and may thus indirectly regulate sexual behavior.

3. THE ROLE OF SEROTONIN IN FEMALE SEXUAL FUNCTION

Pharmacological modulation of 5-HT neurotransmission is a common cause of diminished female sexual activity. Multiple clinical reports show that up to 75% of patients prescribed selective serotonin reuptake inhibitors (SSRIs) experience a treatment-induced detriment in sexual satisfaction, such as delayed or inhibited orgasm, or decreased sexual desire [55-60]. These studies provide evidence for the involvement of 5-HT in diminishing sexual behavior in women, however, without elucidating underlying brain circuitries as 5-HT modulates a wide range of neural, neuroendocrine and behavioral functions [61].

To date, there are 14 known 5-HT receptors, each of which exhibits considerable variation in brain distribution and physiological effects upon activation [61]. The 5-HT receptors are grouped in 7 families (5-HT1-7). Except for the 5-HT_3 receptor subtype, which is a ligand-gated ion channel, 5-HT receptors are G-protein coupled receptors. While serotonergic projections in the brain are diffuse, most of them originate from the raphé nuclei, a neuronal cluster located in the midbrain and the brain stem that contains the cell bodies of 5-HT neurons (Figure 4). A negative feedback mechanism regulates the

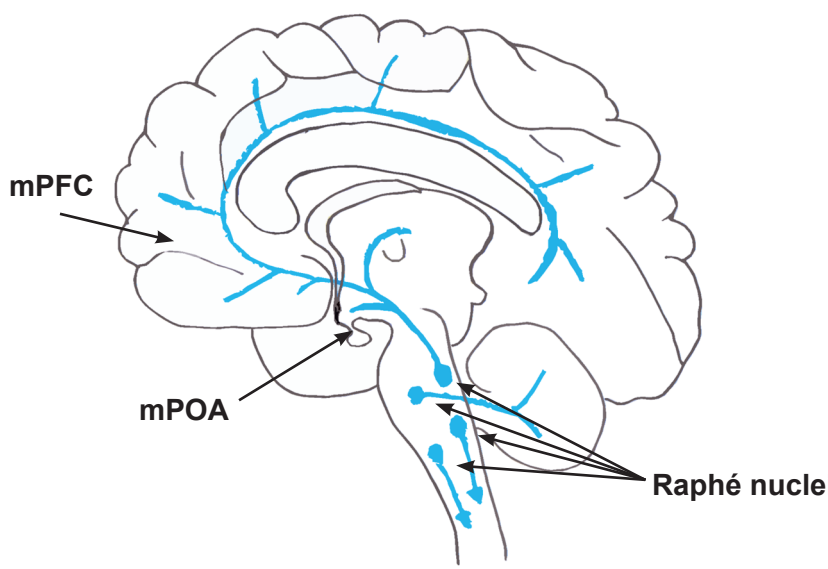


Figure 4. The central serotonin neurotransmitter system. Serotonergic neurons diffusely project from the Raphé nuclei to cortical, limbic and hypothalamic areas. mPFC, medial prefrontal cortex; mPOA, medial preoptic area. Adapted from [35].

activity of serotonergic neurons and acts mostly through presynaptically located autoreceptors of the 5-HT_{1A} and 5-HT_{1B} subtypes. Activation of 5-HT_{1A} receptors located on soma and dendrites opens potassium channels and thus inhibits 5-HT cell firing. Activation of 5-HT_{1B} receptors on the nerve terminals also inhibits 5-HT release. A negative feedback mechanism via postsynaptic 5-HT receptors might also play a role in regulating central 5-HT neurotransmission. This mechanism likely acts via the prefrontal cortex, where an activation of glutamatergic and GABAergic neurons that project back to the raphé nuclei leads to an inhibition of further 5-HT neural firing [62]. Serotonin transporters (5-HTT, or alternatively SERT) also partake in the negative feedback mechanism by actively transporting 5-HT from the synaptic cleft into presynaptic 5-HT neurons. The rapid removal of 5-HT released into the synaptic cleft by 5-HTT allows for the control of magnitude, duration and spatial distribution of signals reaching 5-HT receptors following neuronal stimulation [63].

Serotonin exerts a generally inhibitory tone on female sexual behavior. Studies in female rodents, however, have shown that 5-HT receptor specific agonists or antagonists can either facilitate or inhibit sexual activity, depending on the 5-HT receptor subtype activated. Lordosis is inhibited by 5-HT_{1A}receptor activation and 5-HT_3 receptor antagonism [43, 44, 47, 64], but is facilitated by 5-$HT_{2A/C}$ receptor activation [46, 65]. While the inhibitory effect of 5-HT_{1A} agonists on female receptivity is clear, less is known as to where in the brain these receptors are located. Lesion studies and local administration of the 5-HT_{1A} receptor agonist *R*-(+)-8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) into different brain areas highlight the importance of both pre- and post-synaptic 5-HT_{1A} receptor function in the serotonergic regulation of female sexual behavior, involving a circuitry that comprises the presynaptic raphé nuclei and the postsynaptic VMH, mPOA and PAG [44, 66-71]. The central 5-HT system furthermore interacts with the excitatory DA and NE neurotransmitter systems [72, 73], thus providing the possibility that a manipulation of 5-HT neurotransmission could affect sexual behavior both directly and indirectly by modulation of DA and NE.

The 5-HT system is also intertwined with endocrine systems. Estrogens and glucocorticoids modulate 5-HT receptor density in a receptor subtype and brain region specific manner in both rodents and primates [74-76]. Conversely, the central 5-HT system generally exerts a stimulatory function on HPA axis activity [77-79], and depletion of 5-HT reduces the responsiveness of the HPA axis to stress [80]. Liposits et al. [81] provide an anatomical context for the involvement of 5-HT in activating the HPA axis by showing that serotonergic nerve terminals and corticotropin-releasing hormone (CRH) neurons are

synaptically linked in the paraventricular hypothalamic nucleus (PVN). CRH containing neurons in the PVN that regulate HPA axis activity express 5-HT_{1A} and 5-HT_{2A} receptors [81, 82], and activation of either receptor type stimulates the HPA axis to release ACTH from the pituitary, and cortisol (in primates) or corticosterone (in rodents) from the adrenal cortex [82-85]. Thus, it seems plausible that sexual behavior could be indirectly modulated by 5-HT-regulated HPA axis activity and glucocorticoid secretion.

4. FLIBANSERIN – A NOVEL PHARMACOTHERAPEUTIC APPROACH FOR THE TREATMENT OF HYPOACTIVE SEXUAL DESIRE DISORDER IN WOMEN

Recently, flibanserin (2H-benzimidazol-2-one, 1,3-dihydro-1-[2-[4-[3-(trifluoromethyl) phenyl]-1-piperazinyl]ethyl]), an agonist of 5-HT_{1A} and antagonist of 5-HT_{2A} receptors [86, 87], demonstrates the ability to stimulate female sexual behavior in rats [88] and to improve sexual desire in women with either HSDD or major depressive disorder [89-91]. The positive actions of flibanserin on sexual functioning were first discovered serendipitously while studying flibanserin in patients with major depressive disorder. Flibanserin is ineffective as antidepressant, but increases the scores of women on the Arizona Sexual Experiences Scale [89]. These findings led to further clinical evaluations of flibanserin. In phase-III clinical trials designed to investigate the efficacy of flibanserin as first pharmacotherapeutic treatment of HSDD in women (the *BOUQUET* studies), premenopausal women with HSDD report increased satisfying sexual events, increased desire and decreased distress following chronic flibanserin treatment over several weeks [90, 91]. With exception of the experiments described in this thesis (Chapters 2-5), flibanserin has not been tested in any nonhuman primate.

Flibanserin crosses the blood-brain barrier by diffusion, and brain exposure is therefore expected to be similar to that in blood and peripheral organs. In rodents, flibanserin shows functional preference for cortical 5-HT_{1A} and 5-HT_{2A} receptors over other brain regions [92, 93]. While both flibanserin and 8-OH-DPAT, a prototypical 5-HT_{1A} agonist [94, 95], decrease neuronal firing in the prefrontal cortex (PFC), this decrease is eliminated when the neurons of the dorsal raphé nucleus (DRN) are lesioned in 8-OH-DPAT treated rats, but not in flibanserin treated rats [96]. These findings provide evidence that while 8-OH-DPAT exerts its action on neuronal firing by binding to presynaptic 5-HT_{1A} receptors (autoreceptors), flibanserin seems to activate a postsynaptic mechanism as all 5-HT_{1A} autoreceptors on serotonergic neurons in the DRN have been eliminated by the lesion. In human brain tissue, flibanserin

activates 5-HT_{1A} receptors in the PFC and hippocampus, but does not activate presynaptic 5-HT_{1A} receptors in the DRN [92], thus confirming the postsynaptic binding preference of flibanserin. While in rats flibanserin stimulates female sexual behavior [88], it is of particular interest that 8-OH-DPAT induces the opposite effect and contrasts with flibanserin with regard to female sexual behavior [43, 44, 47].

5. MODELING FEMALE SEXUAL BEHAVIOR IN ANIMALS

Much of our understanding of female sexual function has been gained from the study of animal models that display nonhuman counterparts to desire, arousal, orgasm and satiety [97]. Importantly, these aspects of female sexuality can be assessed in animals both qualitatively and quantitatively. For example, the female rat displays a distinct set of behaviors, referred to as proceptive hops and darts [98], to solicit copulation from the male (sexual proceptivity), while others, such as lordosis, signal her readiness for copulation (sexual receptivity). Sexual proceptivity and receptivity behaviors are flexible in regard to frequency and intensity and can be altered by pharmacological manipulation, a critical prerequisite for a rational approach to pharmacotherapy for female sexual dysfunction.

In animals, sexual desire can be inferred from certain appetitive behaviors that occur during copulation. The occurrence of such behaviors can be experimentally tested and pharmacologically manipulated. For example, female rats that show high motivation to press a lever to gain access to a male, or to run back and forth from behind an opaque barrier, release increased DA in the striatum and nucleus accumbens compared to females with low motivation (reviewed in [97]). The rate at which female rats solicit and pace their copulatory contact with males is another example of an analogous behavior of desire. Pharmacological manipulation of melanocortin and serotonin receptors has been demonstrated to be effective in altering this pacing behavior in female rodents [41, 88]. Furthermore, the willingness to engage in sexual intercourse, expressed as lordosis in female rodents, is mediated by estrogen and progesterone, and modulated by hypothalamic dopamine, norepinephrine, oxytocin and opioid receptor activation [98].

Sexual arousal, as mentioned above in Section 2, additionally involves induction of generalized CNS arousal and genital arousal. While generalized CNS arousal is regulated by the neurotransmitters DA, NE, 5-HT and acetylcholine [99], the genital response includes increased vaginal blood flow, clitoral, labial and vestibular bulb engorgement, and vaginal smooth muscle contraction and relaxation, which are under parasympathetic (cholinergic) and

sympathetic (adrenergic, cholinergic) control and also influenced by vasoactive intestinal polypeptide (VIP), nitric oxide synthase (NOS), neuropeptide Y (NPY), and other mediators (see [97] for a comprehensive review).

Value and importance of animal models to study female sexual function have been recognized, though a vast majority of the studies that aimed to investigate the animal counterparts of human sexual behavior have been performed in rodents. The translation of rodent data to human behavior, however, entails problems. Unlike in humans, certain aspects of female sexual function in rodents are under strict control of hormonal status. Lordosis, for example, is a highly estrogen-dependent behavior. Ovariectomy of the female rat abolishes the behavior, while estradiol and progesterone [100, 101] or estradiol and testosterone replacement [102] restore proceptive and receptive sexual behavior.

In humans, and in primates generally, sex hormones play an important role in the modulation of sexual behavior. They are however not essential [103], and sex in women is abolished neither by menopause nor by a surgical removal of the ovaries [104]. As outlined in Section 1, sexual behavior in women is strongly associated with physical and emotional factors, such as feelings of happiness and satisfaction, quality of their relationship with the partner and lifestyle, and it is influenced by sociocultural, family and personal expectations [2-10]. Sexual behavior in women thus entails the net output of an interplay between hormonal, neural and psychosocial factors and involves, on the neurobiological level, hypothalamic and limbic brain structures that are under close cortical control. In this respect, nonhuman primate models of female sexual function may be a valuable and sensible alternative to rodent models due to their emancipation of sexual behavior from strict hormonal control [103, 105] and their phylogenetic proximity to humans, including advanced corticalization [106, 107]. Considering the advantages of nonhuman primates as animal models for female sexual function and dysfunction, the common marmoset monkey *(Callithrix jacchus)* was chosen as model system for this thesis.

6. THE COMMON MARMOSET MONKEY: A SENSIBLE CHOICE

Figure 5. The common marmoset *(Callithrix jacchus)*. Adult marmosets form stable male-female pair-bonds that are strengthened by affiliative interactions, including allogrooming. Photo: Ivan Mikšík

All experiments presented in this thesis, designed to explore the role of the central 5-HT neurotransmitter system in the regulation of female sexual function, were conducted in the common marmoset monkey (Figure 5). Suitability of the common marmoset to study exogenous compounds regulating female sexual behavior has previously been demonstrated [108]. Unlike the multiple-mating social structures of rats and most nonhuman primates, such as macaques and baboons, marmoset sexual behavior most commonly occurs within stable male-female pairs [109, 110]. The frequency and pattern of sexual behavior can vary greatly between marmoset pairs, as does the quality of their social interactions, as manifest by allogrooming and aggressive behavior [111]. Similar to humans, several aspects in the repertoire of marmoset sexual behavior are controlled by both partners. During most stages of the species-specific sequence of sexual behaviors, including sexual solicitation, acceptance and rejection of the partner's sexual advances, and the execution and permission of an ejaculatory mount, both male and female partner have the ability to promote or to interrupt the sexual interaction. As a further parallel to human sexual physiology, but in contrast to some nonhuman primate [112-115] and many non-primate species [116, 117], the female marmoset's display of sexual

behavior is not limited to the periovulatory period. While marmoset sexual behavior increases across the first half of the ovarian cycle, peaking just prior to ovulation, it also occurs throughout the ovarian cycle, reinforcing the bond between partners [118]. Ovariectomy reduces, but does not abolish sexual behavior or the female's sexual attractiveness to the male [119]. Lesion of the anteromedial hypothalamus, which overlaps with the mPOA, blocks sexual proceptivity in female marmosets, but not sexual receptivity [120]. Finally, the availability of a well-characterized behavioral ethogram of marmoset sexual proceptivity and sexual receptivity [121, 122] permits validated quantification of marmoset sexual behavior.

Female marmosets display sexual proceptivity by rapid protrusion and retraction of the tongue (proceptive tongue flick) and stares directed at the male pairmate while their ear tufts are flattened (proceptive stare). Receptive sexual behaviors include female acceptance of male mounts and mount attempts (accept mount), frequently by pressing herself to the ground in a frozen position (receptive freeze posture, Figure 6), thus permitting the male to intromit his erect penis (erection, intromission), to which the female commonly responds with a head turn and nuzzling or biting of the male (receptive head turn). A successful mount is completed by male ejaculation within the vagina, which is normally followed by the male licking his penis (ejaculation). Grooming behavior between partners (allogrooming) is normally a sign of a stable and close pair-bond between male and female pairmates, while aggressive interactions signal disagreement and conflict between partners. Male marmosets sniff and lick the ano-genital region of the female pairmate (ano-genital sniff/lick), thus obtaining sensory information of the female's ovarian cycle status [123]. Ano-genital sniff/lick also signals sexual interest by the male pairmate (adapted from [121, 122]).

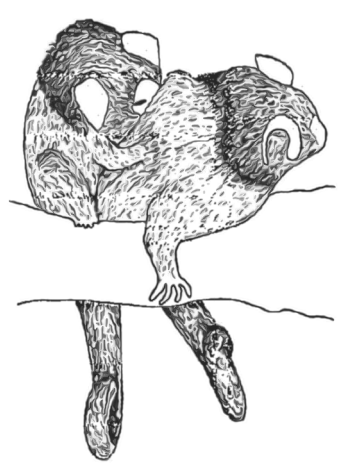


Figure 6. Marmoset copulatory behavior. The female partner (to the right) accpted the male partner's mount attempt. The male is in a mounting position, while the female's posture indicates a receptive freeze posture. Adapted from [121].

7. SCOPE OF THE THESIS

Objectives

The central theme of this thesis is the serotonergic regulation of female sexual behavior in a nonhuman primate that is characterized by a pairmate social setting comparable to humans. The experimental agents employed in the study are (1) flibanserin, a 5-HT1A agonist, 5-HT2A antagonist and putative pharmacotherapeutic treatment for hypoactive sexual desire disorder in women, and (2) 8-OH-DPAT, a prototype 5-HT1A agonist. In the thesis, flibanserin data are presented as they relate to two of the four Objectives key to this thesis (Objectives 1 and 2 in Chapters 2 and 3), while 8-OH-DPAT data are related to all four Objectives (Chapters 2 – 5).

The **First Objective** is to assess the effects of chronic flibanserin and 8-OH-DPAT on sexual and social interactions between female-male marmoset pairs. Based on the results from human clinical trials and rodent experimental data, we hypothesize that female marmoset sexual behavior is facilitated by flibanserin and diminished by 8-OH-DPAT.

The **Second Objective** is to test whether chronic flibanserin and 8-OH-DPAT alter the hypothalamic-pituitary-adrenal axis and thus indirectly modulate female sexual function through a suppressive endocrine mechanism. Central activation of 5-HT1A and 5-HT2A receptors stimulates the HPA axis. We consequently hypothesize that the 5-HT1A agonist 8-OH-DPAT increases HPA axis activity and thus suppresses female sexual behavior, while flibanserin, through concurrent inhibition of 5-HT2A receptors, displays a more moderate effect on HPA axis activity and therefore does not suppress female sexual behavior.

The **Third Objective** is to measure brain activity correlates of 8-OH-DPAT induced alterations of female sexual behavior and social interactions between pairmates. We hypothesize that chronic 8-OH-DPAT alters neural activity in brain regions with high 5-HT1A density, and in regions that have been associated with female sexual behavior: (1) dorsal raphé nucleus (DRN) with its serotonergic cell bodies, (2) medial prefrontal cortex (mPFC) as executive and inhibitory behavioral regulator, (3) medial preoptic area of the hypothalamus (mPOA) as a region essential for the generation of marmoset female sexual behavior, (4) ventromedial hypothalamic nucleus (VMH), a key regulator of female sexual receptivity, and (5) CA1 field of the hippocampus (CA1), involved in sexually relevant memory processes.

The **Fourth Objective** is to investigate the brain region-specific changes in gene expression induced by chronic 8-OH-DPAT to discover neural molecular mechanisms that may underlie suppressed female sexual behavior. We hypothesize that 8-OH-DPAT alters functional gene classes and pathways in

the same brain regions determined in objective 3: (1) DRN, (2) mPFC, (3) mPOA and (4) CA1. The VMH was not studied.

Experimental approach

Sixteen adult common marmoset females will be pair housed with male partners for 8-20 months before study onset. Eight of the 16 females will be assigned to test the effects of flibanserin, while the remaining 8 females will be assigned to test the effects of 8-OH-DPAT. Females will be housed with the same male partner for the entire study and will be ovariectomized and primed with either mid-follicular phase estradiol levels or no estradiol before study onset. Estradiol status will remain the same throughout the study for each individual animal, except for the final 16 weeks prior to brain tissue collection, when all females will be primed with estradiol to increase group size for the gene expression experiment. All in vivo experiments (behavior, Chapter 2; endocrine, Chapter 3; brain imaging, Chapter 4) will be conducted using a counterbalanced, cross-over design that will apply within-subject comparisons to measure the effect of chronic flibanserin and 8-OH-DPAT against respective vehicle treatment. Figure 7 shows a detailed visual representation of the complete study design. The transcriptomic experiment (Chapter 5) will employ a between-subject comparison to measure the effects of 8-OH-DPAT against vehicle treatment.

To investigate the effects of flibanserin and 8-OH-DPAT on pair behavior in **Chapter 2**, behavioral observations will be conducted using a validated experimental setup that encourages a standardized, modest baseline of sexual activity induced by a 90-minute separation of the pairmates prior

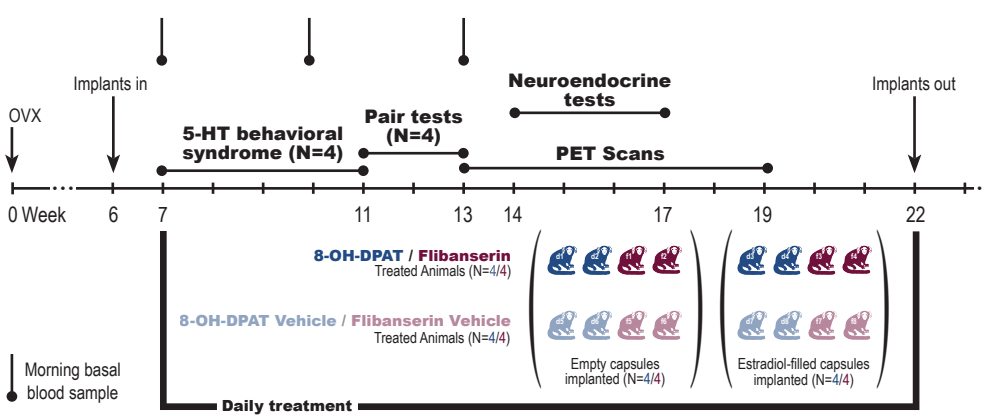
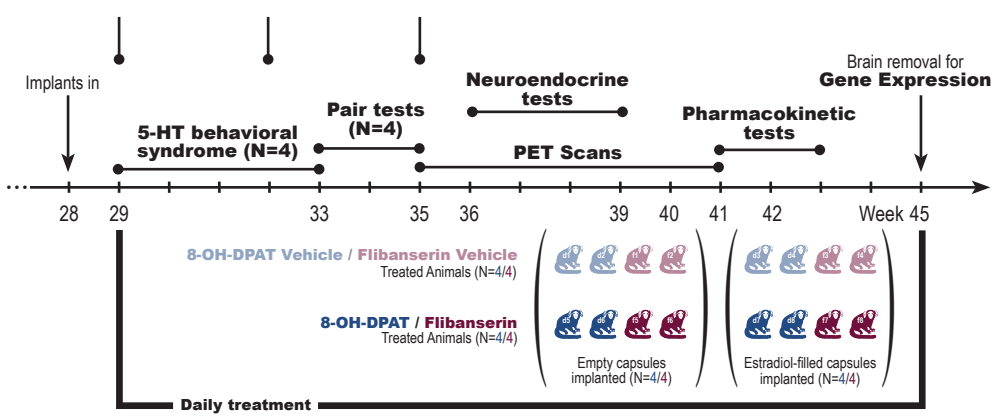


Figure 7. Study design. Drug treatment and estradiol replacement

to a 30-min pair test. In addition to sexual and social behavior observed during the pair tests, additional experiments described in this chapter will also explore behavioral responses to acute flibanserin and 8-OH-DPAT (serotonin behavioral syndrome), and the 24-hour pharmacokinetic profiles of circulating drug levels at 15 min, 30 min, 60 min, 3h, 6h and 24 h of drug administration during the chronic treatment phase.

Chapter 3 will assess female marmoset HPA axis properties following chronic treatment with flibanserin and 8-OH-DPAT. Baseline plasma cortisol levels will be measured before starting chronic treatments, and again after 3 and 6 weeks of daily treatment. Using a 5-HT1A agonist challenge test, it will be determined whether the responsiveness of the HPA axis to 5-HT1A activation will be affected by chronic 5-HT1A activation/5-HT2A inhibition (flibanserin), or by chronic 5-HT1A activation alone (8-OH-DPAT). To further test whether altered HPA axis function may contribute to changes in female interactions with male pairmates, a 30-minute restraint test will be administered to test the marmosets' stress responsiveness after chronic flibanserin and 8-OH-DPAT treatment. Timed blood samples will be drawn for all tests, and plasma ACTH and cortisol levels will be measured using radioimmunoassays.

To associate 8-OH-DPAT induced changes in pair behavior to brain function, a PET imaging experiment will be conducted in **Chapter 4**. Female marmosets will be infused with radiolabeled fluorodeoxyglucose (FDG) immediately prior to a pair test with their male pairmates. The majority of FDG uptake into brain tissue occurs within the first 30 minutes of infusion. The FDG signal will thus reflect glucose metabolism during pair behavior, with the most active brain regions trapping the highest concentration of radiolabeled FDG.



regimens are shown in relation to the timing of experimental events.

After pair test, the females will be anesthetized and imaged by PET. Whole brain normalized PET images will be analyzed with anatomically defined regions of interest derived from overlaid structural MRI images and scanned marmoset brain atlas images, and by whole brain voxelwise mapping. Finally, FDG signal differentials will be correlated with behavioral changes.

In a large-scale gene expression experiment described in **Chapter 5**, transcriptomic changes induced by chronic 8-OH-DPAT will be analyzed in brain tissues of 8-OH-DPAT and vehicle treated female marmosets. Due to great transcriptomic heterogeneity between brain regions, the mPFC, mPOA, CA1 and DRN will be excised using high-precision laser-microdissection. RNA will be isolated, amplified and hybridized to the marmoset-specific EUMAMA microarray. Real-time quantitative PCR (RT-qPCR) will be used to validate the microarray results, and a candidate gene approach will be employed to measure the expression of serotonin receptor and transporter genes by RT-qPCR. Enriched functional gene classes will be determined, possibly representing underlying molecular markers and mechanisms of suppressed female sexual function.

In **Chapter 6**, the combined experimental results from Chapters 2 – 5 will be considered and the abilities of flibanserin and 8-OH-DPAT to regulate female sexual function will be discussed in the context of pharmacological modes of action and future pharmacotherapeutic perspectives for the treatment of hypoactive sexual desire disorder in women.

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CHAPTER 2

Flibanserin and 8-OH-DPAT implicate serotonin in association between female marmoset monkey sexual behavior and changes in pair-bond quality

Yves Aubert[1,2], Morgan L. Gustison[1], Lindsey A. Gardner[1], Michael A. Bohl[1], Jason R. Lange[1], Kelly A. Allers[3], Bernd Sommer[3], Nicole A. Datson[2] and David H. Abbott[1]

[1]Wisconsin National Primate Research Center, University of Wisconsin-Madison, Madison, WI, USA; [2]Division of Medical Pharmacology, Leiden/Amsterdam Center for Drug Research, Leiden University Medical Centre, Leiden, The Netherlands; [3]Department of CNS Diseases, Boehringer Ingelheim, Biberach, Germany

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ABSTRACT

Introduction. Psychopathological origins of personally distressing, hypoactive sexual desire disorder (HSDD) in women are unknown, but are generally attributed to an inhibitory neural regulator, serotonin (5-HT). Flibanserin, a 5-HT_{1A} agonist and 5-HT_{2A} antagonist, shows promise as a treatment for HSDD.

Aim. To test the hypothesis that female marmoset sexual behavior is enhanced by flibanserin and diminished by 8-OH-DPAT, in order to evaluate the efficacy of serotonergic modulation of female sexual behavior in a pairmate social setting comparable to humans.

Methods. Sexual and social behavior were examined in eight female marmoset monkeys receiving daily flibanserin (15 mg/kg), 8-OH-DPAT (0.1 mg/kg), or corresponding vehicle for 15–16 weeks in a counterbalanced, withinsubject design, while housed in long-term, stable male–female pairs.

Main Outcome Measures. Marmoset pairmate interactions, including sexual and social behavior, were scored during weeks 5–6 of daily flibanserin, 8-OH-DPAT or vehicle treatment. 24-hour pharmacokinetic profiles of the drugs and their metabolites, as well as drug-induced acute symptoms of the 5-HT behavioral syndrome were also assessed.

Results. Two-way analysis of variance reveals that flibanserin-treated females attract more male sexual interest ($P = 0.020$) and trigger increased grooming ($P = 0.001$) between partners. In contrast, 8-OH-DPAT-treated females show increased rejection of male sexual advances ($P = 0.024$), a tendency for decreased male sexual interest ($P = 0.080$), and increased aggression with their male pairmates ($P = 0.049$).

Conclusions. While 8-OH-DPAT-treated female marmosets display decreased sexual receptivity and increased aggressive interactions with their male pairmates, flibanserin-treated female marmosets demonstrate increased affiliative behavior with their male pairmates. Such pro-affiliation attributes may underlie flibanserin's effectiveness in treating HSDD in women.

INTRODUCTION

In an estimated 10% of women [1], marked distressand interpersonal difficulty arise from unwanted, persistent or recurrent low sexual desire (hypoactive sexual desire disorder, HSDD; American Psychiatric Association's Diagnostic and Statistical Manual, DSM-IV-TR). Psychopathogenesis of HSDD is not known, but neurotransmitter dysfunction has been proposed involving the excitatory regulators dopamine (DA) and norepinephrine (NE), as well as inhibitory serotonin (5-HT) [2,3]. 5-HT is a key neurotransmitter involved in female sexual inhibition [2]. Pharmacological manipulation of 5-HT commonly results in diminished female sexual satisfaction and activity, particularly in women prescribed selective serotonin reuptake inhibitors (SSRIs) for depression [4]. Animal studies that apply 5-HT receptor subtype specific ligands permit mechanistic examination of 5-HTmediated effects on sexual behavior. There are seven known 5-HT receptor families, each with its own specific brain distribution, as well as effects on behavior and physiology [5]. For example, in rodents, the sexually receptive female lordosis posture is inhibited by 5-HT_{1A} receptor activation [6,7] and 5-HT_3 receptor antagonism [8], but is facilitated by 5-$HT_{2A/C}$ receptor activation [9].

Recently, flibanserin (2H-benzimidazol-2-one, 1,3-dihydro-1-[2-[4-[3-(trifluoromethyl) phenyl]- 1-piperazinyl]ethyl], an agonist of 5-HT_{1A} and antagonist of 5-HT_{2A} receptors [11,12], has been shown to stimulate sexual solicitation and receptivity in female rats [13] and to improve sexual desire in women with major depression [14]. Women with HSDD report increased satisfying sexual events, increased desire and decreased distress following flibanserin treatment [15]. *R*-(+)-8-hydroxy-2-(di-n-propylamino)-tetralin hydrobromide (8-OH-DPAT), however, a selective 5-HT_{1A} receptor agonist [16], diminishes female sexual receptivity in rats [17]. Thus, despite the shared 5-HT_{1A} agonist activity between flibanserin and 8-OH-DPAT, the contrasting effects of the two drugs on rodent female sexual behavior provides us with an opportunity to examine whether such contrasting behavioral outcomes translate to a nonhuman primate model, the common marmoset, in a well-established male– female pairmate social environment.

Female marmoset monkeys present an opportunity to contrast the effects of flibanserin and 8-OH-DPAT in an animal model that readily translates to humans because marmosets form and display modest amounts of sexual behavior [19]. Unlike the multiple-mating social structures of rats and many nonhuman primates, such as macaques and baboons, marmoset sexual behavior most commonly occurs within stable male–female pairs [18,20]. During acceptance or rejection of a pairmate's sexual advances, female marmosets can readily promote, prevent, or terminate sexual interactions [21],

and our recent development of a standardized behavioral testing paradigm permits repeatable, quantitative exploration of neurally active compounds that enhance or diminish female marmoset sexual behavior [19].

AIMS

The aim of the present study was to test the hypothesis that female marmoset sexual behavior is enhanced by flibanserin and diminished by 8-OH-DPAT, in order to evaluate the efficacy of serotonergic modulation of female sexual behavior in a pairmate social setting comparable to humans.

METHODS

Study animals

This study was conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act and its subsequent amendments. All animal procedures were reviewed and approved by the Graduate School Animal Care and Use Committee of the University of Wisconsin-Madison. The Wisconsin National Primate Research Center (WNPRC) is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care as part of the University of Wisconsin-Madison Graduate School. Sixteen adult (age 2–5 years) nulliparous captive-born common marmoset *(Callithrix jacchus)* females were pair housed with similarly aged male partners at the WNPRC for 8–20 months before onset of this study. Females were housed with the same male partner for the entire study, as previously described [22], and were ovariectomized and primed with either mid-follicular phase estradiol levels or no hormone [19] before study onset. This model allows us to provide a repeatable estrogen replete (estradiol capsules implanted) or estrogen deficient (empty capsules implanted) hormonal environment in which sex hormone levels are stable and reflect, respectively, the equivalent of an estrogen-dominant, pre-ovulatory stage in the ovarian cycle or a post-menopausal stage.

Experimental design

A counterbalanced, crossover study that applied within-subject comparisons was designed to examine the effects of chronic (15–16 weeks) daily (12:00pm–2:00pm) administration of flibanserin (N = 8; 15 mg/kg, orally (PO) in 1 mL/kg vehicle; Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach an der Riss, Germany), 8-OH-DPAT (N = 8; 0.1 mg/kg in 0.4 mL/kg vehicle, injected subcutaneously (SC); Sigma-Aldrich St. Louis, MO, USA), or

respective vehicles (for flibanserin, 98.5% of 0.5% hydroxycellulose solution and 1.5% of 1% polysorbate 80 solution, 1.0 mL/kg PO; for 8-OH-DPAT, 0.4 mL saline, SC). The study focused on two major behavioral outcomes: (i) sexual and social interactions between pairmates; and (ii) acute manifestation of the 5-HT behavioral syndrome providing quantitative indication of continued drug efficacy.

As flibanserin was not previously administered to female marmosets, we performed an initial dose response study comparing male–female interactions observed after 5–6 weeks of oral dosing of 10 mg/kg (N = 4) or 30 mg/kg (N = 4) flibanserin to behavior observed during baseline male–female interactions made prior to these flibanserin treatments. We observed changes in selected behaviors (Table S1) in our male–female testing paradigm, described in later discussion, 16–24 hours after daily flibanserin administration, compared to baseline. Blood samples assessing pharmacokinetics of both doses (Figure S1) were obtained after 3–4 weeks. As similar results were obtained from both doses for behavioral and pharmacokinetic assessments, we decided to employ an intermediate dose of 15 mg/kg for the main study. The scaled equivalent in humans of 15 mg/kg flibanserin administered to marmosets is ~2.4 mg/kg [23] and approximately similar to ~1.7 mg/kg flibanserin administered to women in clinical trials (100 mg/ day, assuming 60 kg body weight [24].

8-OH-DPAT has previously been administered to marmosets in i.p. injections of 0.3 mg/kg, resulting in pronounced expression of the serotonin behavioral syndrome immediately following each treatment. 8-OH-DPAT at a dose of 0.3 mg/kg also induces scratching and diarrhea [25]. To minimize the latter responses and to remain within a dose range previously used to consistently diminish female sexual behavior in rats (0.025–1.0 mg/kg, SC or IP [6,17,26], we selected 0.1 mg/kg SC for this study.

Bilateral ovariectomy

Females were injected intramuscularly (IM) with ketamine (15 mg/kg), 0.02–0.04 mg/kg atropine and 0.01 mg/kg buprenorphine, and were maintained on isofluorane (2%; 0.6 liter/min oxygen). Each ovary was isolated through a ventral midline incision and exteriorized for visualization of the fallopian tube and ovarian pedicle. Subsequent histological examination confirmed complete ovarian removal.

Estradiol replacement

One week before the start of daily treatment, females were implanted SC with silastic capsules that were either estradiol-filled (N = 4 per active compound/vehicle) or empty (N = 4 per active compound/vehicle). Plasma

estradiol levels were determined every 2 weeks whenever capsules were implanted. Treatment with active compound/vehicle started at (i) either 7 weeks after ovariectomy or 7 weeks after removal of capsules; and (ii) 1 week after implantation or re-implantation of capsules that occurred at 6 weeks after ovariectomy or 6 weeks after removal of previous capsules, resulting in a constant intertreatment interval of 7 weeks. Estradiol status was maintained throughout treatment for each female.

Estradiol levels in blood samples that were collected by femoral puncture [22] were determined using celite column chromatography and a validated estradiol radioimmunoassay (RIA) for marmoset plasma [27]. Assay sensitivity was 4.6 pg/tube (30.4 pg/mL), and intra- and inter-assay assay coefficients of variation (CVs), respectively, were 5.0% and 14.0%.

Ovariectomized females implanted with estradiol-filled capsules (N = 8) had higher ($P < 0.003$) circulating estradiol levels (396.0 30.6 pg/mL) compared to females implanted with empty capsules (67.5 5.2 pg/mL), and circulating estradiol values in the former females were comparable to those previously reported for female marmosets in the mid-follicular phase of the ovarian cycle [19]. Estradiol replacement is thus below the mid-cycle, peri-ovulatory levels used in some previous studies [28] and thus supports modest [19] rather than maximal [29] expression of female marmoset sexual behavior.

Pharmacokinetic assessment of flibanserin and 8-OH-DPAT administration

Treatment-induced systemic exposure to circulating levels of flibanserin and two common flibanserin metabolites, 1-(3-trifluoromethylphenyl) piperazine (TFMPP) and 6-hydroxy-flibanserin (BIMA 23 BS), and to 8-OH-DPAT, was assessed during study weeks 40 to 42 by validated high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Serial blood samples were collected at 0.25, 0.5, 1, 3, 6, and 24 hours after flibanserin or 8-OH-DPAT administration (N = 4 for each compound).

Behavioral observation of sexual and social behavior

In order to stimulate social and sexual interactions upon reunion [19], females andmales were separated for 90 minutes prior to each of four, 30-minute behavioral tests at 7:00am–1:00pm (16–24 hours after daily administration of active compound/ vehicle), 5–6 weeks after treatment onset. This time window for observations was chosen to assess the chronic changes induced by flibanserin and 8-OHDPAT when circulating levels of drugs were minimal, thus avoiding potential acute effects driven by elevated circulating concentrations of the drug preparations and active metabolites.

Potential behavioral changes are thus consequences of longterm adaptation to treatment that may involve changes in gene and protein expression [30].

At the start of the behavioral test, the male was introduced to the female by remote door operation, and behavior was manually and digitally recorded by two observers from behind a one-way window (Table 1). Behavioral tests were reanalyzed in a random fashion from the digitally stored recordings by two observers blinded with respect to treatment. The reanalyzed data were compared with those obtained on the day of the test to confirm final values and to generate behavioral data not originally scored during live observations (for aggression and self-grooming). Inter-observer reliability scores for behavioral data collection averaged 90.6%, and within-observer reliability scores averaged 96.1%.

Monitoring of the 5-HT behavioral syndrome

Locomotor behaviors indicative of the 5-HT behavioral syndrome, i.e., “random rapid limb movements” and “wet-dog shakes” [16,25], were monitored once per week (0.5 hour) at 0–0.5 hour and at 16–24 hours after administration of active compound/vehicle treatment, during weeks 1 to 4 of treatment (Table 1). Females were placed in a test cage for the duration of the test (8-OH-DPAT/vehicle), or returned to their home cage (flibanserin/vehicle). In addition, sprawling behavior (females lying down in prone position, monitored during flibanserin or respective vehicle treatment only), a possible non-locomotor component of the 5-HT behavioral syndrome [32], and self-scratching behavior, a locomotor behavior not specifically linked to 5-HT neurotransmission, were scored.

Data analysis

Observed levels of female proceptive sexual behavior (Table 1) were too infrequent to permit statistical analysis. Female sexual receptivity was quantified by frequency of female acceptance of male ejaculatory mounts. Female refusal of male sexual advances was quantified by frequency of rejection of male mounts and mount attempts. Sexual arousal of the male was quantified by frequency of penile erection. Analyses of all behavior were performed on transformed frequency data square root (1 + x)] to achieve homogeneity of variance and to increase linearity of data. This transformation generates positive numbers, permitting appropriate analysis of behavioral frequency data as square root transformation in which the variance is independent of the mean [33]. Mean values of the frequencies were analyzed by two-way anova incorporating repeated measures design, with Treatment (active compound, vehicle) and Observations (observation 1–4) as within-subject factors. Data

are presented as backtransformed mean values (95% confidence intervals). A P value less than 0.05 was considered significant.

Initial analyses of behavioral data were performed using the same mixed design anova with Estradiol supplementation and Order of treatment as between-subject factors, and Treatment as within-subject factor. As Estradiol supplementation and Order of treatment consistently failed to affect ($P > 0.05$) any behavioral variable, both factors were omitted in the final analyses reported here.

Table 1 Ethogram and mean ± SEM values for behavioral scores during pairmate tests

Behavior	Flibanserin vehicle	Flibanserin	8-OH-DPAT vehicle	8-OH-DPAT
Proceptive female sexual behavior*				
Proceptive tongue flicking	0	0.03 ± 0.06	0	0
Proceptive staring	0	0	0	0
Proceptive freeze posture/Sprawling position	0.16 ± 0.28	0.12 ± 0.14	0.05 ± 0.06	0.06 ± 0.09
Receptive female sexual behaviors*				
Acceptance of mounts	0.61 ± 0.58	0.75 ± 0.75	0.23 ± 0.27	0.64 ± 0.57
Rejection of mount attempts and mounts**	0.19 ± 0.28	0.25 ± 0.29	**0.62 ± 0.75**	**1.49 ± 1.50**
Receptive freeze posture	0.29 ± 0.28	0.45 ± 0.56	0.23 ± 0.27	0.58 ± 0.55
Receptive tongue flicking	0	0	0	0
Receptive head turning/biting	0.40 ± 0.35	0.35 ± 0.30	0.20 ± 0.23	0.29 ± 0.24
Male sexual behaviors*				
Penile erection	0.78 ± 0.65	1.12 ± 0.98	0.89 ± 0.73	1.05 ± 0.46
Mounting	0.61 ± 0.58	0.71 ± 0.69	0.23 ± 0.27	0.64 ± 0.57
Mounting attempts**	0.19 ± 0.28	0.26 ± 0.27	**0.63 ± 0.83**	**1.63 ± 1.59**
Intromitting	0.43 ± 0.37	0.48 ± 0.43	0.17 ± 0.20	0.35 ± 0.27
Ejaculating	0.28 ± 0.31	0.26 ± 0.27	0.17 ± 0.20	0.21 ± 0.15
Social odors*				
Genital investigation by female	0.03 ± 0.06	0.06 ± 0.08	0.28 ± 0.66	0.08 ± 0.17
Genital investigation by male¶	**1.76 ± 1.54**	**2.62 ± 1.89**	1.12 ± 0.74	0.49 ± 0.39
Scent marking by female	2.54 ± 2.53	3.15 ± 2.67	3.50 ± 3.95	4.36 ± 5.17
Scent marking by male	3.52 ± 4.71	3.39 ± 3.07	5.18 ± 5.23	3.69 ± 2.62
Social interactions*				
Grooming by female	1.29 ± 1.57	2.19 ± 2.15	2.77 ± 3.57	1.79 ± 2.60
Grooming by male¶	**2.21 ± 2.02**	**3.97 ± 3.27**	1.09 ± 0.57	0.76 ± 0.47
Total allogrooming¶	**3.94 ± 2.22**	**6.91 ± 3.20**	4.07 ± 3.74	2.71 ± 2.69
Aggression by female	0.94 ± 1.17	0.78 ± 1.07	0.92 ± 1.03	1.66 ± 1.76
Aggression by male	0.06 ± 0.08	0.17 ± 0.23	0.18 ± 0.18	0.53 ± 0.62
Total aggression**	0.99 ± 1.21	0.91 ± 1.27	**1.09 ± 1.14**	**2.23 ± 1.98**
Contact within arm-length	5.54 ± 1.23	5.42 ± 1.04	7.71 ± 2.04	6.27 ± 1.75
Direct body contact/huddling	8.69 ± 1.72	10.37 ± 2.60	9.42 ± 2.84	9.77 ± 2.48
Total contact	14.30 ± 2.07	15.87 ± 3.07	17.62 ± 1.55	16.19 ± 3.30
Initiating contact by female	11.14 ± 5.67	10.99 ± 3.41	20.13 ± 8.08	16.99 ± 7.84
Breaking contact by female	15.60 ± 5.99	15.03 ± 7.71	21.09 ± 6.46	14.27 ± 7.20
Following by female	1.60 ± 1.66	1.67 ± 1.39	3.00 ± 2.52	2.60 ± 2.33
Avoiding contact by female	1.69 ± 1.15	1.53 ± 1.22	1.60 ± 1.00	1.15 ± 0.61
Self-directed behavior by female*				
Self-grooming¶	**2.75 ± 1.86**	**3.96 ± 1.95**	1.00 ± 0.55	1.89 ± 1.25
Genital inspection/Genital self-grooming¶	**1.87 ± 1.58**	**4.18 ± 2.95**	0.45 ± 0.43	0.33 ± 0.23
Scratching	12.74 ± 8.73	11.50 ± 4.94	8.44 ± 5.09	10.95 ± 3.56
Locomotion and movement by female*				
Mobile in locomotion	2.50 ± 0.74	1.93 ± 0.53	3.07 ± 1.15	2.39 ± 1.03
Mobile while stationary	4.45 ± 1.55	5.33 ± 1.64	4.06 ± 1.95	4.14 ± 1.41
Mobile total	6.97 ± 2.14	7.29 ± 1.92	7.25 ± 2.47	6.67 ± 1.63
Immobile, incl. sprawling position	22.72 ± 2.09	22.47 ± 1.77	22.33 ± 2.59	23.14 ± 1.59
Acute behavioral changes†				
Scratching*	7.32 ± 2.40	5.23 ± 2.32	1.50 ± 0.90	5.56 ± 4.49
Random rapid limb movements‡**	0	0	0	**6.89 ± 4.32**
"Wet-dog" shakes‡**	0.24 ± 0.16	0.11 ± 0.18	0	**17.44 ± 11.90**
Latency to sprawling position§¶	**19.47 ± 4.19**	**14.58 ± 4.71**	Not scored	Not scored

A zero value indicates complete absence of behavior. Bold numbers indicate significant changes compared to vehicle treatment. 8-OH-DPAT, *R*-(+)-8-hydroxy-2-(di-n-propylamino)-tetralin

*Adapted from Stevenson and Poole [21]. Behavior was recorded at 16–24 hours after daily drug/vehicle administration during weeks 5–6 of daily treatment

†Behavior was recorded at 0–0.5 hours after drug/vehicle administration during weeks 1–4 of daily treatment

‡Behavior indicative of the 5-HT behavioral syndrome. Elliott et al. [25]

§Behavior resembling the rodent flat-body posture. Tricklebank et al. [31]

¶Significantly altered by flibanserin administration ($P < 0.05$)

**Significantly altered by 8-OH-DPAT administration ($P < 0.05$)

MAIN OUTCOME MEASURES

Marmoset pairmate interactions, including sexual and social behavior, were scored atweeks 5–6 of daily flibanserin, 8-OH-DPAT or vehicle treatment. In addition, 24-hour pharmacokinetic profiles of flibanserin, TFMPP, BIMA 23 BS, and 8-OH-DPAT, as well as drug-induced acute symptoms of the5-HT behavioral syndrome, were assessed.

RESULTS

Systemic exposure to flibanserin, flibanserin metabolites, and 8-OH-DPAT during chronic drug treatment

Maximal plasma concentrations of flibanserin, TFMPP and BIMA 23 BS (C(max)) of 628 ± 225 ng/mL (mean ± SEM), 46.0 ± 17.0 ng/mL and 149 ± 38.7 ng/mL, respectively, were reached at 0.5–1 hour after oral administration, decreasing rapidly to low levels by 24 hours, providing a circulating half-life for flibanserin of 2.8 hours (Figure 1). For 8-OH-DPAT, C(max) of 69.7 ± 19.8 nmol/L was reached by 0.25–0.5 hour after SC injection, and values dropped rapidly to 3.4 ± 2.6 nmol/L after 3 hours, providing a circulating half-life of 8-OH-DPAT of 0.6 hour (Figure 1). No cumulative effects of chronic 8-OH-DPAT and

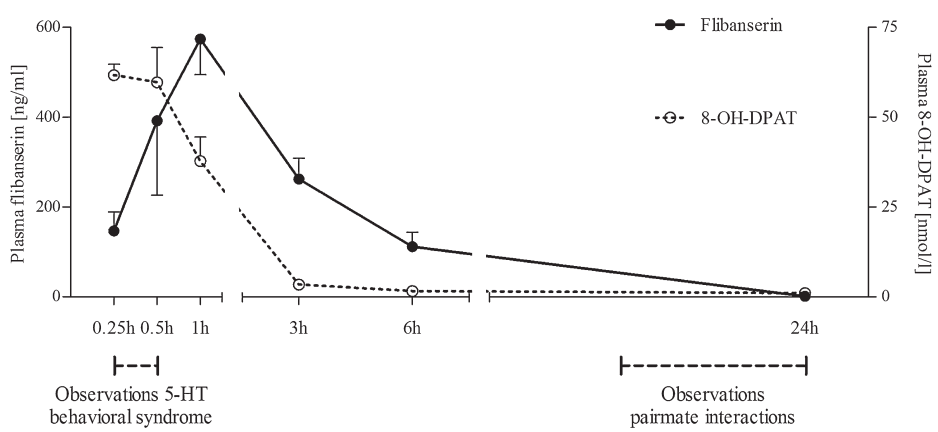

Figure 1. 24-hour pharmacokinetic profiles of flibanserin and 8-OH-DPAT in female marmoset monkeys. Pairmate observations were performed at 16-24 hours after administration, when exposure to circulating drug concentrations was low or absent, while observations of the 5-HT behavioral syndrome was performed at 0-0.5 hour after administration, when circulating drug concentrations were high. 8-OH-DPAT, *R*-(+)-8-hydroxy-2-(di-n-propylamino)-tetralin; 5-HT, serotonin.

flibanserin administration were apparent. The results confirm a substantial systemic exposure to flibanserin, BIMA 23 BS, TFMPP and 8-OH-DPAT during observations for 5-HT behavioral syndrome, but not during pairmate observations. Thus, treatment-induced changes during pairmate observations are most likely attributable to the long-term consequences of flibanserin and 8-OH-DPAT exposure, and not to acute effects elicited by elevated circulating levels of active drug.

Chronic effects of 8-OH-DPAT and flibanserin on sexual, social and self-directed behavior

Five to six weeks of daily oral administration of flibanserin to female marmosets noticeably increased female genital area self-grooming ($F(1,7) = 31.28$, $P = 0.001$; Figure 2A) and male pairmate sniffing/licking of their female's genital area ($F(1,7) = 8.91$, $P = 0.020$; Figure 2B). No other sexually related behavior was altered by flibanserin (Table 1). In contrast, 5–6 weeks of daily SC administration of 0.1 mg/kg 8-OH-DPAT strikingly increased female rejection of male pairmate mount attempts and mounts compared to corresponding vehicle ($F(1,7) = 8.24$, $P = 0.024$; Figure 3). 8-OH-DPAT-induced female rejection of male sexual advances also increased male attempts to mount their female pairmate ($F(1,7) = 6.93$, $P = 0.034$). The male pairmates

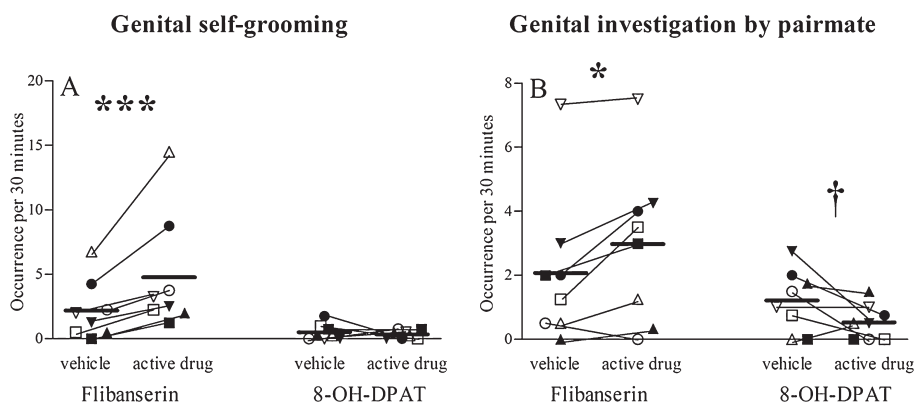

Figure 2. Investigation of female genital area. Frequencies (backtransformed mean) of **(A)** female genital area selfgrooming and **(B)** male inspection of female genital area per 30 minutes during 5–6 weeks following the onset of flibanserin, flibanserin vehicle, 8-OH-DPAT, or 8-OH-DPAT vehicle administration. *** $P = 0.001$ vs. flibanserin vehicle ($F(1,7) = 31.3$), * $P = 0.020$ vs. flibanserin vehicle ($F(1,7) = 8.9$), † $P = 0.080$ vs. 8-OH-DPAT vehicle ($F(1,7) = 4.2$). Each symbol indicates the same individual female marmoset receiving estradiol (solid symbols) or no estradiol (open symbols). 8-OH-DPAT, *R*-(+)-8-hydroxy-2-(di-n-propylamino)-tetralin.

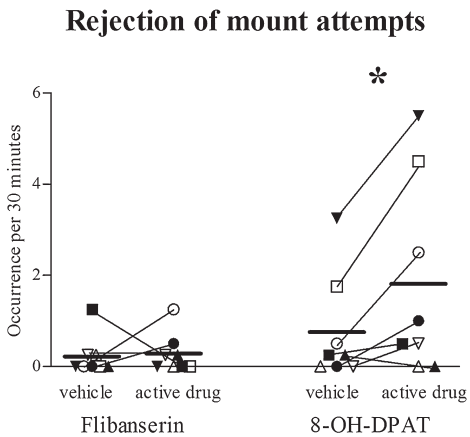

Figure 3. Female sexual rejection. Frequency (backtransformed mean) of female rejection of male mounts and mount attempts per 30 minutes during 5–6 weeks of flibanserin, flibanserin vehicle, 8-OH-DPAT, or 8-OH DPAT vehicle administration. * P = 0.024 vs. 8-OH-DPAT vehicle (F(1,7) = 8.2). Each symbol indicates the same individual female marmoset receiving estradiol (solid symbols) or no estradiol (open symbols). 8-OH-DPAT, *R*-(+)-8-hydroxy-2- (di-n-propylamino)-tetralin.

of 8-OH-DPAT treated females also tended to sniff/lick their female pairmate's genital area less (F(1,7) = 4.20, P = 0.080; Figure 2B), and decreased male genital sniffing was correlated with female rejection of male mount attempts and mounts (r = -0.747, P = 0.033). No other sexually related behavior was altered (Table 1).

Such opposing effects of flibanserin and 8-OHDPAT were also observed in social interactions between male and female pairmates. Flibanserin increased

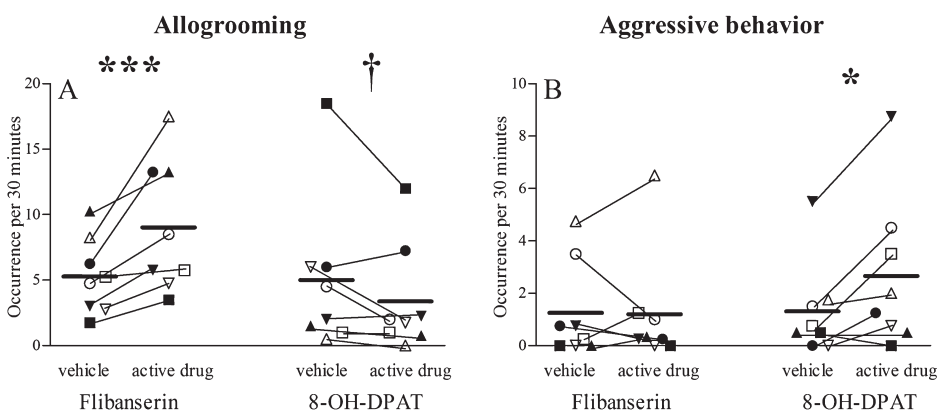

Figure 4. Pairmate allogrooming and aggression. Frequencies (backtransformed mean) of **(A)** allogrooming and **(B)** aggressive interactions between pairmates per 30 minutes during 5–6 weeks of flibanserin, flibanserin vehicle, 8-OH-DPAT, or 8-OH-DPAT vehicle administration. *** P = 0.001 vs. flibanserin vehicle (F(1,7) = 34.2), † P = 0.079 vs. 8-OH-DPAT vehicle (F(1,7) = 4.2), * P = 0.049 vs. 8-OH-DPAT vehicle (F(1,7) = 5.6). Each symbol indicates the same individual female marmoset receiving estradiol (solid symbols) or no estradiol (open symbols). 8-OH-DPAT, *R*-(+)-8-hydroxy-2-(di-n-propylamino)-tetralin.

allogrooming between pairmates (F(1,7) = 34.25, P = 0.001; Figure 4A), mostly male grooming of their female pairmates (F(1,7) = 6.25, P = 0.041). 8-OH-DPAT, in contrast, tended to diminish allogrooming between males and females (F(1,7) = 4.23, P = 0.079; Figure 4A), including a trend toward diminished male grooming of female pairmates (F(1,7) = 4.92, P = 0.062). Frequency of aggressive interactions between the pairmates was increased by 8-OH-DPAT (F(1,7) = 5.65, P = 0.049; Figure 4B), but not by flibanserin (F(1,7) = 0.09, P = 0.778). This 8-OH-DPAT induced increase in aggression positively correlated with increased female rejection of male mount attempts and mounts (r = 0.941, P < 0.001), and correlated negatively with male genital sniffing (r = -0.834, P = 0.010). No other changes in social behavior were induced by flibanserin or 8-OH-DPAT (Table 1).

Female self-grooming behavior was increased by flibanserin (F(1,7) = 7.13, P = 0.032), but not by 8-OH-DPAT (F(1,7) = 3.02, P = 0.126). There were no other behavioral effects of either drug on self-directed behaviors (Table 1). Estradiol supplementation, in both 8-OH-DPAT and flibanserin groups, was without effect on pairmate behavior.

Effects of flibanserin and 8-OH-DPAT on acute induction of the 5-HT behavioral syndrome

During pairmate observations to assess sexual and social behavior (7:00am–1:00pm, 16–24 hours following daily drug administration), neither 8-OH-DPAT nor flibanserin resulted in females displaying symptoms of the potentially disruptive 5-HT behavioral syndrome.

Directly (0–0.5 hour) following administration, however, flibanserin shortened the latency to the first occurrence of sprawling or prone behavior (Figure 5, F(1,7) = 11.90, P = 0.011), without affecting the frequency of this behavior (F(1,7) = 0.44, P = 0.528). Furthermore, in contrast to 8-OH-DPAT, flibanserin first increased (weeks 1–3) female scratching behavior 0–0.5 hour

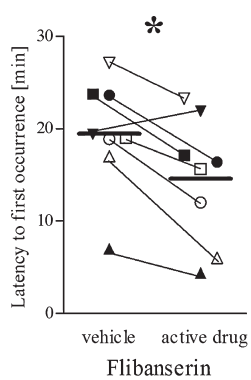

Figure 5. Sprawling behavior. Latency (minutes; backtransformed mean) to the first occurrence of sprawling behavior by female marmosets during pairmate observation after 5–6 weeks of flibanserin or flibanserin vehicle. * P = 0.011 vs. flibanserin vehicle (F(1,7) = 11.90). Each symbol indicates the same individual female marmoset receiving estradiol (solid symbols) or no estradiol (open symbols).

after administration (F(3,21) = 5.64, P = 0.005), and then diminished scratching behavior over the remaining weeks of flibanserin treatment compared to corresponding vehicle treatment (Treatment × Week interaction [F(3,21) = 7.21, P = 0.002]).

In contrast to flibanserin, 8-OH-DPAT consistently induced locomotor components of an acute, transient 5-HT behavioral syndrome in female marmosets 0–0.5 hour after administration, which persisted over the entire time course (15–16 weeks) of chronic treatment. In 8-OH-DPAT-treated female marmosets, the acute 5-HT behavioral syndrome involved displays of "random rapid limb movements" (Figure 6A, F(1,7) = 16.67, P = 0.005) and "wet-dog shakes" (Figure 6B, F(1,7) = 19.81, P = 0.003). In addition, frequency of scratching behavior tended to be elevated 0–0.5 hour after 8-OH-DPAT administration (Figure 6C, F(1,7) = 5.46, P = 0.052), but there was aTreatment × Week interaction (F(1,7) = 15.39, P < 0.001). Post hoc analysis indicated that female marmosets demonstrated an acute onset of scratching behavior during the first week of 8-OH-DPAT treatment (week 1), but not during subsequent weeks. As found with 5-HT behavioral syndrome behaviors, there was no effect of 8-OH-DPAT treatment on scratching behavior at 16–24 hours following drug administration (F(1,7) = 1.75, P = 0.227) when pairmate behavioral observations were conducted.

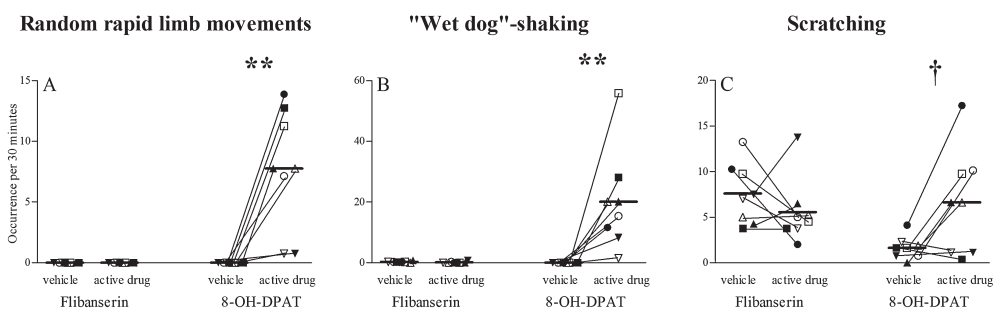

Figure 6. Acute induction of the 5-HT behavioral syndrome. Frequency (backtransformed mean) of female **(A)** "rapid, random limb movements" behavior **(B)** "wet dog shake" behavior, and **(C)** scratching behavior per 30 minutes during 5–6 weeks of flibanserin, flibanserin vehicle, 8-OH-DPAT, or 8-OH-DPAT vehicle and following 0–0.5 hours of treatment administration. ** P < 0.01 vs. 8-OH-DPAT vehicle ("rapid, random limb movements": F(1,7) = 16.7; "wet dog shakes": F(1,7) = 19.8), † P = 0.052 vs. 8-OH-DPAT vehicle (F(1,7) = 5.46). Each symbol indicates the same individual female marmoset receiving estradiol (solid symbols) or no estradiol (open symbols). 8-OH-DPAT, *R*-(+)-8-hydroxy-2-(di-npropylamino)- tetralin.

DISCUSSION

Despite the prevalence of HSDD among women [1,3], its psychopathogenesis is unknown. Pharmacological manipulation of 5-HT, however, commonly induces an HSDD-like condition, especially in women chronically treated with SSRIs for depression [4], likely involving 5-HT_{1A} receptors [34]. Flibanserin, a 5-HT_{1A} postsynaptic receptor agonist and 5-HT_{2A} antagonist, presents a pharmacological opportunity to ameliorate psychosexual distress in women. Flibanserin improves sexual desire in women with major depression [14], and in women with HSDD it has been demonstrated to increase satisfying sexual events, sexual desire, and decreases distress [15]. Female marmosets, already established as a mechanistic model for neural regulation of female sexual behavior [19,28], form stable, long-term, male–female relationships [18] that allow for the examination of the pharmacological efficacy of flibanserin with regard to female sexual behavior.

Chronic administration of flibanserin to female marmoset pairmates stimulates male inspection of female genital area and increases female genital self-grooming. Female sexual behavior, however, is otherwise unaltered, possibly because we employ well-established male–female pairs without a history of infrequent-to-absent sexual behavior. Enhancement of proceptive and receptive aspects of female marmoset sexual behavior can be quantified using our behavioral testing paradigm, as previously shown following administration of gonadotropin releasing-hormone II and respective analogues [19]. In contrast, 8-OH-DPAT (a 5-HT_{1A} pre- and postsynaptic receptor agonist) administered to female marmoset pairmates tends to diminish male inspection of female genitalia and increases female rejection of male-initiated sexual advances, thus diminishing female sexual receptivity toward their long-standing male pairmate. The comparability of behavioral responses in both estrogen and “no hormone replaced” ovariectomized female marmosets used in this study suggests applicability of our results to both pre- and post-menopausal conditions in women, as found in a previous marmoset study [19].

The differential effects of flibanserin and 8-OH-DPAT on female marmoset sexual behavior resemble findings in female rats. Female rats given flibanserin increase expression of proceptive and receptive sexual behavior and receive increased genital sniffing by the male pairmate [13], whereas female rats given 8-OH-DPAT exhibit diminished female sexual receptivity [17]. These contrasting effects of flibanserin and 8-OH-DPAT on marmoset and rodent behavior are surprising since both compounds are described as 5-HT_{1A} agonists. However, while both flibanserin and 8-OH-DPAT activate postsynaptic 5-HT_{1A} receptors, flibanserin is functional only as a postsynaptic

receptor agonist [15], whereas 8-OH-DPAT activates both pre- and postsynaptic 5-HT_{1A} receptors. This difference in biological action results in fundamental differences in pharmacology and the abilities of flibanserin and 8-OH-DPAT to induce functional changes in a brain region-specific manner. For example, flibanserin inhibits forskolin-stimulated cAMP formation in the cortex, while 8-OHDPAT does not affect cortical cAMP accumulation [10]. Flibanserin decreases neuronal firing rate in the rat cortex regardless of whether the presynaptic receptor-containing dorsal raphé nucleus is intact, while the effects of 8-OH-DPAT are dependent upon intact raphé serotonergic neurons [11]. Taken together, flibanserin and 8-OH-DPAT display a different regional selectivity, and they differentially affect neuronal function in 5-HT projection sites.

These functional differences are particularly evident in pyramidal neurons in the prefrontal cortex, a key site for flibanserin's mode of action in affecting female sexual behavior, as these neurons are an important part of regulatory networks that coordinate the release of 5-HT, DA and NE in a brain region-specific manner [15]. Additional 5-HT_{2A} antagonist effects of flibanserin, which 8-OH-DPAT lacks, have been shown to enhance 5-HT_{1A} agonist effects on pyramidal neurons of the prefrontal cortex, thus creating a biochemical environment in flibanserin-exposed prefrontal cortex that is clearly different from 8-OH-DPAT exposure. 8-OH-DPAT is thus not likely to directly affect cortical neurocircuitries [10], but will suppress female sexual behavior by acting on 5-HT_{1A} receptors in postsynaptic hypothalamic areas, such as the ventromedial hypothalamus [35] and medial preoptic area [36], or in presynaptic raphé nuclei [37]. Thus,flibanserin's effects on female sexual behavior are likely mediated by altering cortical neurocircuitries, while 8-OH-DPAT likely inhibits female sexual behavior by activation of hypothalamic or midbrain 5-HT_{1A} receptor populations. The inhibitory effects of 8-OH-DPAT occur immediately and do not require chronic administration in rats [6]. Chronic administration seems not to lead to either tolerance or sensitization of sexual behavior, at least in male rats [38]. Flibanserin, in contrast, might regulate female sexual behavior by affecting pyramidal neurons in the prefrontal cortex and establishing a new monoamine neurotransmitter balance in a brain region-specific manner [15]. Chronic elevations in prefrontal cortex levels of DA and NE in rats are reported after 21 days of repeated flibanserin administration, consistent with the duration needed for flibanserin to facilitate female sexual behavior in rats [13].

Flibanserin's enhancement of interest in treated female marmosets' genitals is reminiscent of its effects when administered twice daily to female rats (45 mg/kg, PO) for 2–3 weeks. Flibanserintreated female rats attract increased male

inspection of their genital area [13]. In both rats and marmosets, female genital odor is important for activation of male sexual arousal [rat: [39]; marmoset: [40]], particularly, female genital odor from the peri-ovulatory period when female sexual proceptivity and receptivity are maximal [rat: [41]; marmoset: [42]]. 5-HT regulates likely mediators of genital olfactory attractiveness, such as oxytocinergic (OT) and vasopressinergic (AVP) neurons [43] that innervate female external genitalia, possibly in conjunction with the central DA neurotransmitter system [44]. Neuro-regulated changes in vaginal odor may also be a consequence of 5-HT-mediated alterations in genital vasodilatation [44] or salt-water regulation [45]. Increased female interest in genital self-grooming and selfgrooming, in general, could involve 5-HT mediated changes in OT, as central administration of OT (and AVP) to female rats increases selfgrooming, particularly of the genital area [46].

Perhaps the most intriguing finding of this marmoset study, the opposing effects of flibanserin and 8-OH-DPAT on the quality of male–female pair interactions, raises the possibility that female sexuality is strongly influenced by the perceived quality of the relationship with their partner (e.g., [3,47]). Social attachment and maintenance of proximity, allogrooming and pair-bonding, function to facilitate reproduction [48] and sexual behavior may contribute toward maintenance of a close male–female relationship [49]. In marmosets, affiliative behavior between females and males co-occurs with sexual behavior, while aggressive behavior between pairmates impedes sexual interaction [50,51]. Thus, the aggression-inducing effect of 8-OH-DPAT and the affiliation-enhancing effect of flibanserin may create relationship environments that either diminish or facilitate sexual interactions between marmoset pairmates, respectively, likely mediated by neurotransmitter systems implicated in the regulation of social behavior, such as 5-HT [52], DA [53], AVP and OT [54,55]. Flibanserin selectively activates postsynaptic 5-HT_{1A} receptors while antagonizing 5-HT_{2A} receptors [11]. Gerretsen et al. [56] recently reported a negative association between the desire for social relationships in humans and 5-HT_{2A} binding in the prefrontal cortex. Furthermore, flibanserin's distinct receptor binding profile induces long-lasting increases in basal DA in the prefrontal cortex, while not changing basal levels in other tested brain areas, including the hypothalamus and nucleus accumbens [57,58]. If replicated in flibanserin-treated female marmosets, such increases in prefrontal cortex DA would be expected to enhance prefrontal cortex-guided attention to male behavioral cues and to more readily elicit female responses based on previous experience [58]. Improved efficiency of DAmediated neural processing is proposed as one aspect of therapeutic efficacy for a variety of drugs that improve disorders of PFC dysfunction, possibly including HSDD [59,60].

Beyond neurobiological factors that influence sexual function, several studies, including the National and Social Life Survey [61], point out that sexual behavior in women is closely linked to psychosocial factors and quality of their relationship with a partner [47]. The complex interplay of these factors with hormonal and neural environments is a challenge for any animal model of female sexual function. The translation of rodent findings to humans, for example, is difficult in light of their strict circadian hormonal control of female sexual function [62]. Studies in non-primate mammals are thus not likely able to capture the multifactorial environment in which sexual behavior is expressed in nonhuman primates and humans [63]. In contrast, our observations in the marmoset monkey emulate the human situation more closely and highlight the importance of pairbond quality in female sexual behavior. Our findings are likely based within an important set of marmoset-typical characteristics, including welldeveloped social behavior, long-term female–male pairings and a degree of emancipation of female sexual behavior from strict hormonal control [28] that permit a significant influence of the social environment on the shaping of sexual behavior.

The strong and consistent, but transient, expression of a 5-HT behavioral syndrome induced in female marmosets by 8-OH-DPAT administration is likely mediated by activation of postsynaptic 5-HT_{1A} receptors [64,65]. Flibanserin, in contrast, does not elicit shaking or rapid limb movements despite its postsynaptic 5-HT_{1A} agonist characteristic. This may be due to flibanserin's 5-HT_{2A} antagonist property, as 5-HT_{1A} and 5-HT_{2A} receptors are functionally linked and show reciprocal modulation [66]. Indeed, pretreatment with 5-HT_{2A} antagonists reduces 8-OH-DPAT-induced 5-HT behavioral syndrome in rats [31]. It is possible that flibanserin increases the expression of brain-derived neurotrophic factor (BDNF) in the cerebral cortex and hippocampus, which, in rats, diminishes the 5-HT behavioral syndrome induced by 8-OH-DPAT [67]. A shortened latency to sprawl induced by flibanserin may be equivalent to the flat-body posture component of the 5-HT behavioral syndrome [32]. Sprawling, however, is also commonly observed when marmosets solicit allogrooming from a partner [21]. Thus, a shortened latency to sprawling behavior may indicate another pro-social effect of flibanserin rather than a flibanserin-induced component of the 5-HT behavioral syndrome. Neither sprawling in flibanserin treated females, nor the 5-HT behavioral syndrome displayed by 8-OH-DPAT treated females, are likely related to 5-HT toxicity (or 5-HT syndrome) described in humans, a potentially life-threatening condition induced by excess 5-HT [68]. 5-HT toxicity can result from combinations of antidepressant treatments, such as monoamine oxidase inhibitors (MAOIs) and SSRIs, causing clonus (involuntary muscle contraction and relaxation)

and hyperthermia [68] apparently due to 5-HT_2 receptor-mediated effects [69]. Activation of 5-HT_{1A} receptors, in contrast, commonly leads to hypothermia in rodents and humans [70,71]. Flibanserin, a 5-HT_{1A} agonist and 5-HT_{2A} receptor antagonist, and 8-OH-DPAT, a 5-HT_{1A} agonist, are thus both unlikely to cause hyperthermic responses. Furthermore, both flibanserin and 8-OH-DPAT administered to rodents selectively decrease 5-HT synthesis in several brain regions [72], further indicating the unlikelihood of either contributing to 5-HT toxicity.

Similar to a previous observation indicating that low-level estradiol supplementation is not required for the efficacy of gonadotropin-releasing hormone II to stimulate sexual behavior in ovariectomized female marmosets [19], our present study, applying the same design in regard to estradiol replacement, demonstrates that behavioral changes due to chronic flibanserin and 8-OH-DPAT treatments are independent of estradiol status. This finding might be somewhat surprising in light of welldocumented interactions of estrogens with the central serotonin system [73] and contrast with a previous study conducted by Kendrick and Dixson [29]. Kendrick and Dixson [29], however, applied high levels of estradiol supplementation equivalent to pre-ovulatory peak levels (~940 pg/mL). The much lower circulating estradiol levels in our study (average with estradiol supplementation: 396 pg/mL), chosen to facilitate a low to modest baseline of sexual behavior, may be responsible for the lack of obvious estradiol-induced behavioral changes in the present study.

CONCLUSIONS

Our findings are the first to demonstrate differential and potentially bi-directional regulation of female sexual behavior (diminished versus unchanged) and social behavior (diminished versus enhanced) in a nonhuman primate through prolonged serotonergic modulation, supporting the central 5-HT system as a promising target for pharmacotherapy of sexual dysfunction in women. Our model applies species-appropriate settings in which female partners can control sexual and social interactions. We show that female marmoset sexual behavior is suppressed by selective chronic stimulation of 5-HT_{1A} receptors by 8-OH-DPAT. In contrast, through its distinctively different 5-HT receptor binding profile, and potential additional abilities to enhance DA levels (and possibly other neurotransmitters) in a brain region specific manner [57,58], flibanserin increases pro-social interactions in male and female marmoset pairmates without obvious enhancement of female sexual behavior. While these findings suggest that flibanserin's effects may not translate from female marmosets to women in every respect, they do show that flibanserin

enhances species-specific, intimate aspects of partner interactions. These are manifest by increased intimate affiliative engagement between marmoset pairmates and improved sexual relationships between women and their partners [14]. Thus, the putative beneficial effect of flibanserin on sexual well-being in women, otherwise distressed about low sexual desire, may arise from its ability to positively influence the quality of relationship with long-term partners. Our observations of female marmosets may therefore suggest the need to integrate mechanistic, neurochemical explanations of female sexual behavior with holistic views of health psychology, including emotional and relational aspects of female sexuality, in order to successfully contribute novel therapeutic approaches to female sexual well-being.

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SUPPORTING INFORMATION

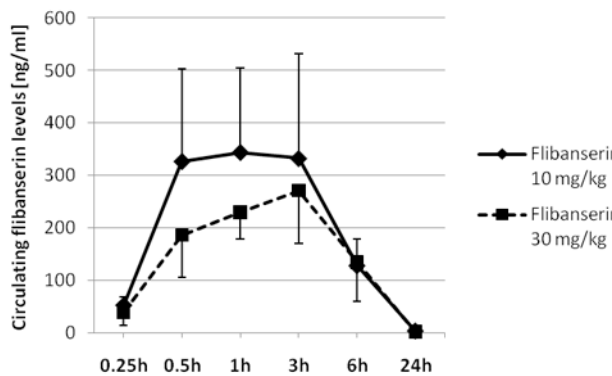

Figure S1. 24-hour pharmacokinetic profiles of 10 mg/kg and 30 mg/kg flibanserin in female marmoset monkeys. Flibanserin was administered PO at doses of 10 mg/kg or 30 mg/kg. Blood samples were taken at 0.25 h, 0.5 h, 1 h, 3 h, 6 h and 24 h after flibanserin administration and analyzed for circulating flibanserin concentrations. Flibanserin doses did not significantly differ ($P > 0.05$; $N = 4$).

Table S1. In a dose-response study, the behavioral effects of 10 mg/kg or 30 mg/kg flibanserin, administered to female marmosets, were compared to respective baseline behavior of each individual. Difference between flibanserin doses were assessed by three-way anova including the dose of flibanserin as between-subject factor.

	Baseline (all)	Flibanserin (all)	Baseline 10	Flibanserin 10	Baseline 30	Flibanserin 30	P-value[1)]
Affiliative Behavior[2)]							
Female Grooms Male	1.72 ± 0.58	2.91 ± 0.76	0.56 ± 0.24	1.81 ± 0.74	2.88 ± 1.07	4.00 ± 1.30	0.037
Female Initiates Contact with Male	10.41 ± 1.05	8.94 ± 1.23	8.81 ± 1.83	7.13 ± 1.59	12.00 ± 0.96	10.75 ± 1.81	n.s.
Female Terminates Contact with Male	18.59 ± 1.80	14.94 ± 1.98	15.00 ± 1.28	8.88 ±1.02	22.19 ± 3.18	21.00 ± 3.21	0.030
Time in Contact	30.41 ± 1.31	24.78 ± 1.74	30.00 ± 1.70	24.19 ± 2.66	30.81 ± 2.05	25.38 ± 2.34	0.020
Sexual Behavior[2)]							
Mount (All)	0.69 ± 0.19	0.66 ± 0.15	0.38 ± 0.22	0.38 ± 0.15	1.00 ± 0.29	0.94 ± 0.25	n.s.
Mount (Ejaculatory)	0.19 ± 0.07	0.19 ± 0.07	0.13 ± 0.09	0.19 ± 0.07	0.25 ± 0.11	0.19 ± 0.07	n.s.
Female Rejection of Male Mounts	0.66 ± 0.24	0.41 ± 0.13	0.25 ± 0.14	0.19 ± 0.14	1.06 ± 0.45	0.63 ± 0.22	n.s.
Self Grooming[2)]							
Non-Genital	4.09 ± 0.80	1.97 ± 0.49	6.31 ± 1.36	2.56 ± 0.85	1.88 ± 0.35	1.38 ± 0.46	0.006
Genital	1.69 ± 0.25	5.31 ± 0.98	1.38 ± 0.29	3.19 ± 0.69	2.00 ± 0.41	7.44 ± 1.71	0.049
Acute Behavior Change[3)]							
Prone/Sprawling Position	0.44 ± 0.16	7.45 ± 1.18	0.19 ± 0.14	7.86 ± 1.89	0.69 ± 0.28	7.03 ± 1.44	0.042
Irregular Locomotion	absent	absent	absent	absent	absent	absent	

[1)] Values reflect Mean ± SEM; Flibanserin 10 = 10 mg/kg; Flibanserin 30 = 30 mg/kg; P-value is shown for main effect of Flibanserin Treatment. There are no significant interaction effects of Flibanserin Treatment x Dose of Flibanserin, indicating no differences between flibanserin doses on marmoset behavior. Effects are reported as significant at $p < 0.05$.

[2)] Behavior was recorded at baseline, and 16-24 hours after daily flibanserin administration during weeks 5-6 of 6 weeks of daily flibanserin treatment.

[3)] Behavior was recorded at baseline, and 0-30 minutes after daily flibanserin administration during weeks 1-6 of 6 weeks of daily flibanserin treatment.

CHAPTER 3

Chronic systemic administration of serotonergic ligands flibanserin and 8-OH-DPAT enhance HPA axis responses to restraint in female marmosets

Yves Aubert[1,2], Michael A. Bohl[1], Jason R. Lange[1], Nicole R. Diol[1], Kelly A. Allers[3], Bernd Sommer[3], Nicole A. Datson[2] and David H. Abbott[1]

[1]Wisconsin National Primate Research Center, University of Wisconsin-Madison, Madison, WI, USA; [2]Division of Medical Pharmacology, Leiden/Amsterdam Center for Drug Research, Leiden University Medical Centre, Leiden, The Netherlands; [3]Department of CNS Diseases, Boehringer Ingelheim, Biberach, Germany

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ABSTRACT

Background. Flibanserin, a novel serotonin (5-HT)$_{1A}$ agonist and 5-HT$_{2A}$ antagonist, has been shown to increase sexual desire and reduce distress in women with Hypoactive Sexual Desire Disorder (HSDD). In marmoset monkeys, flibanserin has demonstrated pro-social effects on male-female pairmates, while the classic 5-HT$_{1A}$ agonist 8-OH-DPAT suppresses female sexual behavior and increases aggressive interactions between pairmates. Activation of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors is known to stimulate the hypothalamic-pituitary-adrenal (HPA) axis. This study aims to characterize the effects of repeated flibanserin and 8-OH-DPAT administration on the marmoset HPA axis and to elucidate endocrine correlates of altered marmoset pair behavior.

Methods. Adrenocorticotropic hormone (ACTH) and cortisol were examined at baseline and during 5-HT$_{1A}$ agonist and restraint challenges in 8 female marmoset monkeys receiving daily flibanserin (15 mg/kg) and an additional 8 female marmosets receiving 8-OH-DPAT (0.1 mg/kg) for 15-16 weeks. Corresponding vehicle treatments were administered in a counterbalanced, within-subject design. All females were housed in stable male-female pairs. Treatment-induced changes in ACTH and cortisol levels were correlated with previously assessed marmoset pair behavior.

Results. While morning basal cortisol levels and HPA responses to a 5-HT$_{1A}$ agonist challenge were not altered by chronic flibanserin or 8-OH-DPAT, both treatments increased the responsiveness of the marmoset HPA axis to restraint. Enhanced ACTH responses to restraint correlated with reduced sexual receptivity and increased aggression in 8-OH-DPAT-, but not in flibanserin-treated female marmosets.

Conclusions. Unaltered HPA responses to a 5-HT$_{1A}$ agonist challenge after chronic flibanserin and 8-OH-DPAT treatments indicate little or no desensitization of the HPA axis to repeated 5-HT$_{1A}$ manipulation. Chronic 8-OH-DPAT, but not flibanserin, leads to aggravated ACTH responses to stress that may contribute to anti-sexual and anti-social behavior between 8-OH-DPAT-treated females and their male pairmates. Despite similar flibanserin and 8-OH-DPAT induced ACTH responses to restraint stress, flibanserin-treated females show unchanged cortisol profiles. This is possibly due to flibanserin's regional selectivity in 5-HT$_{1A}$ activation and concurrent 5-HT$_{2A}$ inhibition. The contrasting restraint-related cortisol responses emulate contrasting behavioral phenotypes of diminished pair-bond of 8-OH-DPAT-treated females compared to the more affiliative pair-bond of flibanserin-treated females.

INTRODUCTION

Hypoactive sexual desire disorder (HSDD), a distressing condition affecting an estimated 10% of women (Clayton, 2010), has no approved pharmacological treatment. Recently, flibanserin (2H-benzimidazol-2-one, 1,3-dihydro-1-[2-[4-[3-(trifluoromethyl) phenyl]-1-piperazinyl]ethyl]), an agonist of serotonin $(5\text{-HT})_{1A}$ and antagonist of 5-HT_{2A} receptors (Borsini et al., 1995a; Borsini et al., 2002), demonstrated an ability to stimulate female sexual behavior in rats (Gelez et al., 2010) and to improve sexual desire in women with either HSDD (Derogatis et al., 2012; Thorp et al, 2012) or major depression (Kennedy, 2010). We have tested the long-term effects of flibanserin on sexual and social behavior in female common marmoset monkeys *(Callithrix jacchus)* housed with long-term male pairmates, and compared flibanserin-induced behavioral outcomes with those induced by *R*-(+)-8-hydroxy-2-(di-n-propylamino)-tetralin hydrobromide (8-OH-DPAT), a classic 5-HT_{1A} agonist (Arvidsson et al., 1981). We found that flibanserin-treated females attracted more sexual interest from male pairmates and enhanced the frequency of grooming interactions between pairmates. In contrast, 8-OH-DPAT-treated females showed increased rejection of male sexual advances and increased aggression with male pairmates (Aubert et al., 2011).

Pharmacological manipulations of the central serotonin neurotransmitter system alter functioning of the hypothalamic-pituitary-adrenal (HPA) axis (Fuller, 1996; Jørgensen, 2007). Such HPA changes play important roles in behavioral modulation (de Kloet 2000; Sapolsky et al., 2000) and may contribute to the alteration of sexual and social behavior observed in our flibanserin and 8-OH-DPAT studies. Stress glucocorticoid levels generally inhibit female reproduction and sexual behavior in most species (De Catanzaro and Gorzalka, 1980; Sapolsky et al., 2000). Interestingly, stress is one of the most commonly perceived factors that contribute to low sexual desire in women diagnosed with HSDD (Maserejian et al., 2010), and disorders of the HPA axis are frequently associated with altered sexual desire and arousal in women (Starkman and Schteingart, 1981; Erichsen et al., 2010).

The central 5-HT system generally exerts a stimulatory function on HPA axis activity (Feldman et al., 1987; Dinan, 1996; Jørgensen, 2007). Activation of 5-HT_{1A} and 5-HT_{2A} receptor subtypes, which are both expressed on corticotropin-releasing hormone (CRH) containing neurons in the paraventricular hypothalamic nucleus (PVN) (Liposits et al., 1987), stimulates the HPA axis to release adrenocorticotropic hormone (ACTH) and subsequently glucocorticoids (Rittenhouse et al., 1994; Osei-Owusu, 2005; Van de Kar et al., 2001). Conversely, the responsiveness of the HPA axis to stress is reduced following 5-HT depletion (Feldman and Weidenfeld, 1998).

5-HT induced regulation of HPA axis activity may thus underlie glucocorticoid influence on female sexual behavior.

The effects of chronic flibanserin administration on HPA axis functioning are not known. Repeated injections of 8-OH-DPAT for 21 days elevate basal circulating corticosterone, but not ACTH levels, in rats (Owens et al., 1990). A single systemic injection of 8-OH-DPAT acutely increases circulating ACTH and corticosterone levels (Owens et al., 1990; Raap et al., 2002) and inhibits sexual behavior in female rodents (Uphouse et al., 1991). No information regarding primate HPA axis function following chronic 8-OH-DPAT administration is currently available.

In female marmosets housed with long-term male pairmates, we previously reported pro-social outcomes during chronic flibanserin treatment in contrast to anti-social and anti-sexual effects of chronic 8-OH-DPAT administration (Aubert et al., 2012). The present study aims to investigate the effects of chronic flibanserin and 8-OH-DPAT (1) on the well-established stimulatory effect of 5-HT1A agonism on HPA function and (2) on the HPA response to restraint stress. Aim (1) will thus determine whether the responsiveness of the HPA axis to 5-HT_{1A} activation is impacted by chronic 5-HT_{1A} activation/5-HT_{2A} inhibition (flibanserin), or by chronic 5-HT_{1A} activation alone (8-OH-DPAT), while aim (2) will determine how flibanserin and 8-OH-DPAT affect marmoset HPA function during restraint stress. Such changes in HPA function may contribute to accompanying changes in female interactions with established male pairmates.

METHODS

Study animals

This study was conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act and its subsequent amendments. All animal procedures were reviewed and approved by the Graduate School Animal Care and Use Committee of the University of Wisconsin-Madison. The Wisconsin National Primate Research Center (WNPRC) is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care as part of the University of Wisconsin-Madison Graduate School. Sixteen adult (age 2-5 yr) nulliparous captive-born common marmoset *(Callithrix jacchus)* females were pair housed with similarly aged male partners at the WNPRC for 8-20 months before onset of this study. Eight of the 16 females were assigned to test the effects of flibanserin, while the remaining 8 females were assigned to test the effects of 8-OH-DPAT. Females were housed with the same male partner for the entire study and

were ovariectomized and primed with either mid-follicular phase estradiol levels or no estradiol before study onset. Estradiol status remained the same throughout the study for each individual animal. Surgical procedures, estradiol priming and study design with regard to estradiol priming were performed as described in Barnett et al. (2006).

Experimental design

A counterbalanced, cross-over study, that applied within-subject comparisons, was designed to examine the effects of chronic (15-16 weeks) daily (12:00h-14:00h) administration of flibanserin (n=8; 15 mg/kg, orally (PO) in 1ml/kg vehicle; Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach an der Riss, Germany) or 8-OH-DPAT (n=8; 0.1 mg/kg in 0.4ml/kg vehicle, injected subcutaneously (SC); Sigma-Aldrich St. Louis, MO, USA), with respective vehicles (for flibanserin, 98.5% of 0.5% hydroxycellulose solution and 1.5% of 1% polysorbate 80 solution, 1.0 ml/kg PO; for 8-OH-DPAT, 0.4 ml saline, SC). The study focused on three aspects of HPA axis functioning: (1) morning basal levels of cortisol, (2) following an acute 5-HT_{1A} agonist challenge, and (3) during and after acute restraint-induced increases in plasma ACTH and cortisol levels.

Blood sampling and hormone assays

Within 3 min of home-cage entry, blood samples were collected by femoral puncture during brief restraint in a marmoset restraint tube (Hearn, 1977). This minimizes circulating ACTH and cortisol responses to the sampling procedures (Saltzman et al., 1994). Samples to be analyzed for ACTH were stored at -80°C while those for cortisol and estradiol were stored at -20°C until hormone assay.

Hormone assays were fully validated previously for use with marmoset plasma (Saltzman et al., 1994; Saltzman et al., 1998; Saltzman et al., 2004). Plasma concentrations of ACTH and cortisol were determined by radioimmunoassay (RIA), and for estradiol by RIA following extraction with 5 ml ethyl ether and celite column chromatography. Assay sensitivity was 0.05 ng/tube (1.0 ng/ml) for ACTH, 18.3 pg/tube (1.8 µg/dl) for cortisol, and 4.6 pg/tube (30.4 pg/ml) for estradiol. Intra- and inter-assay assay coefficients of variation (CVs), respectively, were 4.6% and 12.4% for ACTH, 3.6% and 17.2% for cortisol, and 5.0% and 14.0% for estradiol.

Morning basal cortisol levels (08:45-09:15h)

Basal circulating cortisol was assessed prior to treatment (0 wk) and at 3 and 6 weeks of daily flibanserin, 8-OH-DPAT or respective vehicle treatment.

Neuroendocrine tests

Neuroendocrine tests assessing both cortisol and ACTH responses were performed during week 7 (5-HT_{1A} agonist challenge test) and week 9 (restraint test) of daily treatment, at least 22 h after the previous daily administration of 8-OH-DPAT, flibanserin, or vehicle. The time between 5-HT_{1A} agonist challenge and restraint tests was 13 ± 0.5 days (mean ± SEM).

a) **5-HT_{1A} agonist challenge test.** Blood samples (0.3ml) were taken 0, 15 and 180 min after a SC injection of 8-OH-DPAT (0.1 mg/kg), or vehicle (0.4ml/kg saline), administered at 12:00h-13:00h.
b) **Restraint test.** Animals were restrained for 30 min. A baseline blood sample (0.3ml, 0 min) was drawn immediately prior to restraint onset, and additional samples were taken at t=15 and t=30 min during restraint, and 3 h after return to the home cage (210 min).

Behavioral testing

Sexual and social behavior of the pairmates was observed after a 90-minute separation. Four 30-minute behavioral tests were conducted at 07:00h-13:00h (16-24 hours after daily administration of active serotonergic ligand/vehicle), 5-6 weeks after treatment onset. Pair behavior was stable during the 2 weeks of testing. Procedural details are described in Aubert et al. (2012).

Data analysis

Analyses of circulating ACTH, cortisol and estradiol levels were performed on untransformed data when normality of data distribution was confirmed, or on log transformed data to normalize data distribution. To assess morning basal levels, a 2-way ANOVA with Drug Treatment (test compound, vehicle) and Time (0 wk, 3 wk, 6 wk) as within-subject factors was applied. For the 5-HT_{1A} agonist challenge test, a 3-way ANOVA was employed, with Drug Treatment (test compound, vehicle), Challenge Type (5-HT_{1A} agonist challenge, saline challenge) and Time (0 min, 15 min, 180 min) as within-subject factors. For the restraint test, a 2-way ANOVA was used, with Drug Treatment (test compound, vehicle) and Time (0 min, 15 min, 30 min, 210 min) as within-subject factors. Significant main effects of Time were specified post-hoc by contrast analysis. Data are presented as mean ± S.E.M.

Initial analyses were performed using the same mixed design ANOVA with Estradiol supplementation and Order of treatment as additional between-subject factors. As both factors consistently failed to affect ($p>0.05$) any test variable, they were omitted in the final analyses reported here.

To investigate the relationship between flibanserin and 8-OH-DPAT induced

behavioral changes (Aubert et al., 2012) and HPA axis function, all behaviors that were significantly altered by flibanserin or 8-OH-DPAT were post-hoc correlated to significantly altered endocrine responses, using two-tailed Pearson's tests on the differential [serotonergic ligand – respective vehicle] for each selected variable, after normality of the bivariate distributions was confirmed.

For all results, a p-value below 0.05 was considered significant. Behaviors analyzed are described in Aubert et al. (2012).

RESULTS

Chronic effects of 8-OH-DPAT and flibanserin on HPA axis functioning

Morning basal cortisol

Neither chronic flibanserin (Treatment: $F(1,7) = 0.17$, $p = .694$; Treatment x Time interaction: $F(2,14) = 0.36$, $p = .704$) nor chronic 8-OH-DPAT (Treatment: $F(1,7) = 2.76$, $p = .140$; Treatment x Time interaction: $F(2,14) = 0.99$, $p = .397$) altered morning basal cortisol levels compared to respective vehicle (Tab. 1).

Table 1. Morning basal cortisol levels. Plasma concentrations of cortisol (µg/dl; mean ± SEM) at 0, 3 and 6 weeks of daily flibanserin vehicle, flibanserin, 8-OH-DPAT vehicle and 8-OH-DPAT treatment.

	Flibanserin vehicle	Flibanserin	8-OH-DPAT vehicle	8-OH-DPAT
0 wk	221 ± 30.4	200 ± 27.0	167 ± 19.3	175 ± 23.9
3 wk	234 ± 16.8	235 ± 25.6	156 ± 13.3	196 ± 22.9
6 wk	202 ± 15.4	203 ± 21.9	160 ± 22.9	203 ± 24.0

5-HT$_{1A}$ agonist challenge test

The 5-HT$_{1A}$ agonist challenge test elicited ACTH and cortisol responses in all female groups (Fig. 1A), without influence of chronic daily flibanserin or 8-OH-DPAT. In response to a 5-HT$_{1A}$ agonist challenge, flibanserin-, 8-OH-DPAT- and vehicle-treated female marmosets all exhibited increased plasma ACTH levels at 15 min post-challenge compared to a saline challenge. Plasma cortisol levels were also elevated following 5-HT$_{1A}$ agonist challenge, at 180 min post-challenge (Fig. 1B), without influence of chronic daily flibanserin or 8-OH-DPAT.

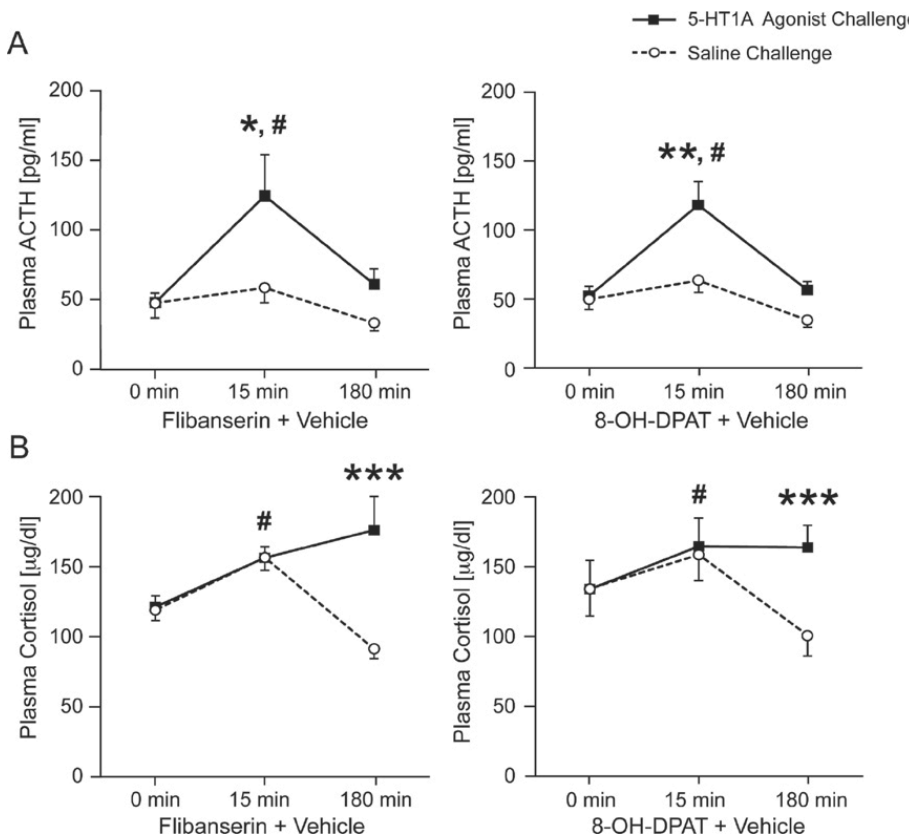

Figure 1. 5-HT$_{1A}$ challenge test, effect of Challenge. Plasma concentrations of **(A)** ACTH (pg/ml; mean±SEM) and **(B)** cortisol (µg/dl; mean±SEM) at 0, 15, and 180 min following an acute 5-HT$_{1A}$ challenge (square dots) at 7 weeks of daily flibanserin, 8-OH-DPAT or corresponding vehicle treatment. ACTH flibanserin group (upper left graph): p=0.045 vs. saline challenge (circular dots; F(1,7) = 6.0); ACTH 8-OH-DPAT group (upper right graph): p=0.008 vs. saline challenge (F(1,7) = 13.5); cortisol flibanserin group (lower left graph): p=0.014 vs. saline challenge (F(1,7) = 8.1), p<0.001 Challenge x Time interaction (F(2,14) = 17.8); cortisol 8-OH-DPAT (lower right graph): p=0.038 vs. saline challenge (F(1,7) = 6.5), p<0.001 Challenge x Time interaction (F(2,14) = 13.9); * p<0.05, ** p<0.01, *** p<0.001 vs. saline challenge (circular dots); # p<0.05 vs. 0min (Time effect). ACTH, adrenocorticotropic hormone; 8-OH-DPAT, *R*-(+)-8-hydroxy-2-(di-n-propylamino)-tetralin.

Restraint test

Thirty minutes of restraint led to acute increases in plasma ACTH levels 15 min and 30 min following restraint onset. Both chronic daily flibanserin

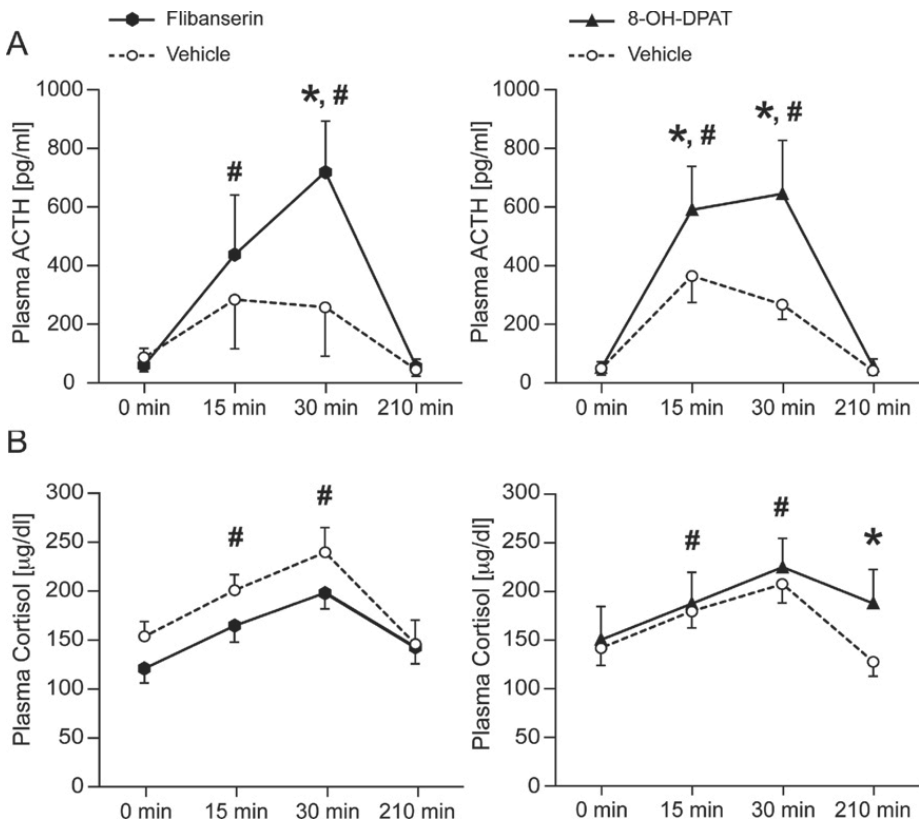

Figure 2. Restraint test. Plasma concentrations of **(A)** ACTH (pg/ml; mean±SEM) and **(B)** cortisol (µg/dl; mean±SEM) at 0, 15, 30 and 210 min during and following 30-min restraint at 0-30 min at 9 weeks of daily flibanserin, 8-OH-DPAT (solid line) or corresponding vehicle (dotted line) treatment. ACTH flibanserin group (upper left graph): p=0.033 Treatment x Time interaction (F(3,21) = 3.5); ACTH 8-OH-DPAT group (upper right graph): p=0.041 Treatment effect (F(1,7) = 6.3), p=0.029 Treatment x Time interaction (F(3,21) = 3.6); cortisol flibanserin group (lower left graph): Treatment x Time interaction non-significant; cortisol 8-OH-DPAT group (lower right graph): p<0.001 Treatment x Time interaction (F(3,21) = 9.5); * p<0.05 vs. corresponding time point control; # p<0.05 vs. 0min (Time effect). ACTH, adrenocorticotropic hormone; 8-OH-DPAT, *R*-(+)-8-hydroxy-2-(di-n-propylamino)-tetralin.

and chronic daily 8-OH-DPAT enhanced restraint-induced increase in plasma ACTH levels (Fig. 2A). 8-OH-DPAT increased ACTH levels at both 15 and 30 minutes of restraint, while flibanserin increased ACTH at 30 minutes only. At 210 min, or 3 h after the animals were returned to their home cages following

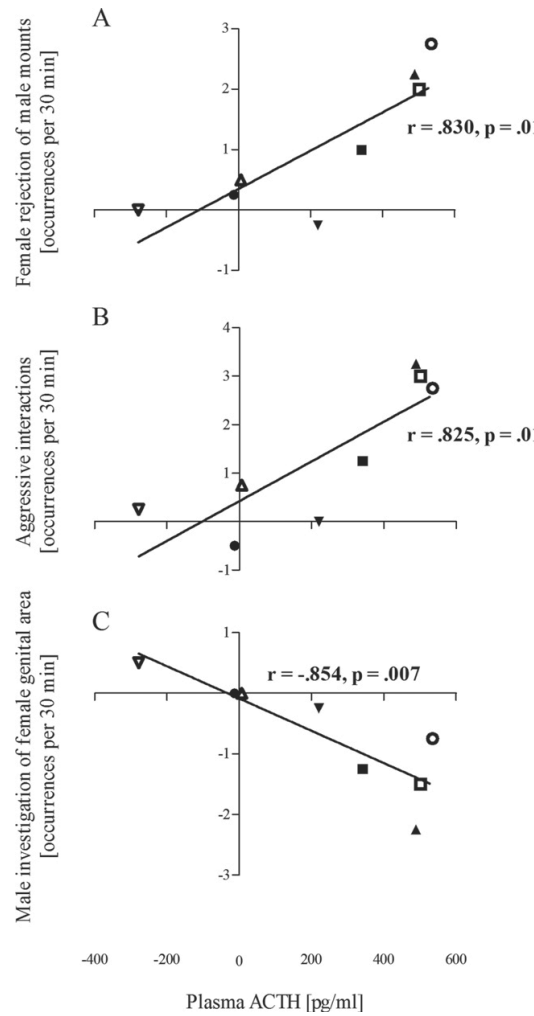

Figure 3. Behavioral correlates of enhanced ACTH response to restraint after 8-OH-DPAT. The magnitude of the 8-OH-DPAT-induced elevation in ACTH responses at 15 min of restraint is positively correlated to female rejection of male mount attempts and mounts **(A)** and aggression between pairmates **(B)**, and negatively correlated to male investigation of the female's genital area **(C)**. The graphs show the differential [8-OH-DPAT – vehicle; n=8] of both ACTH levels (x-axis) and behavior scores (y-axis). Each symbol indicates the same individual female marmoset receiving estradiol (solid symbols) or no estradiol (open symbols). ACTH, adrenocorticotropic hormone; 8-OH-DPAT, *R*-(+)-8-hydroxy-2-(di-n-propylamino)-tetralin.

restraint, plasma ACTH levels in both flibanserin and 8-OH-DPAT treated females were comparable to respective vehicle treated controls.

In flibanserin treated females, elevated ACTH levels at 30 minutes of restraint (the only significantly different time point from vehicle control responses) were not correlated to any behavior altered by flibanserin, including female genital area self-grooming, male investigation of the female's genital area, and allogrooming between pairmates. In 8-OH-DPAT treated females, however, elevated ACTH levels at 15 minutes of restraint were positively correlated to female rejection of male mount attempts and mounts (r = .830, p = .011; Fig. 3A), to unsuccessful male mount attempts (r = .721, p = .044), and to aggressive interactions between pairmates (r = .825, p = .012; Fig 3B),

while they were negatively correlated to male investigation of the female's genital area (r = -.854, p = .007; Fig. 3C). Such correlations were absent at 30 minutes of restraint.

Restraint also led to a stress-induced release of cortisol in all animals. 8-OH-DPAT chronic daily treatment, however, resulted in a significant Treatment x Time interaction, indicating that chronic treatment with 8-OH-DPAT maintained elevated plasma cortisol levels at 210 min, after animals had already been returned to their home cages for 3 h, following cessation of restraint. Chronic daily flibanserin treatment, in contrast, did not induce prolonged elevation of cortisol following cessation of restraint (Fig. 2B). Significantly elevated cortisol levels in 8-OH-DPAT treated females were not correlated to any behavior altered by 8-OH-DPAT.

Estradiol supplementation, during both chronic daily flibanserin and 8-OH-DPAT administration, was without effect on either ACTH or cortisol responses to 5-HT_{1A} agonist challenge or restraint.

DISCUSSION

Comparisons to previous studies

Despite the prevalence of HSDD in women (Clayton, 2010), little is known about the neural and endocrine bases of this disorder. Flibanserin, a novel 5-HT_{1A} agonist and 5-HT_{2A} antagonist, has demonstrated pro-sexual (humans and rodents; Gelez et al., 2010; Kennedy 2010; Stahl et al., 2010) and pro-social (marmosets; Aubert et al., 2011) effects on female behavior, while the classic 5-HT_{1A} agonist 8-OH-DPAT suppresses female sexual behavior in rodents and marmosets (Uphouse et al., 1991; Aubert et al., 2011) and increases aggressive interactions between male-female pairmates of marmosets (Aubert et al., 2011).

In the present study, we demonstrate an increased responsiveness of the female marmoset HPA axis to restraint during chronic flibanserin and 8-OH-DPAT treatments, suggesting that selective manipulation of 5-HT_{1A} receptors, with or without respective 5-HT_{2A} receptor manipulation, sensitizes HPA reactivity. Morning basal cortisol levels, however, are not affected by either treatment and contrast with a study in rats that reports elevated basal plasma corticosterone levels after 21 days of daily 8-OH-DPAT injections (Owens et al., 1990). Differences in species (marmoset vs. rat), dosing (0.1 mg/kg vs. 1.0 mg/kg), route of administration (SC vs. IP) and time of blood sampling in relation to diurnal/nocturnal rhythm (beginning vs. end of active phase in marmoset and rat, respectively), however, make it difficult to directly compare studies. There are no previous studies on flibanserin's effects on HPA axis

function. Both flibanserin and 8-OH-DPAT are agonists on postsynaptic 5-HT_{1A} receptors (Borsini et al., 1995a; Palego et al., 2000), suggesting that the sensitization of the HPA axis to restraint-type of stressors may be mediated by chronic activation of the 5-HT_{1A} receptor in projection areas of 5-HT neurons.

Interestingly, enhanced ACTH responses to restraint correlate with increased aggression and reduced sexual receptivity in 8-OH-DPAT treated female marmosets. In a study in female rats, repeated exposure to a stressful environment is associated with reduced sexual receptivity and increased aggressive behavior (Yoon et al., 2005). ACTH and corticosteroid levels, however, were not measured in the rodent study.

The lack of estradiol effects on HPA axis responses in both flibanserin and 8-OH-DPAT treated marmosets might be somewhat surprising in light of well-documented interactions of estrogens with the central serotonin system in female mammals (Bethea et al., 2002). Estradiol replacement was also without effects on sexual behavior (Aubert et al., 2012). This is in line with our previous studies indicating that follicular phase estradiol supplementation has no effect on circulating cortisol levels (Saltzman et al., 2006) and marmoset female sexual behavior (Barnett et al., 2006), but contrasts with a study conducted by Kendrick and Dixson (1985). Kendrick and Dixson (1985), however, applied mid-cycle levels of estradiol supplementation equivalent to pre-ovulatory peak levels (~940 pg/mL). They also showed that pre-ovulatory estradiol levels are associated with elevated cortisol levels and frequent display of proceptive sexual behavior (Kendrick and Dixson, 1984). The lower follicular phase circulating estradiol levels in our study (~396 pg/mL), chosen to facilitate a modest baseline of sexual behavior, may be responsible for the lack of obvious estradiol-induced HPA-axis and behavioral changes in the present study.

Potential mechanisms of HPA axis sensitization

A neuroendocrine 5-HT_{1A} challenge test, serving as peripheral indicator of central 5-HT_{1A} receptor function (Power and Cowen, 1992; Van de Kar, 1997; Cowen, 2000), was applied to test the responsiveness of the HPA axis to an acute activation of hypothalamic 5-HT_{1A} receptors. It is well known that activation of hypothalamic 5-HT_{1A} receptors stimulates the HPA axis (Przegaliński et al., 1989; Raap et al., 2002; Osei-Owusu et al., 2005). While we show that both circulating ACTH and cortisol levels increase immediately following an injection of 0.1 mg/kg 8-OH-DPAT (SC), neither chronic flibanserin nor chronic 8-OH-DPAT treatments alter ACTH and cortisol responses in this neuroendocrine test (Fig. 1). Thus, repeated administration of flibanserin and 8-OH-DPAT, both agonists at post-synaptic 5-HT_{1A} receptors, did not alter

sensitivity and function of hypothalamic 5-HT_{1A} receptors and the downstream HPA axis response.

Alterations in modulatory (descending) or activating (ascending) systems that regulate CRH/AVP release from the PVN may contribute to stress responses elicited by restraint, as is the case for the limbic system comprising amygdala and hippocampus (Van de Kar et al., 1991; De Kloet et al., 1998), as well as the catecholaminergic system of the brainstem comprising noradrenergic and adrenergic neurons of the nucleus of the solitary tract (Pacák et al., 1993; Pacák and Palkovits, 2001) and locus coeruleus (Vermetten and Bremner, 2002). All of these brain areas express 5-HT_{1A} receptors (Azmitia et al., 1996; Wang et al., 1997; Popova et al., 1998) and project to the PVN (De Kloet et al., 1998; Pacák and Palkovits, 2001), thus becoming potential substrates to flibanserin- and 8-OH-DPAT-induced alterations that modulate HPA axis responsiveness to restraint. In rats, both flibanserin and 8-OH-DPAT have been shown to alter neuronal activity in addition to serotonin, norepinephrine and dopamine neurotransmitter levels in a brain region-specific manner (Lejeune and Millan, 2000; Allers et al., 2010). Chronic flibanserin and 8-OH-DPAT treatments could sensitize the descending and ascending systems described above, leading to enhanced activation of CRH-containing neurons of the PVN during restraint and consequently to the observed exaggerated HPA axis response.

Flibanserin vs 8-OH-DPAT: Differences in pharmacological profiles and HPA responses to restraint

Although both flibanserin and 8-OH-DPAT enhance the excitability of the HPA axis during restraint, subtle differences in ACTH and cortisol response profiles exist between flibanserin and 8-OH-DPAT treated females. Both treatments enhance ACTH responses to the 30-minute restraint stressor, while chronic 8-OH-DPAT, but not flibanserin, also elevates cortisol levels. Increases in circulating ACTH levels relative to vehicle control values occur at both 15 and 30 minutes of restraint in 8-OH-DPAT-treated females, while only at 30 minutes in flibanserin-treated females, indicating a durational difference in the stress response distinguishing a more prolonged HPA axis activation during chronic 8-OH-DPAT compared to chronic flibanserin exposure.

Differences in HPA responses to restraint likely reflect the differences in pharmacological profiles between flibanserin and 8-OH-DPAT. Both flibanserin and 8-OH-DPAT are 5-HT_{1A} agonists. 8-OH-DPAT, however, activates both pre- and postsynaptic 5-HT_{1A} receptors (Palego et al., 2000), while flibanserin activates postsynaptic 5-HT_{1A} receptors (Borsini et al., 1995b) without activating presynaptic 5-HT_{1A} receptors in the raphé nuclei (Marazziti et al.,

2002). Flibanserin thus lacks presynaptic inhibition of 5-HT neurotransmission (Borsini et al., 2002). Flibanserin is additionally an antagonist on 5-HT_{2A} receptors and is possibly a weak partial agonist on dopamine D_4 receptors (Borsini et al. 1995a; Borsini et al., 2002). 8-OH-DPAT is devoid of 5-HT_{2A} (Borsini et al., 1995a) and dopaminergic (Arvidsson et al., 1981) actions, but shows additional 5-HT_7 agonist properties (Shen et al., 1993).

These differences in receptor specificity result in fundamental differences in pharmacology and in abilities of flibanserin and 8-OH-DPAT to induce functional changes in a brain region-specific manner. For example, flibanserin inhibits forskolin-stimulated cAMP formation in the cortex, while 8-OH-DPAT does not affect cortical cAMP accumulation (Borsini et al., 1995a). Flibanserin decreases neuronal firing rate in the rat cortex regardless of whether the presynaptic receptor-containing dorsal raphé nucleus is intact, while the effects of 8-OH-DPAT are dependent upon intact raphé serotonergic neurons (Borsini et al., 1995b). Taken together, flibanserin and 8-OH-DPAT display different regional selectivity in the brain, and they differentially affect neuronal function in 5-HT projection sites.

It is unclear how differences in receptor binding profiles between flibanserin and 8-OH-DPAT translate into a prolongation of cortisol and acceleration of ACTH responses to restraint stress induced by 8-OH-DPAT compared to flibanserin. Additional activation of 5-HT_7 receptors by 8-OH-DPAT may have a stimulatory effect on the HPA axis (Jørgensen et al., 1999), which could explain the increased efficacy in HPA activation by 8-OH-DPAT compared to flibanserin. Alternatively, differences in negative feedback mechanisms may account for the observed differences in hormone levels. The restoration of basal cortisol levels after a stress response is achieved by negative feedback mediated by cortisol binding to glucocorticoid receptors (GR) in limbic structures, PVN of the hypothalamus, and anterior pituitary (De Kloet et al., 1998). Onset of negative feedback, however, varies depending on the modulatory input from the limbic system to the PVN. Genomic GR effects in the hippocampus suppress excitatory β-adrenergic actions and enhance inhibitory effects of 5-HT on the HPA axis (De Kloet et al., 2008). Due to their distinct actions on 5-HT receptor subtypes, flibanserin and 8-OH-DPAT thus may differentially modulate the GR-mediated hippocampal input to the PVN. Indeed, activation of 5-HT_7 increases GR expression in primary hippocampal cell cultures (Laplante et al., 2002), supporting the hypothesis that enhanced cortisol release after restraint may be mediated by chronic 5-HT_7 activation in 8-OH-DPAT-treated females. Another explanation could involve peripheral mechanisms. It is suggested that systemic effects of 5-$HT_{2A/2C}$ agonists may be partially mediated by 5-HT_{2A} receptors in the adrenal cortex (Rittenhouse et al.,

1994; Welch and Saphier, 1994), or through a sympathetic catecholaminergic mechanism (Welch and Saphier, 1994). Flibanserin may reduce the sensitivity of peripheral 5-HT_{2A} receptors to endogenous 5-HT during restraint, or may diminish sympathetic catecholaminergic stimulation of the adrenal cortex, thus preventing a comparable degree of cortisol elevation following restraint induced by 8-OH-DPAT.

Behavioral correlates of enhanced stress reactivity in 8-OH-DPAT-treated marmosets

The difference in ACTH response dynamics between flibanserin and 8-OH-DPAT is of particular significance given the correlation analysis with behavior. 8-OH-DPAT-treated females with the greatest ACTH response at the early measurement point of the restraint test (at 15 minutes) also experienced the most between-partner aggression, attracted the least genital investigation by their male pairmates, and most frequently rejected the pairmate's sexual advances, causing an increased number of unsuccessful male mount attempts (Fig. 3). These associations were absent at the later measurement point of the restraint test (at 30 minutes). Thus, 8-OH-DPAT treated females that rapidly develop aggravated endocrine markers of stress (i.e. elevated ACTH beyond vehicle control values at 15 minutes) also display symptoms of aversive pair behavior proportional to the magnitude of 8-OH-DPAT-induced increase in ACTH responses. Enhanced stress reactivity may thus contribute to decreased female sexual receptivity and heightened state of aggression in 8-OH-DPAT-, but not in flibanserin-treated female marmosets.

While exposure to a stressful environment is associated with impaired sexual behavior and elevated aggression in female rats (Yoon et al., 2005), and personal distress and distress in partner relations are hallmarks of HSDD in women (DSM-IV-TR, 2000), the link between HPA axis activity and aggression is not clear. In some human studies high aggression is associated with low HPA axis activity (Gordis et al., 2006), but others do not confirm such an inverse relationship between aggression and stress hormone levels (Schulz et al., 1997). It is therefore not clear whether the behavioral associations with increased HPA responsiveness to restraint in the current study reflect HPA axis hormone-mediated changes in behavior, or whether both behavior and HPA hormones serve as separate and reliable biomarkers of 8-OH-DPAT-mediated action.

CONCLUSIONS

Our findings are the first to demonstrate enhanced HPA axis responsiveness to a restraint-type stressor after chronic serotonergic modulation in a nonhuman

primate. Considering the receptor binding profiles of flibanserin and 8-OH-DPAT, similarities in their effects on HPA axis sensitization are likely mediated by their shared postsynaptic 5-HT_{1A} agonist property. Chronic 8-OH-DPAT, but not flibanserin, however, leads to rapid aggravated ACTH responses to stress that may contribute to anti-sexual and anti-social behavior between the 8-OH-DPAT-treated female and her male pairmate. Flibanserin, in contrast, dissociates such ACTH-marked stress reactivity from prolonged cortisol responses and aversive pair behavior possibly due to its unique regional selectivity in 5-HT_{1A} receptor activation, combined with concurrent 5-HT_{2A} antagonist activity. Thus, despite endocrine similarities in terms of enhanced ACTH responses to restraint stress, flibanserin-treated females show regular cortisol profiles and strengthening of the affiliative pair-bond with their male pairmates in contrast to the HPA axis and behavioral phenotypes of 8-OH-DPAT-treated females.

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CHAPTER 4

Positron emission tomography assessment of 8-OH-DPAT-mediated changes in an index of cerebral glucose metabolism in female marmosets

Alexander K. Converse[1], Yves Aubert[2,7], Mohammed Farhoud[3], Jamey P. Weichert[3], Ian J. Rowland[4], Nicole M. Ingrisano[5], Kelly A. Allers[6], Bernd Sommer[6] and David H. Abbott[2,5]

[1]Waisman Center, University of Wisconsin-Madison, Madison, WI, USA; [2]Wisconsin National Primate Research Center, University of Wisconsin-Madison, Madison, WI, USA; [3]Department of Radiology, University of Wisconsin-Madison, Madison, WI, USA; [4]Department of Medical Physics, University of Wisconsin-Madison, Madison, WI, USA; [5]Department of Ob/Gyn, University of Wisconsin-Madison, Madison, WI, USA; [6]Department of CNS Diseases, Boehringer Ingelheim, Biberach, Germany; [7]Division of Medical Pharmacology, Leiden/Amsterdam Center for Drug Research, Leiden University Medical Centre, Leiden, The Netherlands

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ABSTRACT

As part of a larger experiment investigating serotonergic regulation of female marmoset sexual behavior, this study was designed to (1) advance methods for PET imaging of common marmoset monkey brain, (2) measure normalized FDG uptake as an index of local cerebral metabolic rates for glucose, and (3) study changes induced in this index of cerebral glucose metabolism by chronic treatment of female marmosets with a serotonin 1A receptor (5-HT_{1A}) agonist. We hypothesized that chronic treatment with the 5-HT1A agonist 8-OH-DPAT would alter the glucose metabolism index in dorsal raphé (DR), medial prefrontal cortex (mPFC), medial preoptic area of hypothalamus (mPOA), ventromedial nucleus of hypothalamus (VMH), and field CA1 of hippocampus.

Eight adult ovariectomized female common marmosets *(Callithrix jacchus)* were studied with and without estradiol replacement. In a crossover design, each subject was treated daily with 8-OH-DPAT (0.1 mg/kg SC daily) or saline. After 42–49 days of treatment, the glucose metabolism radiotracer FDG was administered to each female immediately prior to 30 min of interaction with her male pairmate, after which the subject was anesthetized and imaged by PET. Whole brain normalized PET images were analyzed with anatomically defined regions of interest (ROI). Whole brain voxelwisemappingwas also used to explore treatment effects and correlations between alterations in the glucose metabolism index and pairmate interactions.

The rank order of normalized FDG uptake was VMH/mPOA>DR>mPFC/CA1 in both conditions. 8-OH-DPAT did not induce alterations in the glucose metabolism index in ROIs. Voxelwise mapping showed a significant reduction in normalized FDG uptake in response to 8-OH-DPAT in a cluster in medial occipital cortex as well as a significant correlation between increased rejection of mount attempts and reduced normalized FDG uptake in an overlapping cluster.

In conclusion, PET imaging has been used to measure FDG uptake relative to whole brain in marmoset monkeys. Voxelwise mapping shows that 8-OH-DPAT reduces this index of glucose metabolism in medial occipital cortex, consistent with alterations in female sexual behavior.

INTRODUCTION

This is a study of the effects of chronic serotonergic manipulation in a nonhuman primate model of female sexual behavior (Barnett et al., 2006). This work applied positron emission tomography (PET) to marmoset brain imaging. The goals of this work involved methods development, determination of cerebral glucose metabolic indices, and measurement of the effect of chronic treatment with a serotonin 1A (5-HT_{1A}) receptor agonist on brain regions implicated in female sexual interactions with a male pairmate.

5-HT is thought to play a central inhibitory role in regulating female sexual behavior by modulating satiety and balancing excitatory neuromodulators (Pfaus, 2009). In this marmoset study, the prototypical 5-HT_{1A} receptor agonist *R*-(+)-8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT) was used (Hjorth et al., 1982). In rats, 8-OH-DPAT acutely diminishes female sexual receptivity (Uphouse et al., 1991). Administration of 8-OH-DPAT (1 mg/kg IV) to male rats acutely alters glucose metabolism in a number of brain regions, including decreases in hippocampal areas and increases in motor regions, consistent with the “5-HT behavioral syndrome” (Kelly et al., 1988). Therapeutic efficacy of serotonergic treatments typically requires at least 2 weeks of administration perhaps due to adaptive changes, and therefore it is important to study the chronic effects of pharmacological treatment (Blier and de Montigny, 1994; Blier et al., 1987; Hensler, 2003). The effect of chronic 8-OH-DPAT administration on cerebral glucose metabolism in primates, including humans, has not been reported.

The current study’s focus on PET imaging of female marmoset neural responses to the 5-HT_{1A} agonist 8-OH-DPAT, during male–female pair tests, arises from our interest in understanding the role of 5-HT in regulating female sexual behavior. An estimated 10% of women (Clayton, 2010) show marked distress and interpersonal difficulty because of unwanted, persistent or recurrent low sexual desire (hypoactive sexual desire disorder, HSDD (American Psychiatric Association, 2000)). Psychopathogenesis of HSDD, however, is not known, but neurotransmitter dysfunction has been proposed involving the excitatory regulators dopamine (DA) and norepinephrine (NE), as well as inhibitory 5-HT (Clayton and Hamilton, 2010; Pfaus, 2009). Pharmacological manipulation of 5-HT commonly results in diminished female sexual satisfaction and activity, particularly in women prescribed selective serotonin reuptake inhibitors (SSRIs) for depression (Clayton and Montejo, 2006; Clayton et al., 2002; Patterson, 1993; Rosen et al., 1999; Segraves, 1998). Animal studies that apply 5-HT receptor subtype specific ligands permit mechanistic examination of 5-HT-mediated effects on sexual behavior. There are 7 known 5-HT receptor families, each with its own specific brain

distribution, as well as effects on behavior and physiology (Barnes and Sharp, 1999). We focused on 5-HT_{1A} receptor activation in this study to explore 5-HT involvement in female sexual inhibition, since the sexually receptive female lordosis posture in rats is inhibited by 5-HT_{1A} receptor activation (Ahlenius et al., 1986; Hebert et al., 1995; Uphouse et al., 1992).

Female marmoset monkeys present an opportunity to explore the sexually inhibitory properties of 8-OH-DPAT in a nonhuman primate model that readily translates to humans, as marmosets form stable, long-term, male–female relationships (Abbott et al., 2003) and display modest amounts of sexual behavior (Barnett et al., 2006). Unlike the multiple-mating social structures of rats and many nonhuman primates, such as macaques and baboons, marmoset sexual behavior most commonly occurs within stable male–female pairs (Abbott et al., 2003; Evans and Poole, 1984; Saltzman et al., 2009). By acceptance or rejection of a pairmate's sexual advances, female marmosets can readily promote, prevent or terminate sexual interactions (Kendrick and Dixson, 1986; Stevenson and Poole, 1976), and our recent development of a standardized behavioral testing paradigm permits repeatable, quantitative exploration of neurally active compounds that enhance or diminish female marmoset sexual behavior (Barnett et al., 2006).

Neuroimaging has furthered mechanistic understanding of neural regulation of sexual behavior in both humans and animals (Arnow et al., 2009; Rilling et al., 2004). Recent improvements in PET spatial resolution now permit its application to marmoset brain imaging (Haneda et al., 2007). PET permits longitudinal measures of brain physiology that may be used to study individual differences in treatment response. In the current protocol, pair-bonded female marmosets are observed upon reunion with their male pairmates following a 90-minute separation (Barnett et al., 2006). Measures were performed on the same individuals during chronic 8-OH-DPAT treatment as well as in a control saline treatment condition. To index glucose metabolism during behavioral interactions, we used the trapped tracer [^{18}F]fluorodeoxyglucose (FDG) (Sokoloff et al., 1977). In this protocol, the rate of glucose metabolism during awake, freely moving behavior is reflected by subsequent imaging performed under anesthesia in the PET scanner (Rilling et al., 2001; Tashiro et al., 2001). We identified anatomical location within the marmoset brain using template regions of interest (ROIs) similar to previous methods employed in studies of humans and rats (Lancaster et al., 2007; Rubins et al., 2003). Because template methods may be subject to error due to individual differences in anatomy not accounted for by the coregistration technique, we verified template ROI against individual ROI results.

The goals of this study were to (1) advance methods for PET imaging of

common marmoset monkey brain, (2) measure normalized FDG uptake as an index of local cerebral metabolic rates for glucose, and (3) study changes induced in this index of cerebral glucose metabolism by chronic treatment of female marmosets with a 5-HT_{1A} agonist. We hypothesized that chronic treatment with the 5-HT_{1A} agonist 8-OH-DPAT would yield altered glucose metabolism in the following five ROIs known to have high 5-HT_{1A} density (Moller et al., 2007; Pazos et al., 1987) or to be involved in female proceptive or receptive sexual behavior (Pfaus, 2009): (1) dorsal raphé (DR), containing 5-HT neurons with extensive projections to hypothalamic, limbic, hippocampal and cortical sites, and neurotransmission regulating 5-HT_{1A} receptors (Kakeyama and Yamanouchi, 1996); (2) medial prefrontal cortex (mPFC), involved in executive and inhibitory regulation of behavior, with sexual behavior-relevant contributions from ventral tegmental area dopaminergic projections engaged in responses to sexual and incentive stimuli (Afonso et al., 2007); (3) medial preoptic area of the hypothalamus (mPOA), intimately involved in female proceptive sexual behavior (Dixson and Lloyd, 1988; Graham and Pfaus, 2010; Kendrick and Dixson, 1986), (4) ventromedial nucleus of the hypothalamus (VMH), regulating female sexual receptivity (Dixson and Lloyd, 1988; Griffin and Flanagan-Cato, 2011; Kendrick and Dixson, 1986), and (5) field CA1 of the hippocampus, involved in sexually relevant memory processes (van Wingen et al., 2008).

MATERIALS AND METHODS

Subjects

Female common marmosets were studied (*Callithrix jacchus*, n=8, 425±47 g, age 4.5±0.6 years, mean±standard deviation). Subjects were pair housed with male pairmates at the Wisconsin National Primate Research Center (WNPRC) for 8–20 months before and throughout the duration of the study with lights on during 06:30–18:30 h, as previously described (Barnett et al., 2006). Ovaries were removed and subcutaneous silastic capsules implanted to provide estradiol replacement in 4 of the subjects (serum estradiol during study weeks: 396±184 pg/mL, mid-follicular phase estradiol levels (Barnett et al., 2006)), while the remaining 4 subjects received empty capsules (serum estradiol 68±31 pg/mL, typical of ovariectomized female marmosets (Barnett et al., 2006)). This study was conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act and its subsequent amendments. All animal procedures were reviewed and approved by the Graduate School Animal Care and Use Committee of the University of Wisconsin-Madison. WNPRC, as part

of the University of Wisconsin-Madison Graduate School, is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Experimental protocol

A counterbalanced, cross-over study, that applied within-subject comparisons, was designed to examine the effects of chronic (15–16 weeks) daily (12:00 h–14:00 h) administration of 8-OHDPAT (0.1 mg/kg in 0.4 mL/kg saline, injected subcutaneously (SC); Sigma-Aldrich St. Louis, MO, USA), or 0.4 mL saline SC, with an intervening 6-week washout period.

Behavioral observations of sexual behavior

In order to stimulate sexual interactions upon reunion (Barnett et al., 2006), females and males were separated for 90 min prior to each of four 30-min behavioral tests at 07:00 h–13:00 h (16–24 h after daily administration of 8-OH-DPAT/saline), 5–6 weeks after treatment onset. At the start of each behavioral test, the male was introduced to the female by remote door operation and behavior was manually and digitally recorded by two observers from behind a one-way window (Barnett et al., 2006). Behavioral tests were reanalyzed from the digitally stored recordings in a random fashion by two observers, blinded with respect to treatment.

Brain imaging

Subjects were scanned with FDG PET at 42–49 days following start of treatment with either 8-OH-DPAT or saline. On the day of the PET scan, each female subject was removed from her home cage, placed alone in a test cage for 90 min, injected in the femoral vein with 31.1±6.4 MBq FDG (IBA Romeoville), reunited with her mate after 5.4±1.3 min in a behavioral test cage, and observed for 30 min during pair behavior, as described for behavioral observations above. The subject was then transported to the PET facility, anesthetized with ketamine (10 mg IM), intubated, and maintained on isoflurane (1–3% in O2) for the remaining procedures. Subjects were then positioned in the University ofWisconsin Inveon small animal PET scanner (Siemens, Knoxville), which provides 127 mm axial and 100mm transaxial field of view, 5 μL volumetric resolution, and 6.8% sensitivity (Bao et al., 2009; Constantinescu and Mukherjee, 2009). Subjects were positioned prone and face forward with their heads taped to prevent motion and with their brains located at the center of the field of view. The subjects were warmed with blown air and monitored for heart rate, SpO_2, exhaled CO_2, and rectal temperature. Emission data were acquired for 60 min in list mode with 350–650 keV energy and 3 ns timing windows. Scans started 82.5±6.3 min following FDG injection

and 26.4±5.7 min following ketamine injection. Following the emission scans, a ^{57}Co transmission scan was acquired (120–125 keV).

To obtain anatomical images, each subject was scanned on a separate day under isoflurane anesthesia with the University of Wisconsin small animal 4.7 T MRI (Varian, Palo Alto) using a Varian 72mm ID quadrature coil and a spin echo multi slice sequence (TR=2000 ms, TE=35ms, MA=128×128, FOV=40mm×40mm, slices=40, thickness=1.00 mm, in-plane pixel size=0.31mm×0.31mm).

Brain atlas

A brain atlas of an adult male common marmoset was used to aid anatomical identification (Palazzi and Bordier, 2008). The atlas comprises 48 coronal sections sliced perpendicular to the anterior commissure—posterior commissure line. Uncertainty in the stereotaxic scale due to shrinkage is expected to be within 3% based on comparisons of medial–lateral and anterior–posterior measures of brains before and after freezing. Digital 2D images provided with the atlas were shifted left–right and dorsal–ventral to align the scales between slices (Adobe Illustrator). A slice missing at bregma +0 mm was replaced with a copy of bregma +0.56 mm resulting in 49 slices spaced at 620±45 μmbased on the labeled anteroposterior positions. These 2D images were stacked into a 3D image of 589×584×59 voxels with in-plane pixel sizes of 53.3 μm×53.3 μm and slice thickness of 620.0 μm (ImageJ (Abramoff et al., 2004)).

MR image processing and ROIs

An MR template image was created as follows. The MR image from the first subject scanned (cj1074) was resliced to 0.5 mm cubic voxels and aligned manually (Spamalize, http://brainimaging.waisman.wisc.edu/~oakes) so as to appear symmetric in coronal and axial views and rotated in a sagittal orientation such that the anterior and posterior commissures of the corpus callosum (AC–PC) lay in the same axial plane. A preliminary whole brain mask was drawn on this image. The MR images of the remaining seven subjects were manually coregistered to that of the first subject, and all eight were whole brain normalized and averaged together. Each of the eight images was aligned to this mean image by automated rigid body (6 degrees of freedom, df) coregistration (FSL FLIRT v. 5.5; Oxford Centre for Functional MRI of the Brain) using a normalized mutual information cost function, and whole brain reference and input weights. The resulting eight images were averaged and each was then aligned to this new average by automated affine (9 df) coregistration. The mean of these eight images was taken as the 0.5 mm

cubic voxel MRI template and a whole brain mask was drawn on this template by tracing the inner boundary of the skull, which appears dark in the MRI.

To aid in delineation of ROIs, the 0.5 mm cubic voxel MRI template was resliced and translated manually to match the atlas image, and the same transformation was applied to the individual MRIs. Bilateral ROIs were drawn by two operators on the template and individual MRIs with reference to the atlas for DR, mPFC, mPOA, VMH, and CA1. Inter- and intra-operator comparisons of the drawn ROIs were made by calculating the ratio of the number of overlapping voxels to the average number of voxels between two versions of the same ROI. The second version drawn by the same operator was used for each of the template ROIs. PET scanner resolution effects for each ROI were estimated by smoothing the ROI and its inverse, i.e. Tissue outside the ROI, with a 1.71 mm FWHM Gaussian kernel.

PET image reconstruction

Reconstructions were performed using the scanner vendor's software (Siemens Inveon Acquisition Workplace). Transmission events were sorted into a 2D sinogram (single-slice rebinning, 128 bins, 160 angles, 159 planes) and scaled by a blank sinogram. Emission events were sorted into a single 3D sinogram (128 bins, 160 angles, 159 planes, span 3, ring difference 79, 27 segments, hist.exe v. 2.3.3.8). Images were reconstructed by filtered backprojection with a ramp filter (recon.exe v. 2.222). The resulting images included corrections for accidental coincidences, detector sensitivity, attenuation, and radioactive decay. Consistent with the low scatter fraction expected from the approximately 25 mm diameter marmoset head, scatter correction was found to have a small effect on the whole brain normalized ROI values (e.g. <4% for mPOA), and scatter corrected whole brain normalized radioactivity values were linearly related across subjects to non-corrected values (e.g. r=0.996 for mPOA). Scatter correction was therefore not applied to avoid introducing additional noise. Image dimensions were 128×128×159 with in-plane pixel size 0.776 mm×0.776 mm and slice thickness 0.796 mm.

PET image analysis

Alignment of the PET images to the 0.5 mm cubic voxel MRI template was performed in several steps as follows. The 16 static PET images (n=8 subjects × 2 conditions) were resliced and manually aligned by translations to the MRI template. Each PET image was then automatically aligned to the version of the subject's MRI prior to the final 9 df transformation of that MRI to the template space. This was done with 6 df using a correlation ratio cost function without reference image weighting and then again with weighting

by the template whole brain mask smoothed with a 2 mm Gaussian kernel. For each subject, the 8-OH-DPAT and Saline images were averaged, and the average was aligned by 6 df to the individual MRI. The 8-OH-DPAT and Saline images were again aligned to this aligned average image. As the final alignment step, the 9 df transformation that had been applied to the individual MRI was applied to the 8-OHDPAT and Saline PET images. The PET images were thus aligned within subject and between subject to the 0.5 mm cubic voxel MRI template. The whole brain mask (8601 μL) was trimmed to avoid radioactivity outside the brain by masking it at the edges with a thresholded version of an intermediate mean PET image (8 subjects × 2 conditions, whole brain normalized radioactivity >1.2, trimmed volume 8065 μL). The individual images were then whole brain normalized, i.e. each pixel value was divided by the average value for the individual in the trimmed whole brain mask. Subtraction images, 8-OH-DPAT minus Saline, were then created, and the trimmed whole brain mask was applied to all images.

ROI analysis was performed in the atlas space. All PET images were coregistered to the atlas using the same transformation that had been applied to the 0.5 mm cubic voxel MRI template. The ROIs drawn on the template and individual MRIs were then applied to the PET images. ROI methods were compared by calculating over the eight subjects the root mean square (RMS) differences of PET image values between operators and between individual MRI- and template-based ROIs. As described below in the Results section, template versus individual MRI differences were comparable to inter-operator differences. Use of template ROIs was therefore considered reliable, and template ROI results were used in the subsequent analyses. Paired 2-tailed Student's t tests were performed on the ROI results in the Saline and 8-OH-DPAT conditions. Significance at $p<0.05$ was required for testing the primary hypothesis of a treatment effect in any of the five bilateral ROIs.

Voxelwise whole brain analysis was also performed in the atlas space. To search for a treatment effect, a paired t test was performed between the 8-OH-DPAT and Saline images. To search for correlations between the glucose metabolism index and behavioral response to 8-OH-DPAT (from quantitative observations made during weeks 5–6 described above), a linear regression with zero intercept was performed between the difference images and the behavioral difference scores (8-OH-DPAT minus Saline). In addition, linear regression was performed on the Saline condition images against sexual behavior in the Saline condition. Two-tailed t test or Pearson r thresholds were set corresponding to $p_{uncorrected}<0.05$ at each voxel, and clusters of voxels with significance $p_{corrected}<0.05$ 2-tailed were identified by random field theory (SPM8) (Worsley et al., 1996).

RESULTS

Image alignment and ROIs

As one goal of this study was methods development, we evaluated the image alignment and ROI delineation procedures. In each subject's aligned MRI, visual inspection of the brain contour seen in every coronal slice demonstrated alignment with the mean MRI to within 0.5 mm (Suppl. Fig. 1). The resulting MRI template is shown in Fig. 1. The 16 aligned individual PET images (8 subjects × 2 conditions) agreed to≤0.5 mm between individuals and to higher precision within subjects (Suppl. Fig. 2). There were visible differences between subjects and between conditions in relative FDG uptake in tissue outside the skull, but the trimmed whole brain mask prevented spillover into observed brain regions. Fig. 2 shows the MRI template and mean FDG image

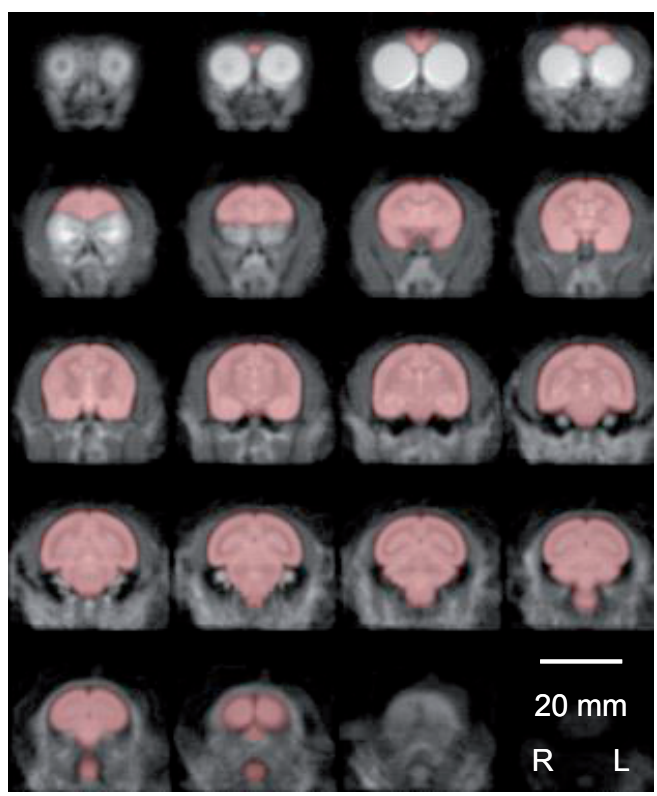

Fig. 1. MRI template. Mean of aligned images (n=8) used as MRI template (Every fourth 0.5 mm coronal slice shown). Whole brain mask is overlaid (red). The template is freely available upon request to the corresponding author.

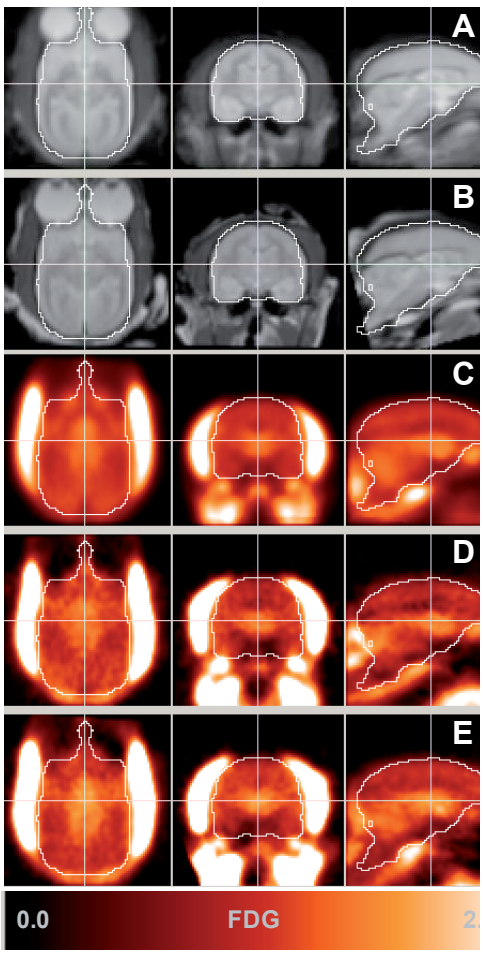

Fig. 2. Aligned images. Axial, coronal, and sagittal slices of MRI template (A, n=8 subjects), MRI of one subject (B, cj1022), mean FDG image (C, n=8 subjects × 2 conditions), and FDG image of one subject (cj1022) in Saline condition D and 8-OH-DPAT condition E. Contour is on whole brain mask trimmed to avoid contributions from radioactivity uptake outside of the brain. Color scale indicates whole brain normalized radioactivity concentration shown in C–E.

(shown in greater detail in Suppl. Fig. 3) along with typical single subject MRI and FDG images in the two conditions. Representative slices of the template MRI aligned to the atlas and the ROIs are shown in Fig. 3.

Methods for delineating ROIs were compared, and resolution effects were estimated. Comparing ROIs delineated on individual MRIs (5 regions × 8 subjects), intra- and inter-operator overlap were 0.86±0.05 (n=40) and 0.85±0.06 (n=40), respectively. Intra- and inter-operator overlap of the five template ROIs were 0.89±0.03 (n=5) and 0.84±0.06 (n=5), respectively. Degrees of differences in FDG values between operators and between methods were highly comparable: 8-OH-DPAT minus Saline values were compared across the eight subjects and the five bilateral ROIs, and the average (n=5) root mean square difference (n=8) was 0.0083±0.0034 for

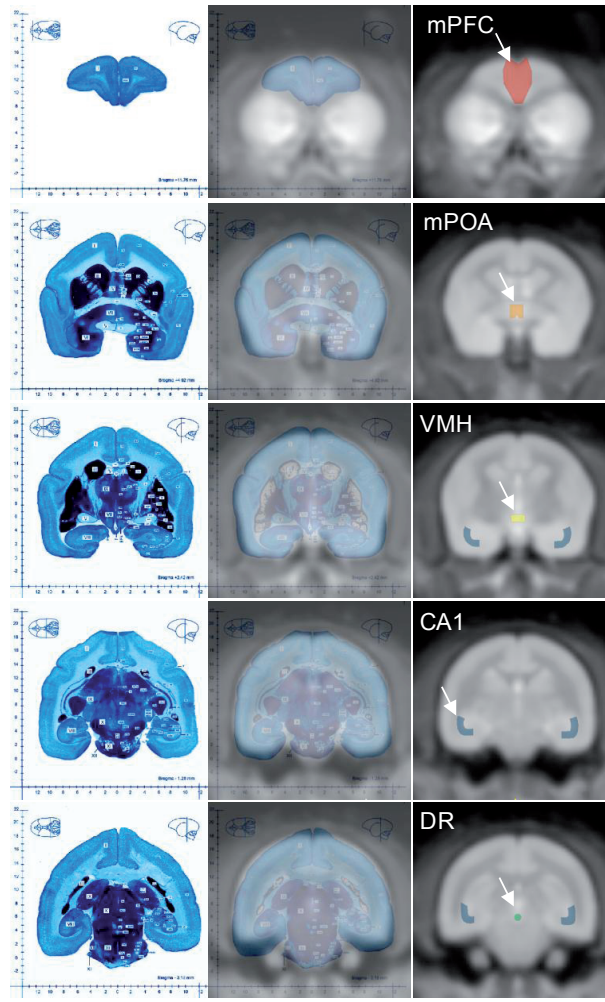

Fig. 3. Anatomical identification. Fusion image (center column) of coregistered brain atlas (left column) and MRI template (right column). Representative coronal slices showing regions of interest (ROIs) for medial prefrontal cortex (mPFC), medial preoptic area of the hypothalamus (mPOA), ventromedial hypothalamic nucleus (VMH), field CA1 of the hippocampus (CA1), and dorsal raphe nucleus (DR). Atlas images courtesy of Springer Science+Business Media (Palazzi and Bordier, 2008).

inter-operator comparisons and 0.0092±0.0034 for individual MRI-determined versus template MRI-determined comparisons. Subsequent analyses were thus performed using only template MRI-determined ROIs. The template ROIs had the following bilateral volumes: DR 1.5 µL, mPFC 100.4 µL, mPOA 4.9 µL, VMH 5.2 µL, and CA1 46.8 µL. Upon simulating the PET scanner resolution (5 µL) with assumed uniform distribution of radioactivity, the activity calculated in the ROI originating in the ROI itself was DR 14%, mPFC 66%, mPOA 29%, VMH 28%, and CA1 36%. No partial volume correction was applied to the measured data, and the reported results must, therefore, be considered best estimates for the delineated ROIs.

Experimental conditions

Experimental conditions for the PET scans are presented in Table 1. There were no significant differences in parameters between treatment conditions. No significant effect of estradiol group was observed in the Saline condition in the ROIs or by voxelwise analysis, nor was there a significant estradiol × 8-OH-DPAT interaction (two way ANOVA with repeated measures). Subsequent results were therefore calculated without reference to estradiol group.

Table 1. Experimental parameters. Mean±SD (n=8), p: paired t test 2-tailed. WB=mean radioactivity concentration in whole brain (MBq/mL), ID=injected dose (MBq), and BW=body weight (g).

	Saline	8-OH-DPAT	p
Age (years)	4.54 ± 0.72	4.54 ± 0.52	1.00
Weight (g)	427 ± 54	422 ± 42	0.44
Injected FDG (MBq)	32.1 ± 7.3	29.9 ± 5.4	0.51
Behavior start–FDG inject (min)	5.4 ± 1.2	5.4 ± 1.5	0.93
Scan start time	11:24 ± 1:09	10:50 ± 1:06	0.15
Scan start–last treatment (h)	22.6 ± 1.3	21.9 ± 1.1	0.14
Scan start–FDG inject (min)	83.1 ± 6.6	82.0 ± 6.3	0.73
Scan start–ketamine inject (min)	27.3 ± 6.5	25.5 ± 5.0	0.50
WB/(ID/BW) (g/mL)	1.06 ± 0.17	0.99 ± 0.13	0.27

Normalized FDG uptake during saline administration and response to treatment

In the Saline as well as the 8-OH-DPAT condition, mean FDG values ranked VMH / mPOA>DR>mPFC / CA1 (Suppl. Fig. 4). Paired t tests indicated no

Table 2. FDG results. Whole brain normalized radioactivity measures in anatomically defined ROIs for Saline condition, 8-OH-DPAT condition, and the difference 8-OH-DPAT minus Saline. (p: paired t test, 2-tailed; n=8).

ROI	V (μL)	Saline	8-OH-DPAT	8-OH-DPAT minus saline	p
DR	1.5	1.164 ± 0.168	1.169 ± 0.113	0.004 ± 0.115	0.922
mPFC	100.4	0.988 ± 0.055	0.981 ± 0.050	−0.007 ± 0.090	0.844
mPOA	4.9	1.292 ± 0.245	1.324 ± 0.249	0.031 ± 0.096	0.387
VMH	5.2	1.349 ± 0.310	1.359 ± 0.232	0.010 ± 0.134	0.832
CA1	46.8	0.987 ± 0.037	0.983 ± 0.045	−0.005 ± 0.042	0.763

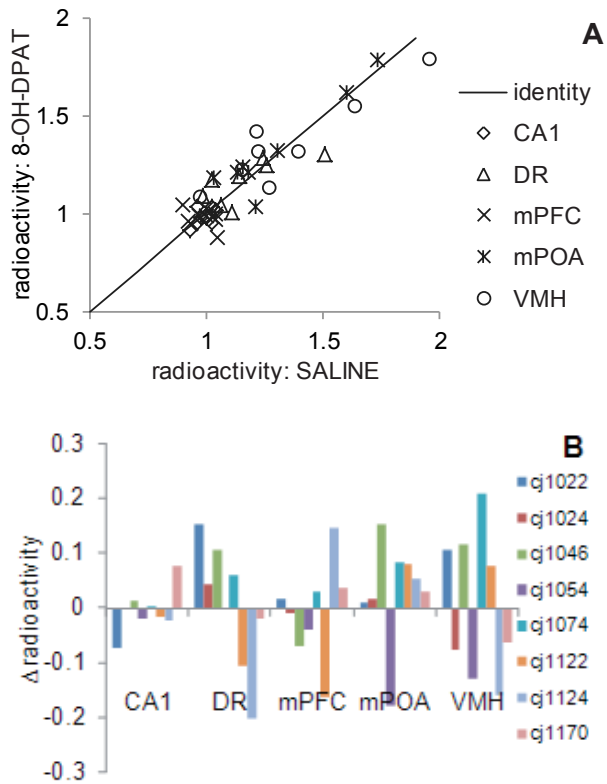

Fig. 4. ROI analysis of response to 8-OH-DPAT treatment. Scatter plot showing individual ROI results (whole brain normalized radioactivity) in 8-OH-DPAT and saline conditions (A). Bar plot shows difference in whole brain normalized radioactivity (8-OH-DPAT minus saline) for each ROI and each subject (B). No ROI shows a significant response to chronic 8-OH-DPAT treatment ($p>0.3$ for each region; paired t test, 2-tailed; n=8).

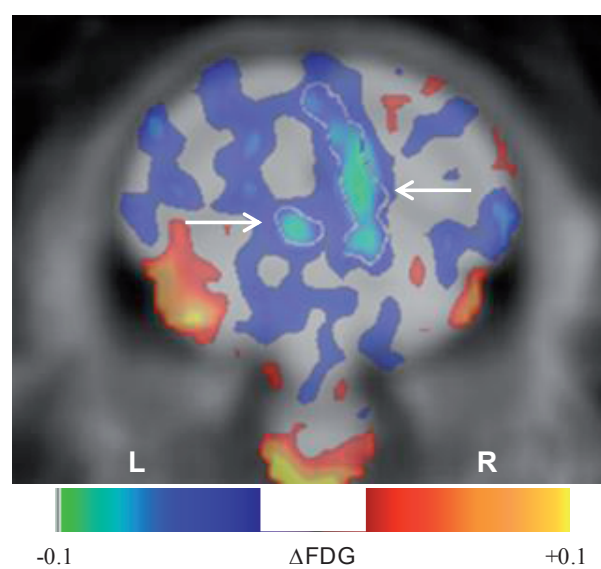

Fig. 5. Glucose metabolism index decreases in medial occipital cortex in response to chronic 8-OH-DPAT treatment. The color scale indicates the mean difference in whole brain normalized radioactivity, ΔFDG = 8-OH-DPAT minus Saline. White contour marked by arrows delineates a significant negative 3D cluster in the corresponding t map, shown at Bregma −11.2 mm (2-tailed paired t test, $p_{uncorrected}<0.05$, extent=60 μL, $p_{corrected}=0.004$). Mean difference arbitrarily thresholded at $0.02<|\Delta FDG|<0.10$.

8-OH-DPAT-induced difference in the magnitude of FDG uptake in any ROI (Table 2 and Fig. 4). Voxelwise mapping of 8-OH-DPAT minus Saline revealed a single cluster in medial occipital cortex of reduced activity (8-OH-DPAT < Saline, $p_{uncorrected}$<0.05, extent=60 µL, $p_{corrected}$=0.004; Fig. 5 and Suppl. Fig. 5).

Behavior

To further confirm the significance of the finding of decreased normalized FDG uptake in medial occipital cortex in response to 8-OHDPAT, we considered alterations in female rejection of male mounts and mount attempts (the most significant 8-OH-DPAT-induced behavioral finding) (Aubert et al., in press). The between-condition difference in FDG uptake was therefore correlated voxelwise against the difference in rejection of mount attempts. Two significant clusters of voxels were found, in which altered normalized FDG uptake correlated with altered rejection of mounts (Fig. 6 and Supp. Fig. 6; $p_{uncorrected}$<0.05; positive correlation: left ventral cerebellum, extent=48 µL, $p_{corrected}$=0.017; negative correlation: medial occipital cortex, extent=72 µL, $p_{corrected}$=0.001). The second cluster, in which decreased metabolism correlated with increased rejection of mounts, overlapped 38% with the cluster of voxels described above that exhibited reduced metabolism during 8-OH-DPAT treatment (Fig. 7 and Suppl. Fig. 7). Subsequent exploration of the relation between normalized FDG uptake and rejection of mount attempts in the Saline condition alone yielded three significant clusters (Table 3, Suppl. Fig. 8), but the mean normalized FDG uptake in these clusters did not reveal a significant response to 8-OH-DPAT (p>0.5, 2-tailed paired t test).

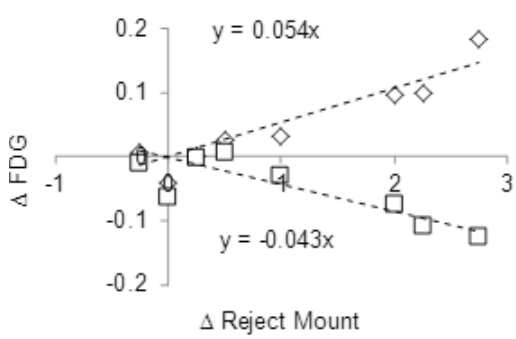

Fig. 6. Rejection of mounts. Between-condition difference in normalized FDG uptake (8-OH-DPAT - Saline) plotted against alteration in behavior for the eight subjects. Voxelwise analysis found two significant clusters (Suppl. Fig. 6), one with a positive correlation (◊ left ventral cerebellum) and one with a negative correlation (□ medial occipital cortex).

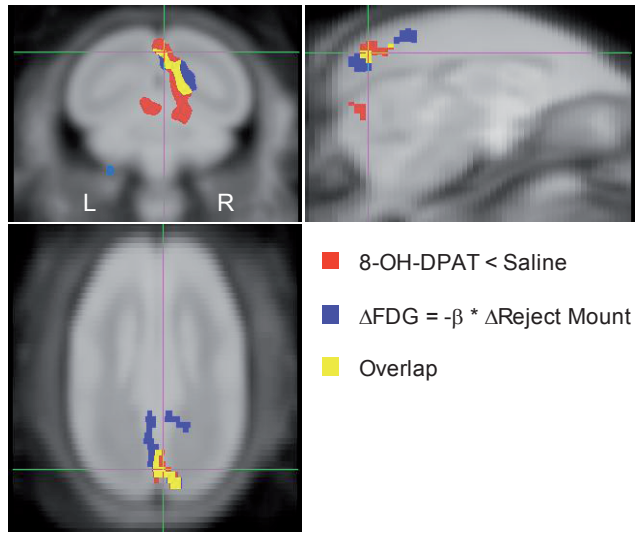

Fig. 7. Glucose metabolism index and behavior respond to 8-OH-DPAT treatment in concert. Orthogonal views of MRI template at medial occipital cortex. Cluster of voxels with significant reduction in FDG radioactivity during 8-OH-DPAT condition compared to Saline condition (red; paired t test, 2-tailed $p_{uncorrected}$<0.05, extent 60 μL, $p_{corrected}$=0.004). Cluster of voxels with significant negative correlation between alterations of FDG radioactivity and rejection of mount attempts between conditions (blue; Pearson r; 2-tailed $p_{uncorrected}$<0.05, extent 72 μL, $p_{corrected}$=0.001). The two clusters overlap (yellow). Part of significant cluster with positive correlation is seen in coronal view (light blue at ventral left edge of cerebellum).

Table 3. Significant clusters identified by voxelwise statistical mapping. Clusters in t maps above threshold at $p_{uncorrected}$=0.05 with extent shown in μL. $p_{corr.}$=p value of cluster corrected for familywise error (SPM8). Position of maximum absolute t value (max. |t|) within cluster indicated in atlas coordinates (mm) (Palazzi and Bordier, 2008): LR=right with respect to midline, AP=anterior w.r.t. bregma, and DV=dorsal w.r.t. interaural line. Atlas origin corresponds to voxel (x,y,z)=(298,30,111).

Test	Region	μL	$p_{corr.}$	max. |t|	LR	AP	DV
Contrast: 8-OH-DPAT minus saline							
	Medial occipital cortex	60	0.004	−14.4	0.6	−11.2	13.5
Correlation: ΔFDG vs Δreject mount							
	Medial occipital cortex	72	0.001	−15.4	1.0	−11.8	12.1
	Left ventral cerebellum	48	0.017	9.7	−4.4	−8.1	0.5
Correlation: FDG vs reject mount (saline)							
	Bilateral ventral cerebellum	161	0.000	11.9	−2.6	−3.7	−1.9
	Bilateral dorsal frontal cortex	115	0.001	16.2	−2.1	5.6	19.0
	Bilateral orbitofrontal cortex	122	0.000	−8.8	8.5	7.4	10.2

DISCUSSION

In this work, we (1) advanced methods for PET imaging of marmoset monkey brain, (2) measured whole brain normalized FDG uptake as an index of local cerebral metabolic rates for glucose, and (3) studied the effect of chronic treatment with a 5-HT1A receptor agonist on brain regions implicated in regulating female sexual interactions.

Anatomical identification

In this work we implemented and validated a novel templatebased method of anatomical identification for PET imaging of marmoset brain. This relied on adequate alignment of the individual MR images to the template space and of PET images to MRIs (Figs. 1 and 2). Alignment of the MRI template to the published histological atlas was within 1 mm, a very satisfactory degree of agreement. While the agreement between the MRI template and the atlas was not sufficient to simply delineate ROIs on the atlas, it aided identification of regions in the MRI by means of relative positions to common features seen in each image (Fig. 3). Despite the excellent alignment between the individual MRIs, as judged by brain contours (Fig. 2), individual anatomical differences in grey-white contrast within the brain may still be significant. Inter-operator variances, however, in geometric overlap and in FDG measures using individual ROIs were comparable to individual- versus template-determined differences. The template ROI method may therefore provide anatomical identification as reproducible as that obtained by delineating regions on individual MRIs. While template ROI location was reliable between subjects, measures attributed to the smaller regions, namely DR, mPOA, and VMH, must be interpreted with caution because of the PET scanner resolution.

Glucose metabolism index and behavior

This is the first report, to our knowledge, of the effect of chronic 8-OH-DPAT administration on cerebral glucose metabolism in primates. Despite relatively large differences in whole brain normalized radioactivity measured between regions and between subjects, FDG measures in the anatomically defined ROIs were generally stable between conditions. Indeed, none of the anatomically defined ROIs exhibited a significant response to 8-OH-DPAT (Table 2 and Fig. 4). On the other hand, voxelwise analysis of 8-OH-DPAT versus Saline identified a significant cluster of reduced metabolism in medial occipital cortex (Fig. 5).

A limitation of this study was the lack of absolute quantification of cerebral glucose metabolism. Because FDG uptake occurred during freely moving behavior, blood sampling would have interfered with the experiment, so

normalized FDG uptake was used as an index of glucose metabolism. The between condition measures reported here therefore only indexed alterations in metabolism relative to whole brain, and interpreting them as changes in absolute glucose metabolism is therefore complicated by potential changes in global cerebral metabolism (Borghammer et al., 2009). The reduction in whole brain normalized radioactivity observed in medial occipital cortex (−5.9%, $p_{corrected}$=0.004) was supported by dimunition of radioactivity in that region normalized to injected dose / body weight (−13.2%, p=0.093, 2-tailed paired t test) suggesting a true reduction in neural activity.

The observed 8-OH-DPAT induced reduction of normalized FDG uptake in medial occipital cortex overlapped 38% with a brain region showing a correlation between 8-OH-DPAT-induced increase in female rejection of male mounts and 8-OH-DPAT-induced reduction in FDG uptake (Figs. 6–7). Such an association between 8-OH-DPAT-induced changes in female sexual behavior and normalized FDG uptake in occipital cortex is perhaps not surprising when considering that primate occipital cortex receives considerable 5-HT innervation (Morrison et al., 1982) associated with processing of visual information involving 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{2A} receptors (Gebhard et al., 1993; Watakabe et al., 2009; Yamamori, 2011).

Our detailed knowledge of within-neuron location of 5-HT receptor subtypes within occiptal cortex derives from rodent studies, in which hyperpolarizing 5-HT_{1A} receptors are found on the soma and axon hillock of layer 5 pyramidal (L5P) neurons (Moreau et al., 2010). L5P neurons, after integrating thalamocortical inputs relayed mostly from L2/3 visual cortex neurons, elaborate cortical outputs from the visual cortex to multiple areas of the cortex (Thomson and Bannister, 1998). Axon hillock 5-HT1A receptors on L5P neurons have been shown to provide crucial inhibitory contributions in balancing excitation and inhibition, possibly by regulating gain control (enhancing weak signals and suppressing excessive responses) (Amargos-Bosch et al., 2004; Moreau et al., 2010; Yamamori, 2011). In this study, therefore, 8-OH-DPAT-mediated chronic overstimulation of axon-hillock 5-HT_{1A} receptors on L5P visual cortical neurons may decrease the likelihood of L5P activity and potentially produce an area-wide overall decrease in glucose metabolism. Such enhanced $5HT_{1A}$-mediated inhibition of female marmoset visual cortex L5P synaptic output may thus distort visually-relevant signals required for timely cortical processing. This disrupted cortical processing could in turn contribute to the 8-OH-DPAT-mediated inhibition of female engagement in sexual behavior with their male pairmates, as the visual cortex projects to, and receives input from, the amygdala (Amaral, 2002) and frontal cortex (Catani et al., 2002; Nieuwenhuys et al., 1988), 5-HT responsive regions that

are intimately involved in attributing salience, and directing visual attention, to negative emotional stimuli (Surguladze et al., 2008).

To identify brain regions associated with female marmoset sexual behavior, we subsequently correlated normalized FDG uptake with female rejection of mounts in the Saline condition alone, which yielded three significant clusters of voxels. A cluster encompassing bilateral orbitofrontal cortex and right insular cortex exhibited increasing FDG uptake across subjects with decreasing female rejection of mounts. This result is consistent with those from human neuroimaging studies, in which women were exposed to erotic visual stimuli (Arnow et al., 2009) or to male faces (Rupp et al., 2009). There was no significant change in FDG uptake observed in any of these three clusters of voxels in response to 8-OH-DPAT. The lack of 8-OH-DPAT related changes in FDG uptake in these voxel clusters suggests that individual differences in these regions are relatively stable and 8-OH-DPAT induced alteration in female mount rejection is mediated by other brain regions, in particular the medial occipital region discussed above.

Taken together, these observations support the notion that the association between 8-OH-DPAT-related changes in behavior and occipital cortex FDG uptake may indicate altered salience of, and visual attention paid to, pairmate interactions contributing to diminished female sexual receptivity. Further planned investigations of the effects of chronic 8-OH-DPAT administration on the primate serotonergic system, particularly upon 5-HT_{1A} and SERT availability, may shed light on this question.

CONCLUSIONS

This work implemented and validated a template method of marmoset brain PET analysis that yielded reliable measures of normalized FDG uptake. Chronic treatment with 8-OH-DPAT (0.1 mg/kg SC daily for 42–49 days) did not produce significant alterations in this glucose metabolism index in the five anatomically defined regions examined (DR, mPFC, mPOA, VMH, and CA1) in awake, behaving, ovariectomized adult female marmosets with or without estradiol replacement. Parametric mapping revealed a significant reduction in the glucose metabolism index in response to 8-OH-DPAT in medial occipital cortex, consistent with alterations in female sexual behavior.

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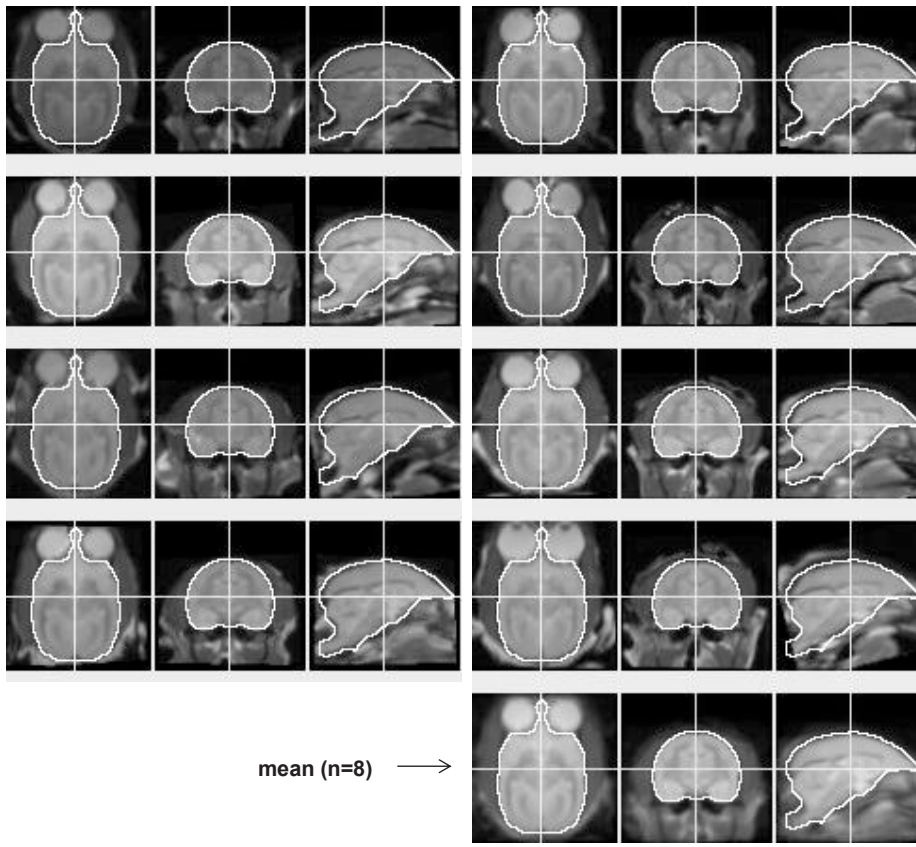
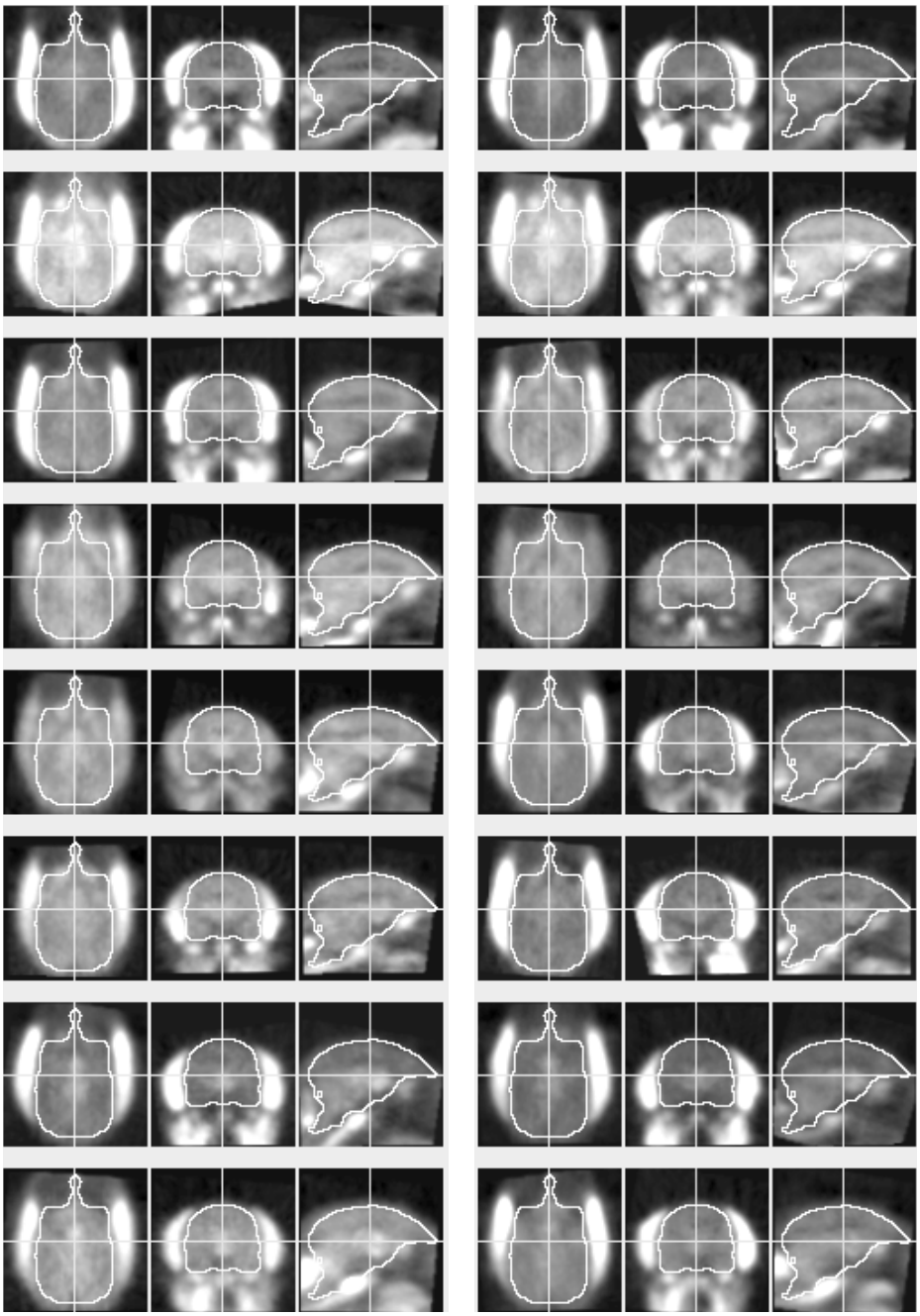
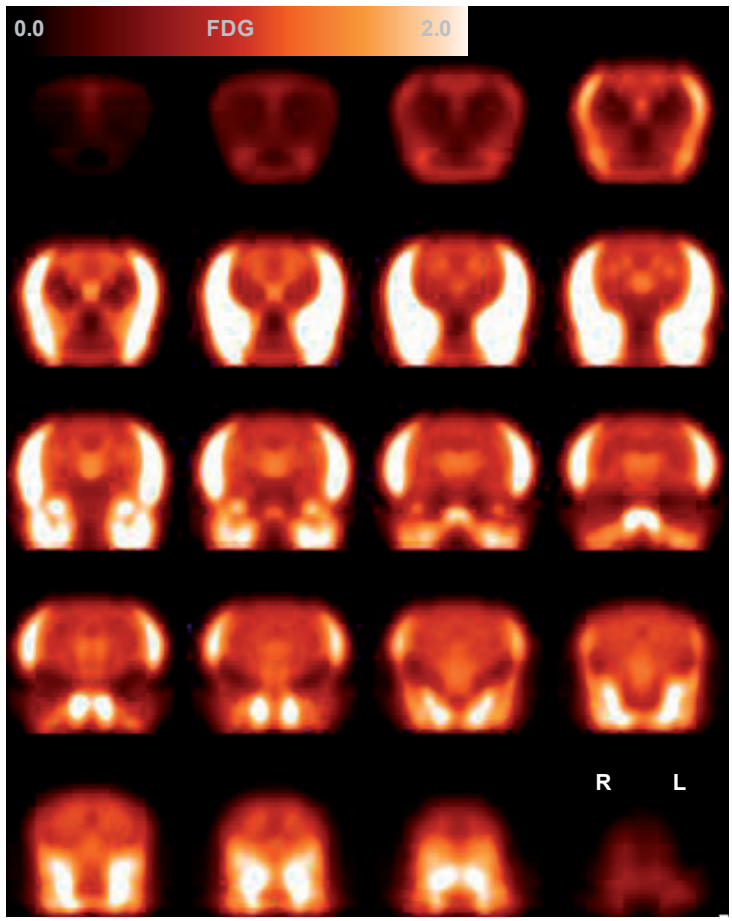
SUPPORTING INFORMATION



Supplementary Figure 1. Alignment of individual MRIs. The first eight images show axial, coronal, and sagittal slices in each subject's aligned MRI at the same position. Individual images have been aligned to the target (cj1074) by 6 degrees of freedom (rigid body) and then by 9 degrees of freedom (rigid body plus zooms). The last image shows the mean image, used as the MRI template. The whole brain contour is the same in all views and demonstrates the quality of the alignments.

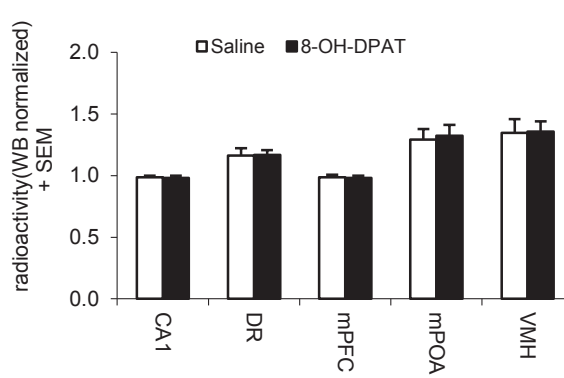


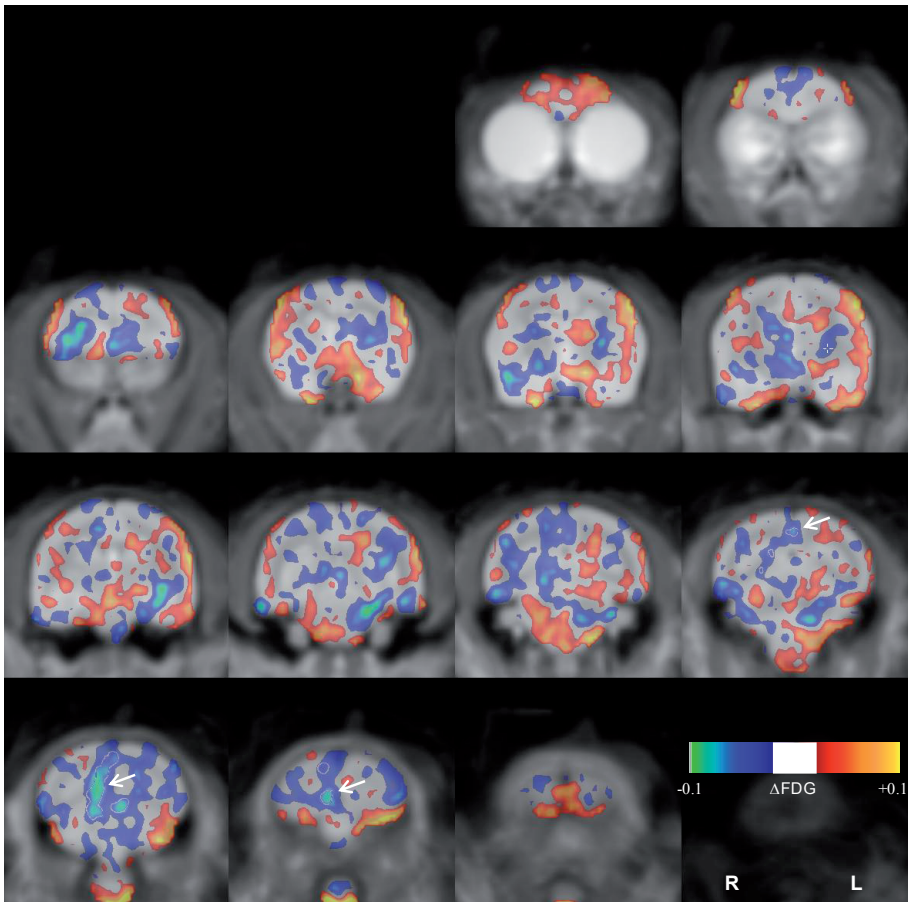
Supplementary Figure 2. Individual FDG images. Each row shows axial, coronal, and sagittal slices of FDG image for one subject in Saline condition (left) and DPAT condition (right). Contour is on whole brain mask modified to avoid contributions from FDG uptake outside of brain.



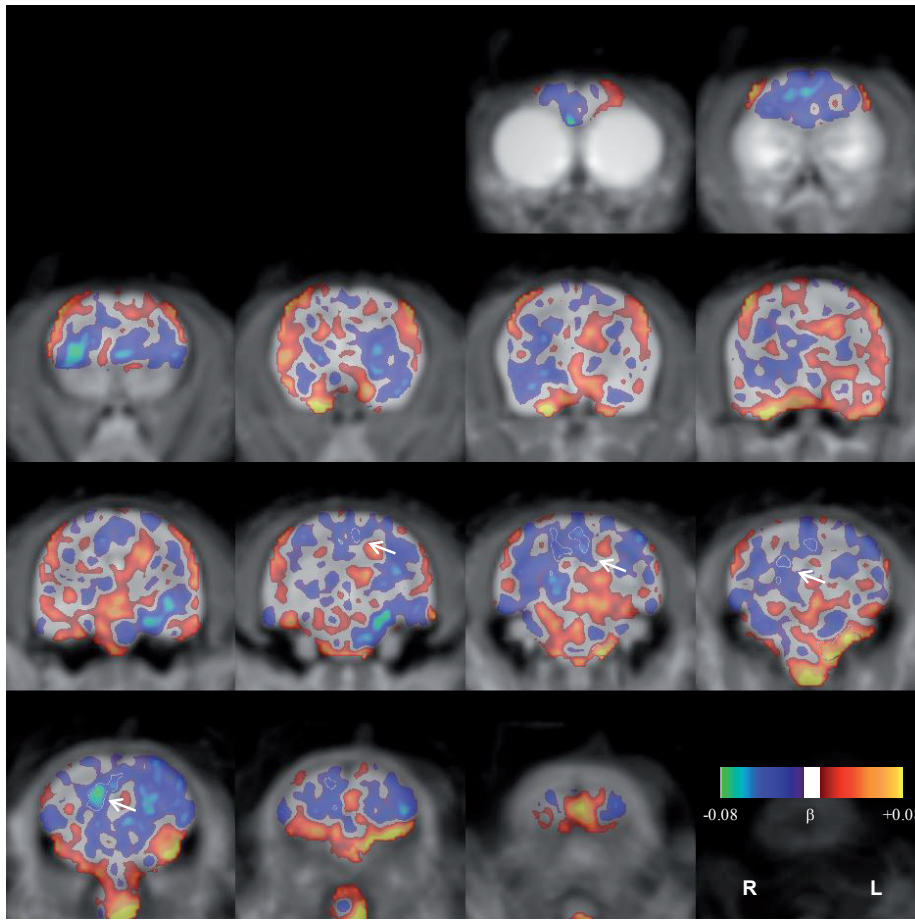
Supplementary Figure 3. Mean FDG Image. Average of 16 (8 subjects x 2 conditions) whole brain normalized images. Every fourth 0.5 mm slice shown.

Supplementary Figure 4. No ROI shows a significant response to chronic 8-OH-DPAT treatment. Mean whole brain normalized radioactivity in each condition (Saline, 8-OH-DPAT) for each ROI and all subjects ($p>0.3$ for each region; paired t test, 2-tailed; n = 8).

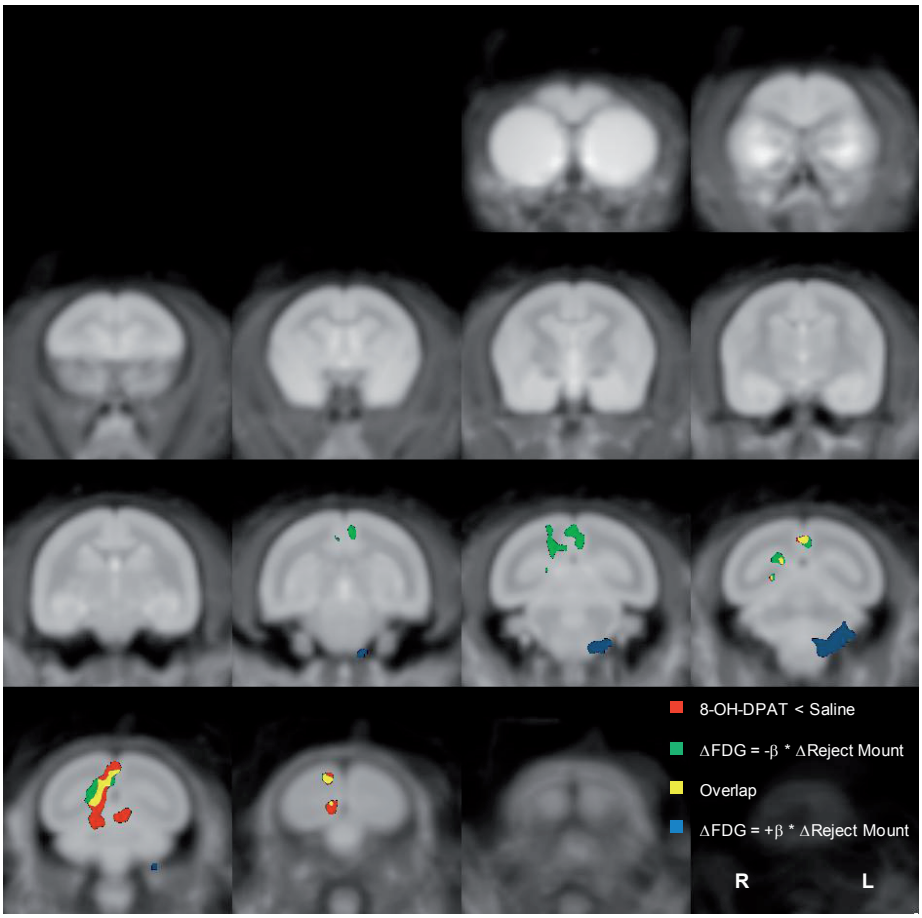




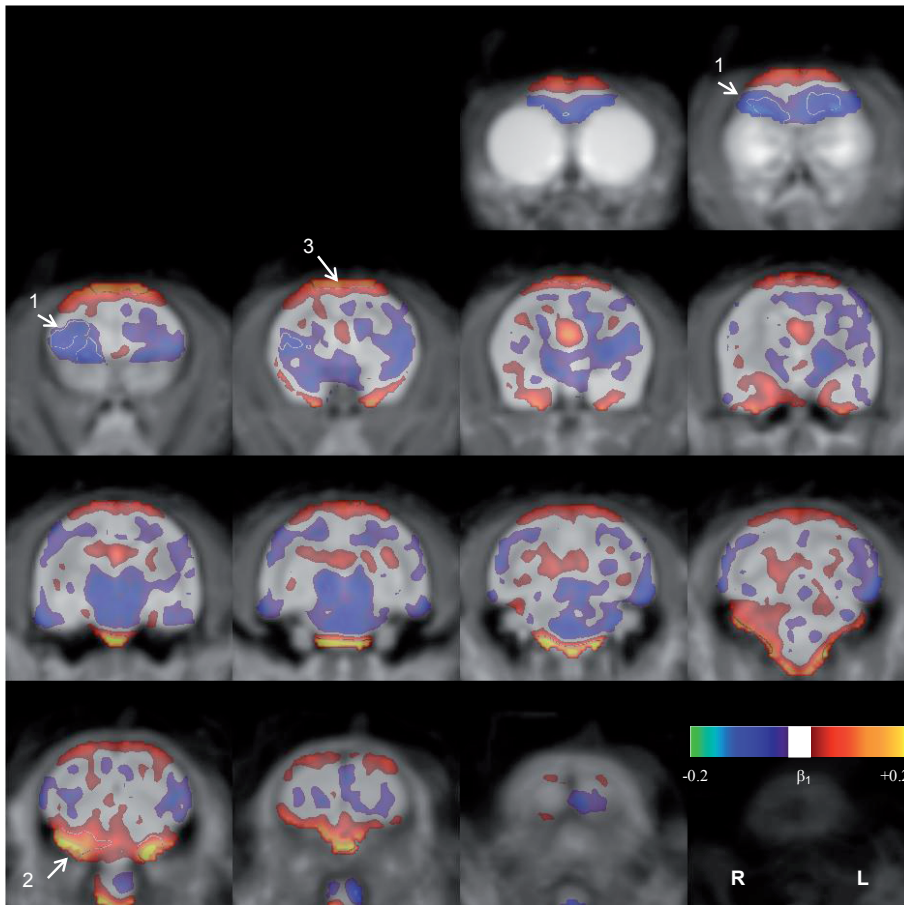
Supplementary Figure 5. Glucose metabolism index decreases in medial occipital cortex in response to chronic 8-OH-DPAT treatment. The color scale indicates the mean difference in whole brain normalized radioactivity, ΔFDG = 8-OH-DPAT minus Saline, thresholded at $0.02 < |\Delta FDG| < 0.10$. The white contour marked by arrows delineates a significant negative cluster (2-tailed paired t test, $p_{uncorrected} < 0.05$, extent = 60 μL, $p_{corrected} = 0.004$). Every fourth 0.62 mm coronal slice shown.



Supplementary Figure 6. Changes in glucose metabolism index correlate with alterations in female rejection of mounts. Between-condition difference in normalized FDG uptake (8-OH-DPAT minus Saline) correlated against alteration in behavior. Color scale corresponds to slope of linear correlation through the origin, $\Delta FDG = \beta * \Delta(\text{Reject Mount})$, thresholded at $0.01 < |\beta| < 0.08$. For Pearson r, 2-tailed $p_{uncorrected} < 0.05$, two significant clusters were identified, one with a positive correlation (left ventral cerebellum; extent = 47 µL; $p_{corrected} = 0.017$) and one with a negative correlation (medial occipital cortex; extent = 72 µL; $p_{corrected} = 0.001$; indicated by arrows). Every fourth 0.62 mm coronal slice shown.



Supplementary Figure 7. Overlap between contrast and correlation clusters shown in Supplementary Figures 5 and 6. Cluster of voxels with significant reduction in FDG radioactivity during 8-OH-DPAT condition compared to Saline condition (**red**; paired t test, 2-tailed $p_{uncorrected} < 0.05$, extent 60 µL, $p_{corrected} = 0.004$). Cluster of voxels with significant negative correlation between alterations of FDG radioactivity and rejection of mount attempts between conditions (**green**; Pearson r; 2-tailed $p_{uncorrected} < 0.05$, extent 72 µL, $p_{corrected} = 0.001$). The two clusters overlap (**yellow**; 23 µL). Significant cluster with positive correlation is seen as well in left ventral cerebellum (**blue**; Pearson r; 2-tailed $p_{uncorrected} < 0.05$, extent 47 µL, $p_{corrected} = 0.017$). Every fourth 0.62 mm coronal slice shown. This image shown in orthogonal views in **Fig. 7**.



Supplementary Figure 8. Glucose metabolism index correlated against female rejection of mounts in Saline condition. Three significant clusters indicated by numbers and arrows. Negative correlation (blue), i.e. less rejection of mounts with increasing FDG uptake: (1) bilateral orbitofrontal cortex and right insular cortex (Pearson r; 2-tailed puncorrected < 0.05, extent 122 μL, pcorrected = 0.000). Positive correlation (red): (2) bilateral ventral cerebellum (161 μL, pcorrected = 0.000) and (3) bilateral dorsal frontal cortex (115 μL, pcorrected = 0.001). FDG = β_1 * Reject Mount + β_2. Arbitrarily thresholded at 0.02 < |β_1| < 0.2. Every fourth 0.62 mm coronal slice shown.

CHAPTER 5

Brain region-specific transcriptomic markers of serotonin-1a receptor agonist action mediating sexual rejection and aggression in female marmoset monkeys

Yves Aubert[1,2], Kelly A. Allers[3], Bernd Sommer[3], E. Ronald de Kloet[1], David H. Abbott[2] and Nicole A. Datson[1]

[1]Division of Medical Pharmacology, Leiden/Amsterdam Center for Drug Research, Leiden University Medical Centre, Leiden, The Netherlands; [2]Wisconsin National Primate Research Center, University of Wisconsin-Madison, Madison, WI, USA; [3]Department of CNS Diseases, Boehringer Ingelheim, Biberach, Germany

Submitted

ABSTRACT

Introduction. In a marmoset model of hypoactive female sexual function, we have shown that chronic administration of the serotonin (5-HT)-1a agonist 8-OH-DPAT inhibits sexual receptivity in female marmoset monkeys and increases aggression towards the male pairmate.

Aim. To investigate gene expression changes induced by 8-OH-DPAT in laser-microdissected brain areas that regulate female sexual function, and to identify genes, functional gene classes and pathways associated with 8-OH-DPAT-mediated inhibition of female sexual receptivity.

Methods. Gene expression was measured in the medial prefrontal cortex (mPFC), medial preoptic area (mPOA), CA1 area of the hippocampus (CA1) and dorsal raphé nucleus (DRN) of four chronically 8-OH-DPAT-treated (0.1 mg/kg) and four vehicle-treated female marmosets using a marmoset-specific microarray (EUMAMA) and validated by Real-time quantitative PCR (RTqPCR). Enriched functional gene classes were determined. In a parallel candidate gene approach, the expression of serotonergic candidate genes, i.e. the 5-HT_{1A}, 5-HT_{2A}, 5-HT_7 receptors and the 5-HT transporter (5-HTT), was measured by RTqPCR.

Main outcome measures. Differential expression of genes between 8-OH-DPAT- and vehicle-treated marmosets.

Results. 8-OH-DPAT affected gene classes important to neural development (mPFC, mPOA and DRN), neurotransmission (mPOA), energy production (mPFC and mPOA), learning and memory (CA1), and intracellular signal transduction (DRN). Oxytocin (OXT) in the mPOA and 5-HTT in the DRN were strongly increased by 8-OH-DPAT. 5-HT_{1A} tended to increase in the mPFC, while 5-HT_7 was decreased in the CA1.

Conclusions. Brain region-specific alterations of gene expression regulating neural circuitries, energy demands and learning processes are associated with 8-OH-DPAT-induced decrease in female sexual receptivity and increase in pairmate aggression. Whether enhanced OXT expression in the mPOA disrupts the oxytocinergic regulation of social and sexual behavior, or presents a pharmacotherapeutic opportunity for the treatment of hypoactive sexual desire disorder (HSDD) in women, remains to be investigated.

INTRODUCTION

Personally distressing hypoactive sexual desire disorder (HSDD) is reported in an estimated 10% of women, but its psychopathogenesis is largely unknown. Causes for HSDD are multifactorial, including hormonal, neurobiological and psychosocial factors [1]. A twin/sibling study investigating the contributions of genetic and environmental influences on domains of the Female Sexual Function Index (FSFI) suggests a modest genetic influence on female sexual function, including desire [2]. Animal research, and a few clinical studies employing novel pharmacological targets to treat HSDD in women, point towards potential genes involved in female sexual function. Such candidates include genes encoding serotonin-1a and -2a receptors (5-HT_{1A}/5-HT_{2A}) [3, 4], dopamine [5] and dopamine D_4 receptor [6], oxytocin (OXT) [7] and oxytocin receptor (OTR) [8], vasopressin and AVP-1a receptor [8], estrogen (E2) and estrogen receptor alpha [9], gonadotropin-releasing hormone II [10], and melanocortin receptors [11]. Genetic research in female sexual dysfunction, including HSDD, however, lags far behind genetic research in other biomedical areas [12], and to the best of our knowledge, neither large-scale gene expression studies nor genome-wide association studies (GWAS) have been undertaken to investigate the genetic substrate of female sexual function and dysfunction.

Female marmoset monkeys *(Callithrix jacchus)* present an opportunity to explore sexually stimulatory or inhibitory pharmacological manipulations in a nonhuman primate model that readily translates to humans. Marmosets form long-term sexual relationships between male-female pairs [13, 14], in contrast to multiple-mating social structures of rats [15] and many nonhuman primates, including macaques [16]. By the display of species-specific proceptive behaviors, and by acceptance or rejection of a pairmate's sexual advances, female marmosets can influence male sexual behavior and readily promote, prevent or terminate sexual interactions [17, 18].

We have established a marmoset model for female sexual function that employs a standardized behavioral testing paradigm, permitting repeatable, quantitative exploration of neurally active compounds that enhance or diminish female marmoset sexual behavior [4, 10]. We have shown that repeated, daily administration of the $5\text{-HT}_{1A/7}$ agonist *R*-(+)-8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) diminishes female sexual receptivity and increases aggression with a male pairmate, as well as modulating other behavioral, endocrine and brain activity parameters, which correlate to the changes observed in sexual receptivity (Table 1; [4, 19, 20]).

We have previously developed a marmoset-specific DNA microarray (EUMAMA), the first microarray designed for this species, representing

Table 1. The effects of chronic 8-OH-DPAT administration on behavioral, endocrine and brain activity parameters in female marmosets. ACTH, adrenocorticotropic hormone; mOCC, medial occipital cortex.

Parameter	Direction of change	Correlation with female sexual receptivity	Ref.
Behavior			
Female sexual receptivity	Reduced	---	[4]
Aggression between pairmates	Increased	Negatively correlated	[4]
Endocrine			
ACTH levels at 15 min of restraint stress	Increased	Negatively correlated	[19]
Cortisol levels 3 h after restraint stress	Increased	Not correlated	[19]
Brain activity			
Glucose metabolism in mOCC	Reduced	Positively correlated	[20]

more than 1,500 transcripts [21]. Using the EUMAMA microarray we have shown that specific marmoset brain regions isolated by laser microdissection (LMD) technology show highly divergent expression profiles, underscoring the importance of characterizing gene expression in precisely isolated brain subregions of interest [21].

In the present study, we take advantage of the well-characterized effects of chronic 8-OH-DPAT administration on marmoset female sexual function to gain insight into 8-OH-DPAT-induced transcriptomic changes that may underlie the observed decrease in female sexual receptivity. Here, we present a gene expression study using large-scale expression profiling on brain tissue of the same female marmosets that were studied for sexual behavior. We applied LMD to isolate discrete brain regions relevant to female sexual behavior [22] or that have high 5-HT_{1A} density [23]. The selected brain regions comprised: (1) medial prefrontal cortex (mPFC), involved in executive and inhibitory regulation of behavior and receiving dopaminergic projections from the ventral tegmental area that are relevant to sexual behavior [24]; (2) medial preoptic area of the hypothalamus (mPOA), intimately involved in female sexual behavior [17, 22]; (3) CA1 field of the hippocampus (CA1), involved in sexually relevant memory processes [25]; and (4) dorsal raphé nucleus (DRN), containing 5-HT neurons

with extensive projections to cortical, hypothalamic and hippocampal sites, and auto-regulating presynaptic 5-HT_{1A} receptors [26].

Parallel to the large-scale microarray study, we followed a candidate gene approach using Real-time quantitative PCR (RTqPCR) to measure expression levels of genes not present on the EUMAMA microarray, but of high relevance to the serotonergic pharmacology of 8-OH-DPAT. The candidate genes included the 5-HT_{1A}, 5-HT_{2A} and 5-HT_{7} receptors, and the 5-HT transporter (5-HTT). Combining this candidate gene approach with the large-scale microarray experiment, we expect to determine a general transcriptomic response to chronic 8-OH-DPAT, mediated by altered expression of specific serotonergic markers that may underlie sexual rejection and aggression in female marmoset monkeys.

AIMS

The aim of the present study was to investigate the transcriptomic changes induced by chronic 8-OH-DPAT in brain areas that play a key role in regulating female sexual function and 5-HT neurotransmission. Identification of genes, functional gene classes and pathways associated with 8-OH-DPAT-mediated inhibition of female marmoset sexual receptivity may reveal neural molecular mechanisms involved in female sexual dysfunction.

METHODS

Animals and treatment

Animal experiments were conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act and its subsequent amendments. All animal procedures were reviewed and approved by the Graduate School Animal Care and Use Committee of the University of Wisconsin-Madison. The Wisconsin National Primate Research Center (WNPRC) is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care as part of the University of Wisconsin-Madison Graduate School. Eight adult (age 2-5 yr) nulliparous captive-born common marmoset *(Callithrix jacchus)* females were pair housed with similarly aged male partners at the WNPRC for 8-20 months before onset of this study. Females were housed with the same male partner for the entire study and were ovariectomized and primed with mid-follicular phase estradiol levels for the entire duration of this study using estradiol capsule implants. Estradiol priming and surgical procedures were performed as described in Barnett et al [10].

Four of the eight females received daily subcutaneous (SC) injections of 8-OH-DPAT (N=4; 0.1 mg/kg in 0.4 ml/kg saline) for 16 weeks, while the remaining four females received daily SC injections of vehicle (N=4; 0.4 ml/kg saline). Animals were euthanized 15-19 hours after the last 8-OH-DPAT or vehicle injection, when circulating 8-OH-DPAT levels were low to absent [4]. Harvesting of neural tissue at a time when circulating 8-OH-DPAT levels were minimal permitted assessment of changes in gene expression induced by repeated dosing of 8-OH-DPAT, while avoiding potential immediate effects of elevated 8-OH-DPAT on transcription. Thus, transcriptomic changes were likely consequences of long-term treatment; effects of single dosing were not assessed in this study.

Between 08:30h-10:30h on the day of tissue harvest, marmosets were anesthetized with an intramuscular (IM) injection of 15 mg/kg ketamine within 1-3 minutes (1.5 min ± 0.3 min) of cage entry, and received a lethal injection of pentobarbital (50 mg/kg) administered intravenously (IV) within 7-13 minutes (10.1 min ± 0.7 min) of cage entry. The brains were snap-frozen in isopentane (-78.5°C) within 6-16 minutes (9.1 min ± 1.1 min) of euthanasia and stored at -80°C until further use.

Laser microdissection (LMD)

Laser microdissection was performed as previously described [27]. Briefly, coronal cryosections (8 µm) containing 1) mPFC, 2) mPOA, 3) CA1, and 4) DRN were prepared according to available marmoset brain atlases [28, 29] at A16.0 – A17.5 (16.0 – 17.5 mm anterior to the interaural level) for mPFC, A10.0 for mPOA, A5.0 – A6.0 for CA1, and A1.5 – A2.0 for DRN samples [28]. Cryosections were mounted on PEN-membrane slides (1440-1000, PALM, Bernried, Germany) and stored at -80 °C. Before use, the sections were stained with hematoxylin and brain regions of interest were laser microdissected (PALM MicroBeam, Bernried, Germany; Figure 1). Per sample, an area of 1.5 – 6.0 x 106 µm2 of the relevant brain region was excised from several slices and pooled.

In one of the four 8-OH-DPAT treated brains, the DRN was damaged, likely due to brain collection/freezing procedures, and not further analyzed, leading to a N=3 8-OH-DPAT vs. N=4 Vehicle comparison for the DRN.

RNA isolation and linear amplification

Total RNA was isolated as described in [27]. 7.5 ng (if 28S/18S ratio >0.7) or 12.5 ng (if 28S/18S ratio <0.7) of total RNA was used as input to the Ovation Pico WTA System kit (NuGEN Technologies, San Carlos, CA, USA) and was subjected to reverse transcription using a mix of random and poly(T) primers,

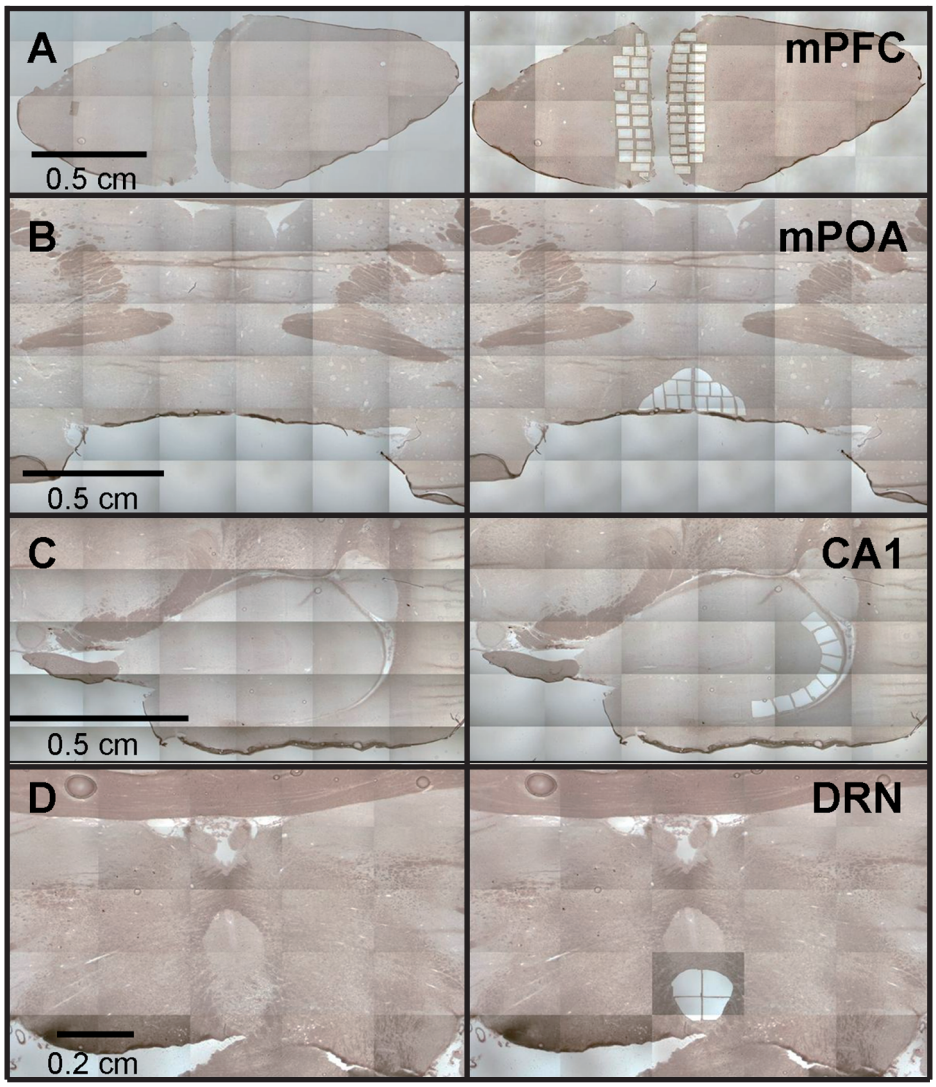


Figure 1. Examples of coronal sections containing mPFC **(A)**, mPOA **(B)**, CA1 **(C)** and DRN **(D)** before and after laser-microdissection. mPFC, medial prefrontal cortex; mPOA, medial preoptic area; CA1, CA1 field of the hippocampus; DRN, dorsal raphé nucleus.

followed by one round of linear amplification. 1.5 µl of amplified cDNA was analyzed and quantified on a NanoDrop ND-1000 spectrophotometer (Isogen Life Science, De Meern, The Netherlands). A total of 3.45 µg of amplified cDNA was fragmented and biotinylated using the Encore Biotin Module kit

(NuGEN Technologies, San Carlos, CA, USA) and subsequently hybridized to our custom EUMAMA marmoset-specific microarray (Affymetrix, Santa Clara, USA; [21]) at the MicroArray Department of the University of Amsterdam, The Netherlands, using an adapted protocol from NuGEN based on the manufacturer's recommendations (Affymetrix, Santa Clara, USA). A total of 31 microarrays were hybridized: four 8-OH-DPAT and four vehicle controls for each mPFC, mPOA and CA1, and three 8-OH-DPAT and four vehicle controls for the DRN.

Data Analysis

Microarray cell intensity files (CEL-files) were subjected to the Affymetrix Expression Console software (Affymetrix, Santa Clara, USA) to estimate signal intensities and signal reliabilities. All microarrays were background-corrected and normalized by a MAS5 statistical algorithm.

A separate probe set-level summarized data file was created for each brain region and analyzed using BRB-Array Tools version 4.2, an integrated package for the visualization and statistical analysis of DNA microarray gene expression data that operates as an add-in to Microsoft Excel [30]. Prefiltering of data was performed to exclude probe sets with unreliable detection in more than 50% of the samples and yielded 1241 (mPFC), 1268 (mPOA), 1221 (CA1) and 1160 (DRN) probe sets that were included in the statistical analysis. Differentially expressed genes between 8-OH-DPAT and vehicle treatment groups were identified per brain region using a univariate two-sample T-test (with random variance model). To increase the information that can be derived from the data regarding patterns of genes and pathways, rather than focusing on single genes, we chose a moderate statistical stringency of analysis, applying a maximal nominal significance level of $P < 0.05$ and no correction for multiple testing. Consequently, individual genes require independent follow-up.

Gene lists containing all significant genes at $P < 0.05$ or a trend towards altered expression ($P < 0.1$), were evaluated using the DAVID 6.7 Functional Annotation Tool [31], particularly suited to determine enriched Gene Ontology (GO) terms and signaling pathways associated with a given gene list.

Validation of microarray results

Real-time quantitative PCR (RTqPCR) was performed to confirm differential expression of genes indicated by the microarray analysis. Primers were designed in the same region of the transcript harboring the Affymetrix probe sets using Primer3 freeware. Primers were checked for specificity using BLAST (NCBI, Bethesda, MD, USA) and for hairpins and self-complementary using oligo 7 (MBI, Cascade, CO, USA; see Table 2, for RTqPCR primer pair

Table 2. RTqPCR primer pair sequences of normalization genes, validated genes and serotonergic candidate genes.

Gene	Forward primer sequence	Reverse primer sequence
Normalization genes		
TLN2	CTC-CAG-CGG-GCC-TGA-GAC-CT	CTC-GGT-GCA-TCT-GCC-CAA-CCA
XRCC6	CCT-AGC-AAT-ACC-AAG-AGG-TCG-CAG	GCC-CAT-GAA-CAT-CAA-ACC-TGG-ATC-A
ACTB	TGG-CAG-TTT-TCC-AGT-GCA-CA	TGG-ACC-CTC-GGT-TTT-CAA-CA
Validated genes		
CASP9	AGG-CTC-TTA-GCA-GCT-TCC-AGA-TTG-T	GCC-ACT-GCT-CAG-GAG-GCC-AT
BRPF3	TGG-CCA-CAC-AGT-GTC-GTT-GAT-TT	AGT-CCA-CAG-CTG-ACC-AAC-TAC-CT
LGI1	CAG-GCA-CCA-AGA-TCA-TTC-ACG-CA	ACA-GAA-TTT-GGT-GTC-TCA-TGC-GCT
OXT	GCC-TCG-CCT-GCT-GTC-TGC-TC	AGG-TCC-AGC-GCG-GCC-CTC-TT
DYRK3	TTT-TAA-GCT-GCC-TCC-GGT-CGT	TGC-ATC-TCT-GGG-TAT-ATC-TCT-GTC-T
FADS3	GCC-CTT-CCT-TAC-TGC-GCT-GGT	TTG-GCT-GCT-GGT-GCC-CTG-AG
P4HB	AGC-CAG-GCT-GGA-CAC-AGT-CAG-T	CCC-ATG-GCC-GGA-AGC-AAC-CA
DNPEP	ACA-AGC-ATG-AGG-AGA-ACC-ATC-GGC	TCA-GGG-CCT-CTG-ACA-CTG-CG
OGDH	GGC-ACA-GAG-GGC-AGC-TTT-GGT	AGG-CTC-CAG-GTC-CTG-TCT-GAG-C
GATAD2A	ACG-CTG-CAG-AGG-CAA-GAC-CT	GGG-CCG-GGA-GCA-AAA-CCA-GC
N-PAC	TTG-CAG-AAG-GAA-GGC-CAG-CA	CCA-ACA-GGC-ATC-TTC-CCT-GCT-CT
MTMR6	TGT-GCC-TTG-GTG-TTC-CAG-TCA	TGA-TTT-CTC-AGT-CCA-TCT-GTG-AGC-A
ADCY1	GGG-AGC-CAA-TTT-GGA-GTG-TGT-GTC-A	CAC-AAC-CCA-AGA-AGG-CCA-CCT-GTC
GMPR2	TCT-GCC-CTC-CCA-AGA-CAC-CAG-T	GGA-GCC-CTC-AGG-AGT-GAG-CCA-A
ENC1	TGT-GTC-ATT-GCG-TGG-CTT-TTG-A	GCC-CAA-CGC-TTA-ACA-CGG-CT
MAP1LC3B	TTC-CCT-CCG-TCT-GGG-CCC-TCT-A	CAA-ACG-CAT-GCA-GCG-GGA-AAG-C
VDAC1	GCT-CGC-TGC-TTG-GTG-GTG-TGT	GCC-CTG-CAG-ACC-TTT-GGT-GCA-A
Serotonergic candidate genes		
5-HTT	TAC-ACG-CGC-CAC-GTT-CTG-CA	TGC-AAA-GGG-CCA-GCT-GCC-AG
5-HT1A	CAG-CTA-GAG-CCT-CTG-AAC-GCC-TTG	AAG-GTA-TCC-CGG-AGG-AGC-AGC-T
5-HT2A	CGT-CAT-CAT-GGC-AGT-GTC-CCT-AGA	CAG-AGG-CCA-CCG-GTA-TCC-ATA-CAG
5-HT7	GCG-TGA-TCA-GCA-TCG-ACA-GGT-ACC-T	ATG-GAG-GCG-GAG-AGA-AGC-CAG-AC

sequences).

A selection of responsive genes was validated in the same experimental cDNA samples that were used to hybridize to the microarray, using RTqPCR on a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and SYBR Green I dye (Applied Biosystems, Bleiswijk, The Netherlands). Standard curves were generated by performing PCR reactions on a serial dilution of the cDNA corresponding to 100%, 25%, 6.25%, 1.56%, 0.39% and 0.10% of 12 ng input cDNA. A negative control (no template control; NTC) was included in all PCR runs, and all samples were measured in triplicates.

A suitable normalization gene for each individual brain region was selected from the microarray data based on high and stable expression in both treatment groups. Stability in expression in controls and 8-OH-DPAT treated animals was confirmed by RTqPCR (data not shown). Talin 2 (TLN2) was used for the mPFC, X-ray repair complementing defective repair in Chinese hamster cells 6 (XRCC6) for the mPOA, and beta actin (ACTB) for both CA1 and DRN. Expression levels of the validated genes were normalized against the expression levels of the respective normalization gene. To test the directionality of the gene expression change predicted by the microarray, a one-tailed, independent-samples T-test was used.

Candidate gene approach by RTqPCR

Gene expression of the main pharmacological targets of 8-OH-DPAT, 5-HT_{1A} and 5-HT_{7}, as well as other serotonergic genes that may alter expression in response to chronic 8-OH-DPAT, 5-HT_{2A}, 5-HTT, were measured by RTqPCR and analyzed as described above. None of these candidate genes are present on the EUMAMA microarray. Primers were designed in the coding region of the transcripts using the same criteria as mentioned above.

RESULTS

Effects of chronic 8-OH-DPAT on transcriptome in mPFC, mPOA, CA1 and DRN

The transcriptional response to chronic 8-OH-DPAT was the largest in the mPOA, with 68 genes significantly ($P < 0.05$) altered, followed by DRN (61), mPFC (55) and CA1 (45). There was only a small overlap of genes altered by 8-OH-DPAT between brain regions (Figure 2), indicating mostly brain region-specific effects of 8-OH-DPAT at the molecular level.

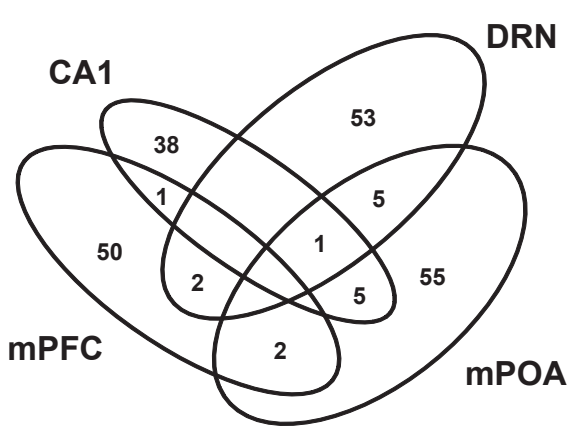


Figure 2. Number of genes per brain region that were significantly altered by 8-OH-DPAT. $P < 0.05$, MAS5 analysis.

In addition, 8-OH-DPAT-induced differential gene expression was most pronounced in the mPOA in terms of magnitude of gene expression change (Figure 3). Approximately 30% of genes altered by 8-OH-DPAT displayed >2-fold changes in expression, while only 20% had fold changes <1.5 (Figure 3B). Expression fold-changes were more moderate in the CA1 (Figure 3C), and even more subtle in the mPFC and DRN (Figures 3A and 3D). A full list of significantly altered genes will be made available online as *Supplementary Materials*.

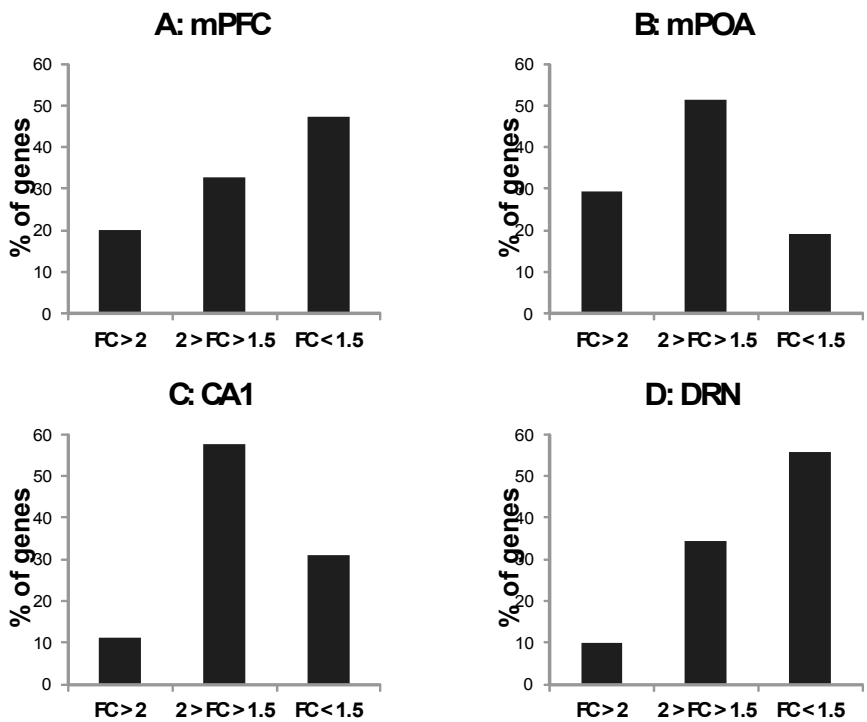


Figure 3. The magnitude of the transcriptional response to 8-OH-DPAT in the mPFC **(A)**, mPOA **(B)**, CA1 **(C)** and DRN **(D)**, showing that the majority of fold-changes are less than 2-fold. FC, fold-change.

Validation of microarray results

A total of 33 genes (7 – 10 genes per brain region) were selected for RTqPCR validation to confirm the 8-OH-DPAT-induced differential expression

predicted by the microarray. Ten genes (30%) were successfully validated by RTqPCR at a significance level of $P < 0.05$. Applying a less stringent criterion ($P < 0.1$), the number of validated genes increased to 17 (52%). There were clear differences between brain regions in terms of validation success rate. While validation was most successful in the mPOA and DRN (57% – 90%; $P < 0.1$), it was less obvious for mPFC and CA1 (25%; $P < 0.1$). A validation summary is presented in Table 3. Overall, microarray and RTqPCR results from the combined analyses of all four brain regions correlated strongly in terms of fold-changes ($r = 0.897$, $p < 0.001$; Figure 4A). When only successfully validated genes ($P < 0.1$) were included, the correlation coefficient was similar ($r = 0.918$, $p < 0.001$; Figure 4B). The successfully validated genes covered a wide range of different functions, ranging from regulation of gene transcription, protein phosphorylation and metabolism to regulation of neurogenesis, cognition and behavior. Oxytocin (OXT), however, clearly stood out from all other successfully validated genes in terms of comparative magnitude of the 8-OH-DPAT-induced change in expression. Microarray analysis predicted a >10-fold increase in OXT expression, and this magnitude difference was confirmed by RTqPCR. A full list of the validated genes with their associated functions is presented in Table 4.

Table 3. Overview of validation of microarray predictions by RTqPCR. The numbers between parentheses indicate the number of genes validated.

Region	Validation ($p<0.05$)	Validation ($p<0.1$)
mPFC	13% (1/8)	25% (2/8)
mPOA	50% (5/10)	90% (9/10)
CA1	0% (0/8)	25% (2/8)
DRN	57% (4/7)	57% (4/7)
All	**30% (10/33)**	**52% (17/33)**

Gene ontology classes enriched in the differentially expressed genes

8-OH-DPAT induced changes in gene expression ($p<0.1$) were analyzed using the DAVID 6.7 Functional Annotation Tool. Table 5 lists a selection of clusters of GO terms altered by 8-OH-DPAT. *Supplementary Materials* with a

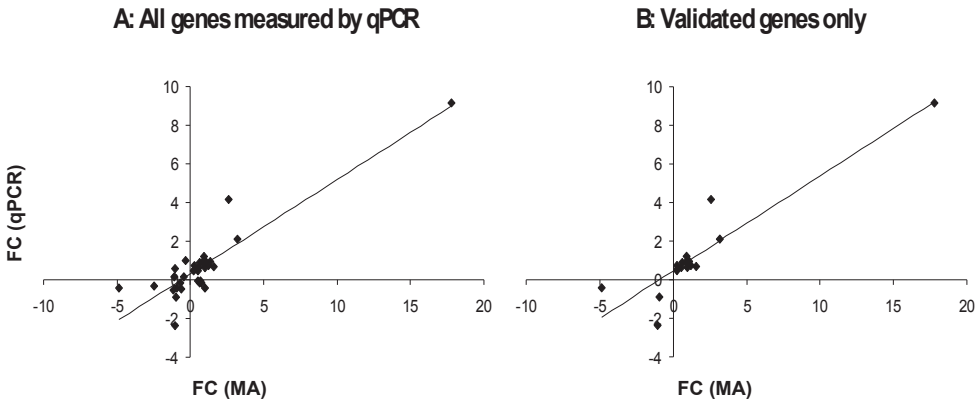


Figure 4. Linear regression analysis of fold-changes from microarray and RTqPCR data of all four brain regions combined, including **(A)** all genes tested, or **(B)** successfully validated genes only. FC, fold-change; MA, microarray.

Table 4. List of validated genes. P, p-value; FC, fold-change; MA, microarray; Dir, direction of change.

Gene ID	Region	P		FC		Dir.	Gene name	Gene Function
		qPCR	MA	qPCR	MA			
Casp9	mPFC	0.03	0.001	3.4	2.0	↓	Caspase 9	Induction of apoptosis
Brpf3	mPFC	0.06	0.005	2.0	2.1	↑	Bromodomain and PHD finger containing, 3	DNA-dependent regulation of transcription
Lgi1	mPOA	0.01	<0.001	1.9	2.0	↓	Leucine-rich repeat LGI family, member 1	Predisposes to epilepsy; expression reduced in brain tumors (metastasis suppressor?)
Oxt	mPOA	0.03	0.015	10.2	18.8	↑	Oxytocin-neurophysin	Involved in cognition, tolerance, adaptation and complex social, sexual and maternal behavior
Dyrk3	mPOA	0.09	0.001	1.7	2.2	↑	Dual-specificity tyrosine-(Y-)phosphorylation regulated kinase 3	Peptidyl-tyrosine phosphorylation
Fads3	mPOA	0.06	0.002	1.7	2.6	↑	Fatty acid desaturase 3	Fatty acid biosynthesis
P4hb	mPOA	0.02	0.010	1.6	1.6	↑	Prolyl 4-hydroxylase, beta polypeptide	Protein folding; electron carrier activity
Dnpep	mPOA	0.10	0.011	1.9	1.6	↑	Aspartyl aminopeptidase	Proteolysis
Ogdh	mPOA	0.04	0.012	3.1	4.2	↑	Oxoglutarate dehydrogenase (lipoamide)	Glycolysis; metabolism
Gatad2a	mPOA	0.04	0.002	2.2	1.9	↑	GATA zinc finger domain containing 2A	Negative regulation of transcription; transcription factor activity
N-pac	mPOA	0.07	0.009	1.9	1.8	↑	Cytokine-like nuclear factor n-pac	Phosphogluconate dehydrogenase (decarboxylating) activity
Mtmr6	CA1	0.09	0.005	1.4	5.9	↓	Myotubularin related protein 6	Protein amino acid dephosphorylation; protein tyrosine phosphatase activity
Adcy1	CA1	0.10	0.004	1.6	2.0	↑	Adenylate cyclase 1 (brain)	cAMP biosynthesis; intracellular signaling; calmodulin binding; overexpression enhances recognition memory and LTP in mouse
Gmpr2	DRN	0.01	0.004	1.5	1.3	↑	Guanosine monophosphate reductase 2	GMP catabolism; GMP reductase activity
Enc1	DRN	<0.01	0.006	5.2	3.6	↑	Ectodermal-neural cortex 1	Proteolysis; actin binding; cysteine-type endopeptidase activity; expression strongly induced by p53; interacts with Rb1
Map1lc3b	DRN	<0.01	0.004	1.7	1.3	↑	Microtubule-associated protein 1 light chain 3 beta	Ubiquitin cycle; autophagy; microtubule-based process; neurogenesis
Vdac1	DRN	0.01	0.006	1.5	1.2	↑	Voltage-dependent anion channel 1	Ion transport; apoptosis; synaptic transmission; learning; behavioral fear response; voltage-gated ion-selective channel activity

Table 5. A selection of GO terms and genes, grouped into functionally related clusters that were altered by 8-OH-DPAT (p-values calculated by DAVID 6.7 Functional Annotation Tool). GO, gene ontology; BP, biological process; CC, cellular compartment.

GO Term ID	GO Term Description	mPFC	mPOA	CA1	DRN
Energy production					
GO:0006091 (BP)	Generation of precursor metabolites and energy	0.004	0.010		
GO:0045333 (BP)	Cellular respiration	0.025	0.003		
GO:0006099 (BP)	Tricarboxylic acid cycle	0.010	0.008		
Mitochondrion					
GO:0005739 (CC)	Mitochondrion			0.029	0.009
Neural development					
GO:0000902 (BP)	Cell morphogenesis	0.028			0.009
GO:0043005 (CC)	Neuron projection	0.020	0.043		0.022
GO:0007409 (BP)	Axonogenesis				0.009
GO:0030424 (CC)	Axon	0.018	0.012		
Neurotransmission					
GO:0032844 (BP)	Regulation of homeostatic process		0.030		
GO:0050804 (BP)	Regulation of synaptic transmission		0.047		
Protein transport					
GO:0015031 (BP)	Protein transport		0.005		0.028
Intracellular signal transduction					
GO:0010627 (BP)	Regulation of protein kinase cascade				0.001
GO:0009967 (BP)	Positive regulation of signal transduction				0.003
GO:0080135 (BP)	Regulation of cellular response to stress				0.030
GO:0043408 (BP)	Regulation of MAPKKK cascade				0.035
Ion channel activity					
GO:0022836 (MF)	Gated channel activity			0.024	
Learning and memory					
GO:0007611 (BP)	Learning or memory			0.006	

complete list of terms will be published online. A significant P-value indicates that genes related to a given term are overrepresented in the list of differentially expressed genes.

Both in mPFC and mPOA, 8-OH-DPAT affected the expression of gene classes important to energy production and neural development. In the mPOA, the GO term generation of precursor metabolites and energy includes genes such as fatty acid desaturase 3 (FADS3) and oxoglutarate dehydrogenase (OGDH), which were both validated by RTqPCR. Additional gene classes altered in the mPOA, but not in the mPFC, are related to neurotransmission and protein transport. Altered expression of both OXT, implicated in neurotransmission [32] and complex sexual and social behaviors [33], and leucine-rich, glioma inactivated 1 (LGI1), which plays a role in the regulation of synaptic transmission, was validated in the mPOA by RTqPCR.

8-OH-DPAT-induced changes in gene expression in the CA1 were mostly unique to this brain region and included gene classes important to mitochondrial function, ion channel activity and learning and memory. The expression change of myotubularin related protein 6 (MTMR6, validated by RTqPCR), part of the GO term ion channel activity, and of adenylate cyclase 1 (ADCY1), encoding an important enzyme that is activated by G-protein signaling and related to the overrepresented GO term learning or memory, were confirmed by RTqPCR.

Similar to mPFC and mPOA, 8-OH-DPAT affected gene classes related to neural development in the DRN. Other gene classes altered by 8-OH-DPAT in the DRN are related to mitochondrial function (including the validated gene voltage-dependent anion channel 1; VDAC1), protein transport and intracellular signal transduction. While mitochondrial function and protein transport were also affected in other brain regions (see Table 5), changes in intracellular signal transduction were specific to the DRN.

Expression of candidate genes

Expression of 5-HT_{1A}, 5-HT_{2A}, 5-HT_{7} and 5-HTT was measured in all four brain regions and compared between treatments (Figure 5). There was a trend for an increase in 5-HT_{1A} transcripts in the mPFC ($p<0.06$), while expression of 5-HT_{1A} autoreceptors in the DRN was not altered after chronic 8-OH-DPAT treatment. There was also a trend for decreased 5-HT_{7} expression in the CA1 ($p<0.08$). 8-OH-DPAT elevated the expression of 5-HTT in the DRN ($p<0.05$). The increased 5-HTT expression was pronounced (approx. 15x), particularly considering the relatively subtle changes in expression in other genes. 5-HT_{2A} expression was not altered in any of the brain regions analyzed.

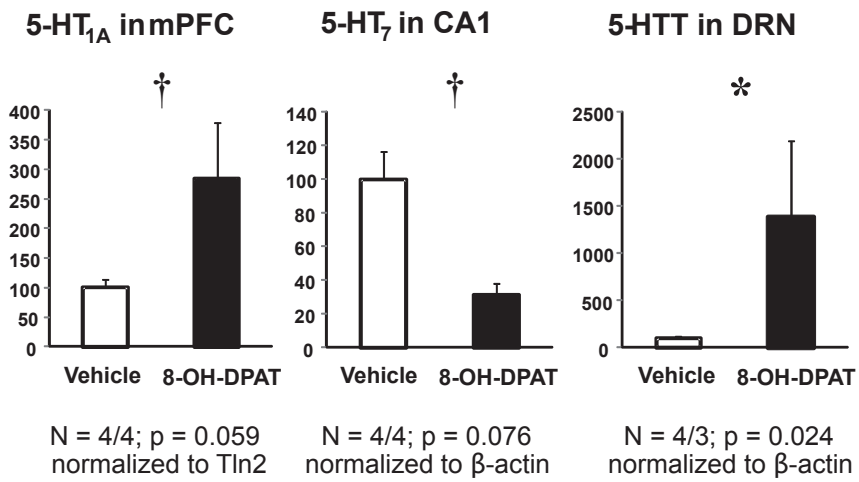


Figure 5. The expression profiles of 5-HT$_{1A}$ (in the mPFC), 5-HT$_{7}$ (in the CA1) and 5-HTT (in the DRN) in response to chronic 8-OH-DPAT, as measured by RTqPCR. The expression levels are normalized to brain area specific normalization genes and are presented relative to those in the vehicle group (100%).

DISCUSSION

Despite the relative prevalence of personally distressing HSDD, nonhuman primate studies targeting potential genomic mechanisms of female sexual dysfunction are lacking. Studies using novel pharmacotherapeutic targets have so far only provided indirect evidence for potential candidate genes involved in HSDD, such as serotonin and dopamine receptors [3, 5]. To the best of our knowledge, we present the first large-scale transcriptomic study to investigate the sexually suppressive effects of a pharmacological manipulation on gene expression of more than 1500 genes in the nonhuman primate brain.

In rodents, microarray experiments have focused on the estrogenic regulation of gene expression in the hypothalamus [34]. Estrogen function in female rodents is intimately linked to sexual behavior. Estrogen facilitates sexually proceptive (appetitive) and receptive (consummatory) behavior in both rodents [35, 36] and nonhuman primates [37]. The studies show that estrogen priming in female rats leads to activation of genes that code for neurotransmitter receptors (α_{1B} adrenoreceptor and muscarinic receptors) or facilitate neuronal growth in the ventromedial hypothalamic nucleus (VMH) [38]. Female rat estrogen priming also increases OXT receptor density in the VMH [39] and stimulates OXT release from dendrites or soma [40] of neurons

in the mPOA/medial basal hypothalamus [41] and magnocellular neurons of the paraventricular and supra-optic nuclei [40, 42]. The translation of rodent findings to humans, however, is difficult in light of the strict hormonal control of female sexual function in rodents [34] compared to humans [43]. In contrast to rodents, but in line with human studies, marmosets permit studies of female sexual behavior in the context of pair-bonds [4, 10]. Moreover, marmosets emulate humans in demonstrating a degree of emancipation of female sexual behavior from estrogen determination [10, 44].

Hypothalamic oxytocin is over-expressed after chronic 8-OH-DPAT

Probably the most intriguing result of this study is the strong increase in OXT mRNA expression in the mPOA after chronic 8-OH-DPAT. This finding is of particular interest as the function of hypothalamic OXT has been closely associated with social and sexual behavior in rodents, non-human primates and humans [45-47]. OXT expression in the paraventricular (PVN) and supraoptic nuclei (SON) of the hypothalamus can be induced by activation of 5-HT_{1A} receptors [42]. While the mPOA samples collected in the present study contain neither PVN nor SON, oxytocin expression in areas overlapping with the mPOA has previously been demonstrated in the female marmoset monkey [48]. The magnitude of the OXT increase in the present study (greater than 10-fold) is particularly striking, considering that the magnitude of 8-OH-DPAT induced changes in gene expression was mostly below 2-fold.

There is strong evidence for functional interaction of the serotonergic and oxytocinergic systems. Immunocytochemical and double-immunofluorescent techniques reveal that the density of 5-HTT fibers follows the distribution of OXT-containing neurons in the PVN and SON in macaques [49]. The 5-HT_{1A} agonist alnespirone [50], the 5-HT releasers p-choloroamphetamine and fenfluramine [51] as well as acute and chronic SSRI administration [52] stimulate OXT secretion in rats. Collectively, these studies support the notion that OXT synthesis and release is stimulated by 5-HT via $5HT_{1A}$ receptors.

Interestingly, there is an apparent discrepancy between the present marmoset data, which associates overexpression of hypothalamic OXT with reduced female sexual receptivity, increased aggression and increased stress responsiveness, and the large body of literature that reports OXT function as supporting pro-social and pro-sexual behavior, and reducing stress reactivity [46, 53-54]. A possible explanation might be that while chronic 8-OH-DPAT treated females exhibit increased OXT expression in the mPOA, as well as likely increased estradiol-supported, 8-OH-DPAT-induced OXT release from the dendrites or soma of oxytocinergic neurons in the PVN and SON [42], such OXT increases may be part of a compensatory mechanism for 8-OH-DPAT-

mediated sexual and social impairment. Evidence for increased OXT action, however, such as increased social or sexual behavior, is absent, suggesting diminished OXT signaling through OTR. In rodents, estrogen depletion due to ovariectomy reduces or even abolishes OTR density in a brain region-specific manner [39, 55], which might not be fully restored by the low, mid-follicular phase level estradiol replacement used in our marmoset model. In addition, chronic 8-OH-DPAT diminishes estradiol receptor expression in the hippocampus of ovariectomized rats [56]. Estrogen priming is indeed necessary for OXT-induced facilitation of female sexual receptivity, at least in rodents [57]. Thus, with minimal expression of its receptor due to chronic 8-OH-DPAT in combination with relatively low circulating estradiol levels compared to mid-cycle and luteal phase levels in ovary-intact females, enhanced hypothalamic OXT expression may have little functional impact. Validation of this hypothesis requires further studies of OTR expression and binding in the marmoset brain. The potential for pharmacotherapeutic treatment of HSDD, however, may arise through development of a serotonergic compound that increases hypothalamic OXT without impairing OTR function. We recently reported that female marmosets chronically treated with flibanserin, a 5-HT_{1A} agonist and 5-HT_{2A} antagonist, attract more male sexual interest and show increased social grooming between partners, contrasting the behavioral effects of 8-OH-DPAT [4]. In this context, it would be of particular interest to investigate whether the pro-sexual and pro-social effects of flibanserin in the female marmoset are mediated by increased hypothalamic OXT release that is not diminished by impaired action.

An alternative explanation for the paradoxical association of increased hypothalamic OXT expression and reduced sexual receptivity, increased aggression and stress responsiveness may come from the magnitude of the OXT elevation in the mPOA. While studies that link increased social behavior to elevated OXT mRNA levels report small fold-changes, typically below 1.5-fold [42, 58], the more than 10-fold increase in hypothalamic OXT transcription in 8-OH-DPAT treated marmosets might reflect a major disruption of a finely tuned balance in the oxytocinergic regulation of female social and sexual behavior, and stress responsiveness. OXT furthermore binds with a low affinity to vasopressin-1a receptors (AVP-1a) [59], including marmosets [60] and humans [61]. A more than 10-fold increased OXT expression might thus lead to enhanced AVP-1a activity in the mPOA of 8-OH-DPAT, but not in vehicle treated animals, and result in sexual rejection [62] and aggression [63].

Brain region-specific effects of chronic 8-OH-DPAT

In agreement with Figure 1, the effect of 8-OH-DPAT on the expression

of individual genes is mostly region-specific, with only a small number of differentially expressed genes overlapping between brain regions. This finding was expected, given the vast differences in basal molecular make-up of different brain regions [27], including differential 5-HT_{1A} densities that likely underlie the region-specific, 8-OH-DPAT induced transcriptomic changes. The regional specificity of 8-OH-DPAT-induced transcriptomic changes is also reflected in brain region-specific alterations of several classes of functionally related genes (Table 3), such as a mPOA-specific change in neurotransmission (GO term regulation of synaptic transmission), CA1-specific changes in ion channel activity (GO term gated channel activity) and learning and memory (GO term learning or memory), and a DRN-specific change in intracellular signal transduction (GO terms regulation of protein kinase cascade, positive regulation of signal transduction and regulation of MAPKKK cascade).

Not surprisingly, 8-OH-DPAT-induced alteration of the GO term learning or memory was restricted to the hippocampal CA1 subregion, a structure that is intimately implicated in episodic and semantic memory processes [64, 65]. In mice, overexpression of ADCY1 leads to elevated long-term potentiation (LTP), increased recognition memory and slower rates of extinction of contextual memory [66]. 8-OH-DPAT increased transcription of ADCY1 in the marmoset CA1, suggesting that memory function could play a role in altered pairmate interaction dynamics. These changes might be caused by 8-OH-DPAT-induced downregulation of 5-HT_7 receptors in the CA1 (Figure 4), a hypothesis that is supported by rodent studies that implicate 5-HT_7 function in contextual learning [67] and memory consolidation [68].

Brain region-overlapping effects of chronic 8-OH-DPAT

Interestingly, and despite the small number of overlapping genes between brain regions, 8-OH-DPAT affected several gene classes that are similar between brain regions and comprise identical or closely related GO terms. Energy production, for example, including the GO terms generation of precursor metabolites and energy, cellular respiration and tricarboxylic acid cycle, was affected by 8-OH-DPAT in mPFC and mPOA, which are both major postsynaptic projection areas of serotonergic neurons originating from the raphé nuclei [69]. Changes in energy production may reflect altered energy demands due to a changed state of brain activation in the mPFC and mPOA following 8-OH-DPAT [20, 70], which may be mediated by OXT [71] and may underlie the observed changes in sexual and social behavior after chronic 8-OH-DPAT.

As another example of a more global effect of 8-OH-DPAT, alterations of gene classes related to neural development in mPFC, mPOA and DRN, and to

neurotransmission in the mPOA, implicate that chronic administration of 8-OH-DPAT leads to a global modification of neural circuitries and communication between neurons. The GO terms axon/axonogenesis and neuron projection were significantly altered in all three regions. Increased expression of genes involved in axon formation, axon guidance and neuron migration in the mPFC and mPOA, including OXT, suggests that chronic activation of 5-HT_{1A} receptors by 8-OH-DPAT likely leads to a structural reorganization of neural circuits within the serotonergic projection areas. In rodents, altered expression of genes involved in neuronal growth has been described and linked to altered sociosexual behaviors [72].

Candidate gene approach: Changes in serotonin receptor and transporter expression may underlie altered signal transduction, neural development and oxytocin function

The 5-HT_{1A} and 5-HT_7 receptors are the primary pharmacological targets of 8-OH-DPAT and are both coupled to G-proteins to induce intracellular signal transduction cascades upon activation [73]. The DRN is characterized by high presynaptic 5-HT_{1A} and moderate 5-HT_7 density [74, 75]. It is therefore not surprising that 8-OH-DPAT affected GO terms associated with intracellular signal transduction in the DRN. The 5-HT_{1A} receptor couples negatively via G-proteins (α_i) to adenylate cyclase and promotes inward rectifying K^+ currents producing hyperpolarization [76], while the 5-HT_7 receptor couples to $G\alpha_s$ and activates both G_s-sensitive and G_s-insensitive adenylate cyclase isoforms. 5-HT_{1A} receptors in transfected cell lines decrease intracellular Ca^{2+} and activate phospholipase C, while 5-HT_7 receptors increase intracellular Ca^{2+} (reviewed in [73]). Given the high density of 5-HT_{1A} receptors on the presynaptic soma of serotonergic neurons, the present results indicate that 8-OH-DPAT may alter MAPKKK cascades in the DRN via inhibition of adenylate cyclase to modify downstream mitochondrial function (GO term mitochondrion), protein transport [77] and neural development (GO terms cell morphogenesis, neuron projection and axonogenesis). Changes in the postsynaptic projection areas (mPFC, mPOA and CA1) could either be indirect results of diminished serotonergic neurotransmission due to altered DRN function, or due to direct activation of postsynaptic 5-HT_{1A} and 5-HT_7 receptors by 8-OH-DPAT.

8-OH-DPAT drastically increased 5-HTT expression in the DRN, and tended to increase 5-HT_{1A} expression in the mPFC (Figure 4). Activation of 5-HT_{1A} autoreceptors in the DRN by 8-OH-DPAT likely suppresses serotonergic activity [73], which might trigger a compensatory upregulation of 5-HTT in the DRN and of 5-HT_{1A} in the mPFC to restore the serotonergic tone. In contrast, the expression of 5-HT_7 in the CA1 tended to be decreased by chronic 8-OH-

DPAT, indicating a brain region-specific desensitization of 5-HT_7 receptors. Repeated, 8-OH-DPAT induced stimulation of normally expressed 5-HT_{1A} receptors in the mPOA might present a direct mechanism for increased hypothalamic OXT expression [42].

Methodological considerations

The current study presents a transcriptomic conclusion of a long-term marmoset experiment designed to explore the effects of chronic serotonergic manipulation on female sexual and social behavior [4], endocrine function [19] and neural activity parameters [20]. In contrast to our previous in vivo studies in which different treatments were administered to the same subjects, the nature of this transcriptomic study requires a between-subject comparison. Due to small group sizes (n=4), and to decrease variation in expression profiles, we did not experimentally explore the potential effects of contrasting 8-OH-DPAT induced changes in gene expression in females with and without estradiol replacement, as we provided estradiol replacement to all test subjects for the entire duration of the study. The estradiol regimen chosen for this study provides the ovariectomized female marmosets with estradiol levels comparable to the physiological levels previously reported for female marmosets in the mid-follicular phase of the ovarian cycle, supporting modest expression of female marmoset sexual behavior [10]. Estradiol priming in rodents is necessary for the full expression of female sexual behavior [36], including oxytocin-induced facilitation of sexual receptivity [57], and is also required for the activation of genes coding for oxytocin, norepinephrine and acetylcholine receptors [38, 39, 55]. Thus, estradiol replacement was the regimen of choice for this study, despite that it has surprisingly been without effects on in vivo results in the female marmoset [4].

Validation of microarray predictions by RTqPCR was successful in 52% of the measured genes (including seven genes that were just below significance), with notable differences between brain regions. Given the small responses with the majority below two-fold, validation success rates were reasonably high for mPOA and DRN (57 – 90%). Validation was however low at 25% for mPFC and CA1, indicating that microarray data should be cautiously interpreted for these two regions. A 1.5 – 2-fold change is commonly considered as cutoff, below which microarray and RTqPCR data begin to lose correlation [78]. Despite the small changes, the correlation between microarray and RTqPCR data in this study was very strong ($r = 0.897$, including non-validated genes, all four brain regions combined; Figure 3), reinforcing the reliability of the microarray analysis.

CONCLUSIONS

In a marmoset model of hypoactive female sexual function, we demonstrate that chronic 5-HT_{1A} activation leads to lasting, brain region-specific transcriptomic changes. Analysis of classes of functionally related genes revealed that modification of neural circuitries in the mPFC, mPOA and DRN, altered energy demands in the mPFC and mPOA, and altered learning and memory may underlie 8-OH-DPAT-induced decrease in sexual receptivity, increase in aggression towards the male pairmate, and higher stress reactivity. 5-HTT expression in the DRN is increased, possibly via alterations in intracellular signal transduction within the DRN induced by chronic 5-HT_{1A} autoreceptor activation. Chronic post-synaptic 5-HT_{1A} activation in the mPOA could be responsible for augmented hypothalamic OXT expression in 8-OH-DPAT treated female marmosets. Enhanced OXT expression in the mPOA may disrupt the oxytocinergic regulation of social and sexual behavior.

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CHAPTER 6

General Discussion

TABLE OF CONTENTS

The main goal of this thesis is to investigate serotonergic (5-HT) regulation of female sexual behavior in marmoset monkeys using the 5-HT_{1A} agonist/ 5-HT_{2A} antagonist flibanserin and the 5-HT_{1A} agonist 8-OH-DPAT. The specific objectives are to assess the effects of chronic flibanserin and 8-OH-DPAT treatments on (1) sexual and social behavior, (2) hypothalamic-pituitary-adrenal (HPA) axis function, (3) brain activity, and (4) gene transcription in the brain.[1]

1. OVERVIEW OF THE EFFECTS OF FLIBANSERIN AND 8-OH-DPAT IN THE FEMALE MARMOSET MONKEY

Table 1 presents an overview of the effects of repeated daily administration of flibanserin (15 mg/kg, PO) and 8-OH-DPAT (0.1 mg/kg, SC) on ovariectomized female marmosets. More detailed information regarding the timing of experiments with respect to treatment duration and regarding estradiol supplementation is shown in Chapter 1, Figure 7, *study design*.

1.1 Sexual and social behavior

The main objective in **Chapter 2** was to quantify the effects of chronic flibanserin and 8-OH-DPAT on sexual and social interactions between treated females and their untreated male pairmates. Repeated observations of the pairmates' interactions at reunion following a 90 minute separation revealed remarkable and contrasting differences between flibanserin and 8-OH-DPAT on marmoset pair behavior, despite the drugs' shared 5-HT_{1A} agonist properties.

Chronic flibanserin treatment increased the female's sexual attractiveness to her male pairmate (more frequent male inspection of the female's genital area) and grooming between pairmates. While increased grooming indicates a flibanserin-induced strengthening of the pair bond between marmoset partners, female sexual behavior *per se* was not altered by flibanserin. Chronic flibanserin also enhanced female genital and non-genital self-grooming. Notably, flibanserin administration did not cause an acute occurrence of the serotonin (5-HT) behavioral syndrome, commonly induced by other 5-HT_{1A} agonists, as well as by 5-$HT_{2A/2C}$ agonists [1-3].

In contrast to flibanserin, 8-OH-DPAT (0.1 mg/kg, SC, 4-16 weeks) transiently induced an acute 5-HT behavioral syndrome, which persisted during the course of chronic administration. Chronic 8-OH-DPAT decreased

1 Flibanserin data are not available for brain activity (analyses ongoing) and gene transcription (not assessed as per agreement with Boehringer Ingelheim, proprietor and developer of flibanserin).

Table 1. Summary of chronic flibanserin and 8-OH-DPAT actions in the female marmoset monkey (cumulated data from Chapters 2-5). ↑, increased function; ↓, decreased function; ---, not changed; n/a, data not available; *, parameter correlated with female sexual receptivity; 8-OH-DPAT, *R*-(+)-8-hydroxy-2-(di-n-propylamino)tetralin; 5-HT_{1A}, serotonin-1A receptor; 5-HT_{2A}, serotonin-2A receptor; 5-HT_7, serotonin-7 receptor; 5-HTT, serotonin transporter; OXT, oxytocin; mPFC, medial prefrontal cortex; mPOA, medial preoptic area; VMH, ventromedial hypothalamic nucleus; CA1, CA1 field of the hippocampus; DRN, dorsal raphé nucleus; mOCC, medial occipital cortex.

Parameter		Flibanserin	8-OH-DPAT	Chapter
Behavior				2
Female sexual proceptivity		---	---	
Female sexual receptivity		---	↓*	
Female attractiveness to male		↑	(↓)*	
Allogrooming		↑	(↓)	
Aggression		---	↑*	
Female self-grooming		↑	---	
Serotonin behavioral syndrome		---	↑	
HPA axis				3
Morning basal cortisol		---	---	
5-HT_{1A} agonist challenge	ACTH	---	---	
	Cortisol	---	---	
Restraint test	ACTH 15 min	---	↑*	
	ACTH 30 min	↑	↑	
	ACTH 210 min	---	---	
	Cortisol 15 min	---	---	
	Cortisol 30 min	---	---	
	Cortisol 210 min	---	↑	
Cerebral glucose metabolism				4
mPFC		n/a	---	
mPOA		n/a	---	
VMH		n/a	---	
CA1		n/a	---	
DRN		n/a	---	
mOCC		n/a	↓*	
Gene transcription in the brain				5
5-HT_{1A} in mPFC		n/a	(↑)	
5-HT_{1A} in mPOA, CA1, DRN		n/a	---	
5-HT_{2A} in mPFC, mPOA, CA1, DRN		n/a	---	
5-HT_7 in CA1		n/a	(↓)	
5-HT_7 in mPFC, mPOA, DRN		n/a	---	
5-HTT in DRN		n/a	↑	
5-HTT in mPFC, mPOA, CA1		n/a	---	
OXT in mPOA		n/a	↑	
OXT in mPFC, CA1, DRN		n/a	---	
Molecular functions				5
Neural development		n/a	mPFC, mPOA, DRN	
Neurotransmission		n/a	mPOA	
Energy production		n/a	mPFC, mPOA	
Mitochondrial function		n/a	CA1, DRN	
Protein transport		n/a	mPOA, DRN	
Learning and memory		n/a	CA1	
Ion channel activity		n/a	CA1	
Intracellular signal transduction		n/a	DRN	

female sexual receptivity and increased aggressive interactions between male-female pairmates, while tending to decrease female sexual attractiveness and grooming between pairmates.

The data described in Chapter 2 resemble studies performed in female rodents that show that flibanserin increases the female's sexual attractiveness and display of proceptive and receptive sexual behavior [4], while 8-OH-DPAT inhibits lordosis [5], an indicator of female sexual receptivity in rodents. Chapter 2 thus presents the first study to confirm the sexual attractiveness-enhancing effects of flibanserin and sexually inhibitory effects of 8-OH-DPAT in a non-human primate. In humans, flibanserin improved satisfying sexual events, desire and the female sexual function index, and reduced sexual distress [6, 7]. While flibanserin did not alter the frequency of female sexual behavior in the marmoset, enhanced female sexual attractiveness and strengthened pair-bond between marmoset pairmates suggest that the observed beneficial effects of flibanserin on female sexual function in humans might be rooted on improved partner interactions and intimacy in the relationship between long-term sexual partners.

1.2 HPA axis function

In **Chapter 3**, the impact of chronic flibanserin and 8-OH-DPAT on HPA axis function was investigated to delineate the possibility of a HPA axis-mediated mechanism in the serotonergic regulation of female sexual behavior.

Chronic flibanserin neither affected circulating morning basal cortisol levels, nor ACTH and cortisol responses to an acute 5-HT_{1A} agonist challenge. Flibanserin treated females, however, displayed an increased ACTH response at 30 minutes during a restraint test, while cortisol levels were not altered during the 30 minutes of restraint, nor at 3 hours after return to the home cage.

Similar to flibanserin, neither circulating morning basal cortisol levels nor ACTH and cortisol responses to an acute 5-HT_{1A} agonist challenge were altered by chronic 8-OH-DPAT. The restraint-induced increase in ACTH was enhanced by chronic 8-OH-DPAT at both 15 and 30 minutes, while cortisol was elevated at 3 hours after return to the home cage, in comparison to vehicle control treatment. In 8-OH-DPAT, but not in flibanserin treated females, exaggerated ACTH responses at 15 minutes of restraint were associated with reduced female sexual receptivity, reduced sexual attractiveness and increased aggression between pairmates.

Importantly, enhanced ACTH responses to restraint were correlated with increased aggression and reduced sexual receptivity in 8-OH-DPAT treated female marmosets, supporting the hypothesis that increased stress reactivity might contribute to inhibition of sexual behavior. Such correlations were

absent in flibanserin treated females, suggesting no inhibitory role of HPA axis reactivity on female sexual function.

1.3 Cerebral glucose metabolism

In **Chapter 4**, a PET/MRI functional brain imaging experiment was described that was designed to measure cerebral glucose metabolism, an indicator of neural activity, in chronic 8-OH-DPAT or vehicle treated female marmosets during sexual and social interactions after a 90 minute separation from their male pairmates. Radiolabeled [^{18}F]fluorodeoxyglucose (FDG) was infused to the femoral vein immediately prior to a 30 minute pair test. The females were subsequently imaged by PET under isofluorane anesthesia. Structural MRI scans were recorded and overlaid on the PET images to improve the visualization of anatomical structures. PET scans using 5-HT$_{1A}$- and 5-HTT-specific radiotracers were also performed. Data processing and analysis of the latter scans are still ongoing, and the results are not within the scope of this thesis.

8-OH-DPAT decreased glucose metabolism, an indicator of neural activity, in the females' medial occipital cortex (mOCC) while interacting with their male partners. Decreased neural activity in the mOCC was associated with reduced female sexual receptivity, suggesting 8-OH-DPAT induced altered female perception of interactions with their male pairmate. However, glucose metabolism in pre-defined regions of interest (ROI), the medial prefrontal cortex (mPFC), medial preoptic area (mPOA), ventromedial hypothalamic nucleus (VMH), CA1 field of the hippocampus (CA1) and dorsal raphé nucleus (DRN), hypothesized to play a central role in sexual function and serotonin neurotransmission, were not altered.

1.4 Gene transcription in the marmoset brain

In **Chapter 5**, the transcriptomic effects of chronic 8-OH-DPAT administration to female marmoset monkeys were reported, complementing an open, hypothesis-generating microarray approach using the marmoset-specific EUMAMA microarray, with a candidate gene approach using real-time quantitative PCR (RT-qPCR) to measure serotonin receptor and transporter transcripts. In keeping experimental parameters consistent between chapters, the same brain regions of interest (mPFC, mPOA, CA1, DRN) as in Chapter 4 were selected, with exception of the VMH, which was not analyzed in Chapter 5.

Chronic 8-OH-DPAT, in a brain region-specific fashion, altered the expression of gene clusters linked to neural development (mPFC, mPOA, DRN), neurotransmission (mPOA), energy production (mPFC, mPOA),

mitochondrial function (CA1, DRN), protein transport (mPOA, DRN), learning and memory (CA1), ion channel activity (CA1), and intracellular signal transduction (DRN), suggesting global changes in gene regulation of neuronal function induced by 8-OH-DPAT that involve changes in cellular metabolism, communication between neurons and axonal growth.

Furthermore, 8-OH-DPAT increased 5-HT transporter (5-HTT, or SERT) mRNA expression in the DRN and oxytocin (OXT) mRNA expression in the mPOA. There was a statistical trend towards increased 5-HT_{1A} expression in the mPFC and towards reduced 5-HT_7 expression in the CA1. Activation of 5-HT_{1A} autoreceptors in the DRN by 8-OH-DPAT likely suppressed serotonergic activity [8], which may have triggered a compensatory upregulation of 5-HTT in the DRN and of 5-HT_{1A} in the mPFC to restore the serotonergic tone. In contrast, decreased 5-HT_7 expression in the CA1 may reflect a brain region-specific desensitization of 5-HT_7 receptors. As 8-OH-DPAT directly interacts with 5-HT_{1A} and 5-HT_7 receptors, their brain region-specific expression changes may present the most upstream molecular mechanisms underlying sexual and affiliative suppression induced by chronic 8-OH-DPAT.

While resolution was a limiting factor in the brain imaging study (Chapter 4), the gene expression experiments described in Chapter 5 benefitted from high anatomical precision provided by laser-microdissection techniques, which were used to dissect the ROIs prior to RNA isolation. Both Chapter 4 and Chapter 5, however, indicate that the selected ROIs (mPFC, mPOA, CA1 and DRN) may not comprehensively cover the brain areas involved in 8-OH-DPAT induced modulation of female sexual and social behavior. For example, Chapter 5 highlights increased oxytocin expression in the mPOA, but does not investigate expression levels in oxytocin-rich areas such as the paraventricular and supraoptic nuclei of the hypothalamus. Future studies should also target the expression of oxytocin receptor in marmoset brain areas with high receptor densities, including the basal forebrain cholinergic nuclei, nucleus basalis of Meynert, the diagonal band of Broca, and the amygdala [9, 10], areas that were not part of the pre-selected ROIs.

2. POTENTIAL MECHANISMS OF ACTION OF FLIBANSERIN AND 8-OH-DPAT IN THE REGULATION OF FEMALE SEXUAL AND SOCIAL BEHAVIOR

In assessing how flibanserin and 8-OH-DPAT may affect female sexual function, the data collected in Chapters 2-5 point towards a complex, multi-modal mechanism of serotonergic regulation of female sexual function. Thus, flibanserin's and 8-OH-DPAT's potential modes of action in regulating female

sexual behavior will be discussed in the framework of a model that involves four distinct, but interactive modules (Figure 1).

The core component of this model entails a hypothalamic unit, referred to as the *regulatory module of female sexual and social behavior*, which integrates relevant sensory information and produces sexual and social behavioral responses. Sexual and social behaviors are joined into a single unit, consistent with the results obtained in Chapter 2 that highlight the significance of pair-bond quality for female sexual behavior. This module is influenced by the other three modules. A *monoamine regulatory module* can exert either excitatory or inhibitory leverage, as outlined in Chapter 1, Figure 3: *Excitatory-Inhibitory model of female sexual behavior* (adapted from [11]). A *HPA axis module* accounts for the potential influence of stress hormones on female sexual behavior, while a *pair-bond, experience and memory module* applies a more integrative, holistic approach and emphasizes the important findings

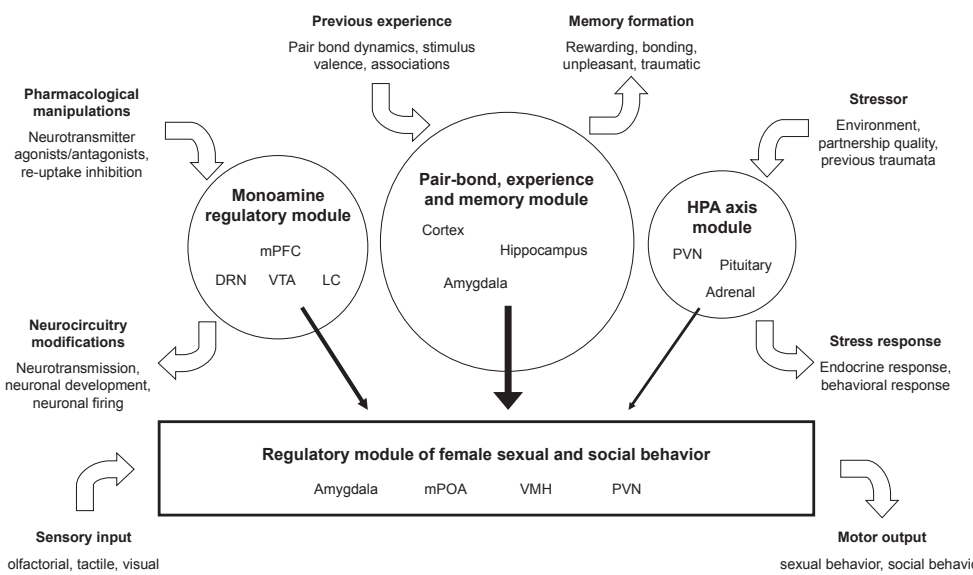

Figure 1. Four interacting modules through which flibanserin and 8-OH-DPAT modulate female sexual and social behavior. Circle and arrow size indicate relative impact of the respective module on female sexual and social behavior according to the observations described in Chapters 2-5 and additional data reported in [12, 13]. HPA, hypothalamus-pituitary-adrenal; mPFC, medial prefrontal cortex; DRN, dorsal raphé nucleus; VTA, ventral tegmental area; LC, locus coeruleus; PVN, paraventricular hypothalamic nucleus; mPOA, medial preoptic area; VMH, ventromedial hypothalamic nucleus.

of Chapters 2, 4 and 5 that implicate relationship dynamics and cognitive processes in the regulation female sexual behavior. The four modules are briefly described in the following sections.

2.1 Monoamine regulatory module

Pharmacological approaches based on manipulation of serotonin receptors, particularly of the 1A and 2A subtypes, have recently emerged in animal models of female sexual dysfunction [4, 14, 15] and as putative treatments for HSDD in women [6, 7]. The monoamine regulatory module integrates evidence derived from rodent studies, as modified from Stahl et al [16], in which flibanserin and 8-OH-DPAT selectively alter serotonergic, dopaminergic and noradrenergic neurotransmission in a brain region-specific manner. Pyramidal neurons in the prefrontal cortex (PFC) process sensory and cognitive inputs to the PFC and send glutamatergic projections to, among others, brainstem monoamine centers for 5-HT (DRN), for DA (ventral tegmental area, VTA) and for NE (locus coeruleus, LC). These connections can either be direct, resulting in monoamine release, or indirect via inhibitory GABAergic (γ amino butyric acid) interneurons, in which case monoamine release decreases.

Flibanserin activates 5-HT_{1A} and inhibits 5-HT_{2A} receptors on PFC pyramidal neurons, leading to increased DA and NE, but decreased 5-HT levels in the mPFC [12], purportedly by selective inhibition of pyramidal neurons that excite brainstem serotonergic neurons (decrease in 5-HT) and GABAergic interneurons in the VTA and LC (increase in DA and NE) [16]. Interestingly, we found that neural activity was indeed decreased in the mPFC of flibanserin treated female marmosets [AK Converse and DH Abbott, personal communication], consistent with the hypothesized inhibition of pyramidal neurons by flibanserin.

In contrast to flibanserin, 8-OH-DPAT preferentially binds to 5-HT_{1A} receptors located on GABAergic interneurons, but not pyramidal neurons of the PFC, causing enhanced pyramidal cell firing in the PFC [17]. Increased activation of dopaminergic and noradrenergic neurons in the VTA and LC through these pyramidal neurons would explain the cortical increase in DA and NE that is observed after systemic administration of 8-OH-DPAT [18]. Compared to flibanserin, however, the increase in DA is more modest [11]. 5-HT levels have been shown to remain unaltered [13, 18], possibly due to direct activation of counteractive presynaptic 5-HT_{1A} autoreceptors in the DRN, or due to upregulated 5-HTT function in the DRN in response to chronic 5-HT_{1A} activation, as suggested by the marmoset data described in Chapter 5.

As proposed by the *Excitatory-Inhibitory model of female sexual behavior* (Chapter 1, Figure 3, [11]), DA and NE activate the regulatory module of

female sexual and social behavior (section 2.4) to facilitate sexual behavior, while 5-HT has an inhibitory effect. Increased DA and NE and decreased 5-HT after flibanserin is thus consistent with increased sexual attractiveness and affiliative behavior observed in flibanserin treated female marmosets (Chapter 2). 8-OH-DPAT likely increases excitatory DA and NE inputs to the regulatory module of female sexual and social behavior, but more moderately compared to flibanserin, without affecting the inhibitory 5-HT input. The expected net effect of 8-OH-DPAT on sexual and social behavior in the female marmoset would thus be slightly pro-sexual and pro-social, which contrasts the results shown in Chapter 2. This discrepancy will be discussed in section 2.4 below.

Projected module effect on sexual and social behavior:
Flibanserin ↑; 8-OH-DPAT (↑) [2]

2.2 HPA axis module

Pharmacological manipulations of serotonin receptors commonly activate the HPA axis. The HPA axis is a key neuroendocrine component in the physiological response to stress that generally suppresses female reproductive function [19]. Cortisol interacts with the hippocampus and amygdala which indirectly regulate the HPA axis by modulating the processing of stressful information, involving inhibitory GABAergic neuron projections to the PVN [20]. Adrenergic and noradrenergic projections from the nucleus of the solitary tract (NTS) [21, 22] and from the LC [23] can modulate the HPA axis responsiveness to stress.

In Chapter 3, evidence was presented for flibanserin and 8-OH-DPAT induced HPA axis sensitization in response to a stressor. In 8-OH-DPAT, but not in flibanserin treated females, increased ACTH responses to restraint were positively correlated with increased sexual rejection. Furthermore, only 8-OH-DPAT, but not flibanserin, elevated cortisol levels several hours after termination of the stressor.

Chronic flibanserin might enhance the HPA axis response to a stressor by inhibiting the modulatory, inhibitory GABAergic inputs from the hippocampus or amygdala, or by activating the stimulatory noradrenergic inputs from the NTS and LC, causing exaggerated ACTH release to restraint stress. Chronic 8-OH-DPAT might enhance the ACTH response to restraint stress in a similar way as flibanserin by changing modulatory and/or activating inputs to the PVN. Such stress-induced rises in ACTH are likely independent from acute

2 Upwards pointing arrows indicate that the module exerts pro-sexual and pro-social influence, while downwards pointing arrows indicate that the module exerts anti-sexual and anti-social influence.

5-HT_{1A} actions of flibanserin and 8-OH-DPAT [24]. In contrast to flibanserin, 8-OH-DPAT also elevates cortisol levels following a stressor. Elevated cortisol might suppress female sexual receptivity and induce aggression, consistent with the observation that exaggerated ACTH responses to a stressor correlate with agonistic and sexual rejection behavior in 8-OH-DPAT treated female marmosets (Chapter 3).

Projected module effect on sexual and social behavior:
Flibanserin unaltered; 8-OH-DPAT ↓

2.3 Pair-bond, experience and memory module

In humans, psychosocial factors such as partnership quality, previous sexual experiences and personal, social and cultural expectations [25-31] are important determinants of women's sexuality (see Chapter 1). Interpersonal distress is in fact a central component in the diagnosis of hypoactive sexual desire disorder (HSDD) in women [32]. It is in this context that marmoset data on flibanserin's actions, outlined in Chapters 2-5, may add the most important contribution to understanding flibanserin's action on HSDD in women. The pair-bond, experience and memory module links the effects of flibanserin and 8-OH-DPAT on female sexual behavior to experimental evidence that partner interactions (Chapter 2), stress reactivity (Chapter 3), cortical glucose metabolism (Chapter 4) and learning and memory processes (Chapter 5) may all partake in regulating female sexual function.

In Chapter 2, it was demonstrated that both flibanserin and 8-OH-DPAT not only affected the treated female's behavior towards her partner, but also altered the untreated male's social behavior towards the female, clearly implicating altered relationship dynamics between the partners. As outlined in Chapter 1, sexual behavior in women entails a complex interplay between psychosocial, hormonal and neural factors and requires hypothalamic, limbic and cortical brain structures for its regulation [11].

Similar factors may play a role in the female marmoset monkey. For 8-OH-DPAT, such psychosocial, hormonal and neural factors were determined in Chapters 2-5. Reduced female sexual receptivity was associated with more frequent agonistic interactions between pairmates (Chapter 2), increased endocrine stress reactivity (Chapter 3) and reduced glucose metabolism in the occipital cortex (Chapter 4). Reduced glucose metabolism in the occipital cortex suggests an 8-OH-DPAT-induced impairment in the perception of visual salience in social interactions with the long-term sexual partner. Agonistic social interactions, as well as increased stress reactivity, may cause sexual signals from the male partner to be negatively interpreted and rejected,

thus establishing a negative association with the sexual partner. Chapter 5 highlights that 8-OH-DPAT affects the expression of hippocampal genes that are linked to learning and memory. Learning negative associations with the sexual partner and remembering previous agonistic interactions could indeed impair the pair-bond between marmoset pairmates and negatively affect female sexual function.

For flibanserin, behavioral data indicate a positive shift in social and sexual behavior that is built on improved pair bonds (Chapter 2). Brain activity and transcriptomic data, however, are not yet available.

Projected module effect on sexual and social behavior:
Flibanserin ↑; 8-OH-DPAT ↓

2.4 Regulatory module of female sexual and social behavior

This module relates to a key finding described in Chapter 5, that 8-OH-DPAT affects the transcription of hypothalamic oxytocin, to a previous marmoset study employing the same study design [33], and places it in context of a theoretical framework proposed by Choleris et al [34], the four-gene micronet of social behavior regulation, described below.

The hypothalamus, specifically the mPOA and VMH, plays a central role in the regulation of female sexual behavior by integrating sexually-relevant sensory inputs with excitatory and inhibitory neurotransmitter and sex hormone signals, thus controlling female sexual behavior (reviewed in [11, 35]. The essential function of the mPOA and VMH in the generation of sexual behavior in female and male marmoset monkeys is confirmed by lesion and functional imaging studies [36-39]. In rodents, oxytocin immunoreactivity and mRNA expression in the mPOA has been associated with social behavior [40, 41], and infusion of oxytocin into the mPOA increases female sexual receptivity [42]. It is however possible that the detection of oxytocin mRNA in the mPOA may reflect mRNA molecules stored in dendritic projections of oxytocin neurons located in the PVN [43].

The mPOA receives a large number of projections from the PVN, which also sends efferents to the amygdala [44]. The PVN is a major site of oxytocin production, which can act centrally as a neuropeptide through oxytocin receptors expressed in the mPOA and amygdala to regulate maternal, social and sexual behavior [45-49]. Oxytocin thus serves as a prominent connection between the pair-bond, experience and memory module (Section 2.3), and the regulatory module of female sexual and social behavior presented in this section.

Choleris et al [34] propose a four-gene micronet, involving oxytocin,

oxytocin receptor and the estrogen receptors alpha and beta (ERα, ERβ), in the regulation of social recognition and ultimately in the regulation of affiliation, pair bonds, aggression and sex [50]. In this model, estradiol plays a crucial role in the expression of oxytocin in the PVN and PVN dendrites that extend to other hypothalamic areas, and of oxytocin receptors in the amygdala. Signaling through the oxytocin receptor initiates a cascade of events (described in [35, 51]) that facilitate social recognition and promote or prevent the display of sexual behavior.

Chronic 8-OH-DPAT increases the expression of oxytocin in the mPOA (Chapter 5) and likely in the PVN [52]. 8-OH-DPAT, however, downregulates the expression of estrogen receptors [53], which are essential for oxytocin receptor function in the amygdala [34]. Ovariectomy may further impair oxytocin receptor expression [54]. Thus, and despite elevated hypothalamic oxytocin expression, the cumulative effects of chronic administration and ovariectomy might suppress oxytocin signaling through its receptor and cause sexual rejection and aggression between marmoset pairmates (Chapter 2; discussion in Chapter 5). Furthermore, the impact of increased excitatory input from the monoamine regulatory module on the regulatory module of sexual and social behavior, proposed in Section 2.1, may also be without functional consequence due to the lack of downstream mediators.

Although no molecular data are available for the effect of flibanserin on oxytocin and oxytocin receptor expression, there is ample indirect evidence that flibanserin, similar to 8-OH-DPAT, may increase oxytocin in the hypothalamus. Flibanserin supports pro-social and pro-sexual behavior in female marmosets (Chapter 2), behaviors that are strongly associated to increased central oxytocin function [55-57]. Flibanserin also increases genital and non-genital self-grooming in female marmosets (Chapter 2), a behavior that is induced by central oxytocin administration in female rats [58]. Furthermore, flibanserin increases norepinephrine (NE) levels in the mPFC and mPOA in female rats [59]. Oxytocin induces NE release in the VMH [60], while NE conversely induces oxytocin release in the PVN and the supraoptic hypothalamic nucleus (SON) [41]. Unlike 8-OH-DPAT, flibanserin has not been reported to impair oxytocin receptor function. Thus, according to Choleris' model [34], flibanserin-induced increases in oxytocin in the mPOA and PVN could lead to enhanced activation of the amygdala and promote pro-social [41] and pro-sexual behavior [11, 61].

A flibanserin-induced increase in oxytocin signaling may also stimulate sexual and social behavior through the release of gonadotropin-releasing hormone (GnRH). Oxytocinergic neurons in the mPOA and PVN project to GnRH containing neurons in the mPOA to stimulate GnRH release [62]. In the marmoset monkey, GnRH II has been shown to stimulate female sexual

behavior by binding to the GnRH receptor type II, which is expressed in the mPOA and VMH [33]. Noradrenergic, dopaminergic and serotonergic afferents from the brainstem monoamine centers play an important regulatory role on oxytocin and GnRH release in the hypothalamic nuclei [60, 62] and serve thus as interface between the monoamine regulatory module described in Section 2.1, and the regulatory module of female sexual and social behavior.

Net effect on sexual and social behavior:
Flibanserin ↑; 8-OH-DPAT ↓

3. FLIBANSERIN AND HYPOACTIVE SEXUAL DESIRE DISORDER (HSDD) IN WOMEN: WHERE NEXT?

Parallel to the marmoset study presented in this thesis, flibanserin underwent large Phase III clinical trials termed the *BOUQUET Study Program*, conducted by Boehringer Ingelheim [63]. The seven trials involved over 5,000 pre-menopausal women with HSDD and were conducted in North America and Europe, with the aim to evaluate the efficacy and safety of flibanserin. Trial participants suffered from primary generalized acquired HSDD for a period of more than 24 weeks. Inclusion criteria were, among others, that the participants lived in a stable, communicative, heterosexual relationship of at least one year, with a sexually functioning partner and that they experienced major personal distress due to sexual dysfunction.

The co-primary endpoints of the *BOUQUET* studies were the change from baseline to study end in the number of satisfying sexual events (SSE) in all trials and a sexual desire score measured using a daily electronic diary (eDiary). Sexual desire was measured using both the daily eDiary and the desire domain of the Female Sexual Function Index (FSFI). Secondary endpoints assessed to provide data on sexual functioning and the alleviation of distress, a hallmark of HSDD, using the Female Sexual Function Index (FSFI) and Female Sexual Distress Scale (FSDS-R).

The North American trials demonstrated significantly increased numbers of sexually satisfying events at the highest flibanserin dose of 100 mg/day (from 2.7/month to 4.5/month) compared to 2.7/month versus 3.7/month, respectively, in the placebo group. Flibanserin also improved the secondary endpoints (FSFI, FSDS-R) and was thus effective in decreasing interpersonal distress. The eDiary desire scores were, however, not significantly increased by flibanserin, when compared to placebo treatment [6, 7]. The latter result, together with some reported adverse events (sleepiness, dizziness and fatigue in > 10% of women treated with flibanserin), led to a negative review

for flibanserin by an advisory panel of the US Food and Drug Administration (FDA) in August 2010. Boehringer Ingelheim, as a consequence, withdrew the drug from development in October 2010. Controversy regarding the usability of eDiary and SSE as suitable primary endpoints remains after the withdrawal of flibanserin, as the development of meaningful and valid end points that capture the complexity of women's sexual responses is unsolved [64].[3]

The marmoset flibanserin data presented in this thesis suggest the possibility that flibanserin might indirectly affect female sexual function by improving the relationship with a partner. Increased female sexual attractiveness and strengthened pair-bond between marmoset pairmates reflect improved partner interactions and intimacy in the relationship between long-term sexual partners. Allogrooming in nonhuman primates is essential to lasting social bonding and the creation of a psychological environment of trust between partners [66], likely involving central actions of oxytocin. Flibanserin's main effect in the marmoset study was to increase allogrooming in established, long-term marmoset pairs, while its main effect in the clinical BOUQUET trials was to increase sexual satisfaction and decrease distress in women in a relationship with a long-term sexual partner. Considering these main outcomes, it seems plausible that flibanserin's therapeutic effect in the treatment of HSDD may be mediated by improving relationship quality and reducing sexual distress between partners. Flibanserin-induced improvements in sexual, social and emotional bonding between partners may underlie the efficacy of flibanserin in increasing sexual satisfaction and decreasing interpersonal distress in women suffering from HSDD.

For future clinical HSDD trials, the marmoset findings suggest that it would be valuable to consider parameters that characterize the intimacy of a relationship and emphasize on the role of the partner as trial end-points, thus exploring novel perspectives in the pharmacotherapeutic treatment of HSDD. In addition, the role of hypothalamic oxytocin in the regulation of female sexual behavior merits special attention in future studies of flibanserin. It would be of great interest to measure oxytocin and oxytocin receptor expression in the available brain tissues of chronic flibanserin treated female marmoset monkeys. Regions of interest should entail PVN, mPOA and amygdala, as well as other brain regions with high oxytocin and oxytocin receptor expression. A large-scale microarray study using the EUMAMA microarray would provide the transcriptomic correlates of the behavioral findings. Furthermore,

3 On July 13, 2012, it was reported that the startup company Sprout Pharmaceuticals, who acquired flibanserin from Boehringer Ingelheim in 2011, plans to resubmit a flibanserin drug application to the FDA in early 2013 without additional clinical studies. Flawed metrics in looking at the data were cited that led the FDA to deny flibanserin approval [65].

neuroimaging tools for oxytocin receptor binding are currently being developed [67] and may soon provide the opportunity to detect oxytocin receptors in vivo in both marmoset and human flibanserin trials.

4. THE *BOUQUET* FINALE

A *bouquet* of concluding statements highlights new insights gained from experiments conducted as part of this thesis and their added value to the flibanserin pivotal phase-III clinical trials *(BOUQUET Study Program)*.

- Pair bond quality determines sexual attractiveness and activity in female marmoset monkeys (Chapter 2).
- Chronic 5-HT_{1A} activation by 8-OH-DPAT causes a phenotype characterized by reduced female sexual receptivity and attractiveness, increased aggression between pairmates, increased stress reactivity and reduced neural activity in the medial occipital cortex (Chapters 2, 3, 4).
- Oxytocin may be the pivot of serotonergic regulation of female sexual behavior, pair-bond and pharmacotherapy of HSDD (Chapters 2, 5).
- Marmoset data suggest that flibanserin-induced improvements in partner interactions may underlie increased sexual satisfaction and reduced interpersonal distress in the *BOUQUET* studies, thus presenting unexplored perspectives to investigate HSDD and its future pharmacotherapy.

5. DISCLAIMER

Experiments described in the thesis were sponsored by Boehringer Ingelheim, proprietor and developer of flibanserin. Selection of experiments, as well as experimental design, was under full control of the Principal Investigators of the studies. Experiments described in Chapters 2, 3 and 4 were conceived and designed by Prof. David Abbott (PI), Dr. Alexander Converse and Yves Aubert. Experiments described in Chapter 5 were conceived and designed by Dr. Nicole Datson (PI) and Yves Aubert.

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6

SUMMARY

SUMMARY

Sexual dysfunctions in women are highly prevalent. Hypoactive sexual desire disorder (HSDD), as the most frequently diagnosed condition, is found in approximately 1 out of 10 women, causing distress and interpersonal difficulty that arise from unwanted, persistent or recurrent low sexual desire. Etiology and underlying neurobiological mechanisms of HSDD are not well understood, but neurotransmitter dysfunction has been proposed involving the excitatory regulators dopamine and norepinephrine, as well as inhibitory serotonin (5-HT). The objective of the research described in this thesis was to investigate the serotonergic regulation of female sexual behavior in a nonhuman primate model of female sexual function. Due to its pairmate social setting comparable to humans, the common marmoset monkey *(Callithrix jacchus)* was the animal model of choice. Marmosets form stable male-female relationships that are strengthened by partner-directed social interactions, including grooming behavior. Females can signal interest for sexual interactions (proceptivity) and control the occurrence of sexual intercourse by accepting (receptivity) or rejecting the pairmate's sexual advances.

A behavioral testing paradigm was developed to quantitatively assess the behavioral expression of proceptive and receptive sexual states of the female marmoset, as well as other parameters of pairmate interactions that give information about the pair-bond quality between the sexual partners. This paradigm was used to test the chronic effects of the serotonergically active substances flibanserin, a 5-HT_{1A} agonist and 5-HT_{2A} antagonist, and 8-OH-DPAT, a 5-HT_{1A} agonist. Flibanserin is currently being developed as a potential pharmacotherapeutic treatment of HSDD in women.

Chapter 1 introduced female sexuality and provided an overview of the classification of female sexual dysfunctions. The current knowledge of the neurobiology of female sexual function and dysfunction in both humans and animal models was presented. A concept was introduced that sexual behavior is centrally regulated by both excitatory and inhibitory factors. Such factors include steroid hormones, neuropeptides and neurotransmitters. On the excitatory side, estradiol, testosterone, oxytocin, melanocortin, dopamine and norepinephrine play an important role, while on the inhibitory side, opioids, cannabinoids and serotonin exert a suppressive function. The central serotonin neurotransmitter system was introduced in more detail, and the stimulatory or inhibitory function of different serotonin receptor subtypes was highlighted. The effects of flibanserin and 8-OH-DPAT on female sexual behavior in rodents (8-OH-DPAT) and in humans (flibanserin, 8-OH-DPAT) were described in this context. Chapter 1 also outlined how female sexuality can be modeled in animals, and why the common marmoset monkey is a

particularly suitable model species to explore female sexual behavior and to test the chronic effects of flibanserin and 8-OH-DPAT on behavior and its underlying neurobiological substrate.

The main objective of **Chapter 2** was to quantify the effects of chronic flibanserin and 8-OH-DPAT on sexual and social interactions between treated females and their untreated male pairmates. Repeated observations of the pairmates' interactions at reunion following a 90 minute separation revealed remarkable and contrasting differences between flibanserin and 8-OH-DPAT on marmoset pair behavior, despite the drugs' shared 5-HT_{1A} agonist properties. While pairmates with flibanserin-treated females were more frequently engaged in sexually-related and affiliative, pro-social interactions, the pair-bond between 8-OH-DPAT-treated females and their male partners was impaired as evidenced by increased female rejection of male sexual advances, a tendency for diminished female sexual attractiveness, reduced affiliative and increased agonistic interactions. We proposed that the drugs' effects on social interactions and pair-bond between the pairmates play an important role in determining female sexual behavior in the marmoset. Chapter 2 furthermore described the pharmacokinetic 24-hour profiles of circulating flibanserin and 8-OH-DPAT levels during chronic administration. The results showed that blood levels of flibanserin and 8-OH-DPAT were low or absent during the time of behavioral tests, as well as during endocrine, brain imaging and gene expression experiments described below.

In **Chapter 3**, the impact of chronic flibanserin and 8-OH-DPAT on HPA axis function was investigated to delineate the possibility of an HPA axis-mediated mechanism in the serotonergic regulation of female sexual behavior. Chronic flibanserin and 8-OH-DPAT neither altered circulating morning basal cortisol levels nor ACTH and cortisol responses to an acute 5-HT_{1A} agonist challenge. Activation of hypothalamic 5-HT_{1A} receptors stimulates the HPA axis, and a neuroendocrine 5-HT_{1A} challenge can thus serve as peripheral indicator of central 5-HT_{1A} receptor function. In response to 30 minute restraint stressor, the ACTH response was enhanced by flibanserin and 8-OH-DPAT, suggesting sensitized HPA axis reactivity to stress after chronic flibanserin and 8-OH-DPAT treatment. Cortisol was elevated after 8-OH-DPAT, but not after flibanserin treatment. Enhanced ACTH responses to restraint were correlated with increased aggression and reduced sexual receptivity in 8-OH-DPAT treated female marmosets, supporting the hypothesis that increased stress reactivity may have contributed to inhibition of sexual behavior. Such correlations were absent in flibanserin treated females, suggesting no inhibitory effect of HPA axis reactivity on female sexual function after flibanserin.

Chapter 4 described the results of a PET/MRI functional brain imaging experiment that was designed to measure cerebral glucose metabolism, an indicator of neural activity, in chronic 8-OH-DPAT or vehicle treated female marmosets during sexual and social interactions with their male pairmates. Radiolabeled [^{18} F]fluorodeoxyglucose (FDG) was infused immediately prior to a 30 minute pair test, and the females were subsequently imaged by PET under isofluorane anesthesia. Structural MRI scans were recorded and overlaid on the PET images to improve the visualization of anatomical structures. In predefined regions of interest that were chosen for their involvement in mediating female sexual behavior and their high 5-HT$_{1A}$ density (mPFC, mPOA, VMH, CA1 and DRN), chronic 8-OH-DPAT was without effect on FDG uptake. Whole brain voxel-wise mapping, however, showed significantly reduced neural activity in a cluster located in the medial occipital cortex (mOCC), overlapping with a cluster derived from correlations of behavioral scores of female rejection behavior with FDG signals. This finding suggests that reduced neural activity in the mOCC may underlie the 8-OH-DPAT induced reduction in female sexual receptivity.

Chapter 5 described a large-scale gene expression experiment using the marmoset-specific EUMAMA microarray, as well as a candidate gene approach using real-time quantitative PCR that targeted mRNA expression of the 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{7}$ and 5-HTT genes. The same brain regions of interest were targeted as those studied in the PET imaging experiment (Chapter 4), with exception of the VMH, which was not analyzed.

Functional annotation clustering of microarray data showed that chronic 8-OH-DPAT specifically altered genes associated with neurotransmission in the mPOA, while gene clusters associated to ion channel activity and learning and memory were affected in the CA1. Genes involved in intracellular signal transduction were affected in the DRN. Gene clusters involved in neural development were altered in the mPFC, mPOA and DRN, energy production was affected in the mPFC and mPOA, mitochondrial function in the CA1 and DRN, and protein transport in the mPOA and DRN. We thus proposed that transcriptomic changes related to neural plasticity, energy production and learning and memory processes in cortical, hippocampal and hypothalamic brain areas may have contributed to sexual impairment in response to chronic 8-OH-DPAT administration. The microarray analysis also revealed a greater than 10-fold increased expression of oxytocin (OXT) in the mPOA of 8-OH-DPAT treated females. This finding is of particular interest as the function of hypothalamic OXT has been closely associated with social and sexual behavior in rodents, nonhuman primates and humans. Serotonergic regulation of marmoset sexual and social behavior might thus be mediated by hypothalamic

OXT. However, increased hypothalamic oxytocin is normally attributed to pro-sexual and pro-social behavior, contrasting the finding reported in Chapter 5. Assessment of oxytocin receptor expression and signaling will be essential to shed more light into this apparent contradiction.

The candidate gene approach revealed that the serotonin transporter gene (5-HTT) was strongly upregulated in the DRN. The expression of 5-HT_{1A} autoreceptors in the DRN was not altered by chronic 8-OH-DPAT, but there was a trend to increased 5-HT_{1A} expression in the mPFC. Expression of 5-HT_{2A} was not affected by 8-OH-DPAT in any of the investigated brain regions, while there was a trend for decreased 5-HT_{7} expression in the CA1 area of the hippocampus. We proposed that activation of 5-HT_{1A} autoreceptors in the DRN by 8-OH-DPAT likely suppressed serotonergic activity, triggering a compensatory upregulation of 5-HTT in the DRN and of 5-HT_{1A} in the mPFC to restore the serotonergic tone.

In **Chapter 6,** a synthesis of the experimental findings described in Chapters 2-5 was proposed. Contrasting effects of flibanserin and 8-OH-DPAT on female sexual and social behavior (Chapter 2) were discussed in context with enhanced HPA axis responsiveness to stress (Chapter 3), and brain region-specific alterations in glucose metabolism (Chapter 4) and gene transcription (Chapter 5). In light of the discipline-spanning data set presented in this thesis and the multifactorial etiology of female sexual dysfunctions (see Chapter 1), we proposed that flibanserin and 8-OH-DPAT regulate female sexual behavior through four separate, but interactive modules.

In a monoamine regulatory module (i), flibanserin and 8-OH-DPAT alter serotonergic, dopaminergic and noradrenergic neurotransmission, causing altered excitatory or inhibitory input on female sexual behavior. Through this module, flibanserin was proposed to exert both an excitatory and dis-inhibitory influence on female sexual behavior. An HPA axis module (ii) accounts for sexually inhibitory effects due to enhanced activation of the endocrine stress system. This module could play a role in increased sexual rejection behavior of chronic 8-OH-DPAT treated female marmosets. A pair-bond, experience and memory module (iii) was proposed as impactful mechanism through which flibanserin and 8-OH-DPAT may exert their pro-sexual and pro-social (flibanserin), or anti-sexual and anti-social (8-OH-DPAT) actions. Oxytocin might play a key role in linking this module to a regulatory module of female sexual and social behavior (iv), which integrates the inputs from modules (i)-(iii) and ultimately determines the expression of female sexual and social behavior.

In conclusion, the synthesis of experimental data within the proposed theoretical framework highlighted the importance of pair-bond quality on the

expression of female sexual behavior and called for further investigation of the role of oxytocin in the serotonergic regulation of female sexual function. The thesis was concluded by suggesting that flibanserin's therapeutic effect in women with HSDD may be rooted on improvements in sexual, social and emotional bonding between partners. In translating the marmoset findings to humans, the thesis also suggested that future HSDD trials should consider the inclusion of parameters that characterize the intimacy of a relationship and emphasize on the role of the partner as clinical end-points.

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SAMENVATTING

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Sexueel dysfunctioneren komt bij vrouwen veel voor. Bij ongeveer 1 op de 10 vrouwen is er sprake van “Hypoactive sexual desire disorder (HSDD)”, een aandoening waarbij het libido regelmatig of aanhoudend sterk verlaagd is, hetgeen voor problemen zorgt in de persoonlijke en relationele sfeer. Hoe HSDD ontstaat en wat de onderliggende neurobiologische mechanismen zijn, is nog grotendeels onbekend, maar mogelijk speelt dysfunctie van de excitatoire neurotransmitters dopamine en norepinephrine en de inhibitoire neurotransmitter serotonine (5-HT) een rol. Doel van het onderzoek beschreven in dit proefschrift was om de rol te onderzoeken van het serotonine systeem in de regulatie van vrouwelijk sexueel gedrag. Hierbij werd gebruik gemaakt van het penseelaapje *(Callithrix jacchus)* als diermodel, omdat de paarvorming in penseelaapjes sociaal gezien veel lijkt op die van de mens. Penseelaapjes vormen namelijk stabiele man-vrouw koppels waarin sociaal gedrag tussen de partners, zoals bijvoorbeeld elkaar vlooien, een belangrijke rol speelt. Vrouwtjes geven hun interesse in sexuele interactie aan en reguleren ook wanneer dit plaatsvindt door actief sexuele avances te accepteren of af te wijzen.

Een gedragstest is ontwikkeld waarmee de belangstelling van het vrouwtje voor en het toelaten of afwijzen van de sexuele avances van het mannetje kwantitiatief bepaald kan worden. Daarnaast meet deze gedragstest ook andere parameters die informatie geven over de interactie tussen beide partners van een koppel en de kwaliteit van deze interacties. Deze gedragstest werd vervolgens gebruikt om de chronische effecten te bepalen van twee verschillende serotonerge stoffen: flibanserin (een 5-HT_{1A} agonist en 5-HT_{2A} antagonist) en 8-OH-DPAT (een 5-HT_{1A} agonist). Momenteel is flibanserin in ontwikkeling als potentieel geneesmiddel voor de behandeling van HSDD in vrouwen.

Hoofdstuk 1 gaf een inleiding in vrouwelijke sexualiteit en een overzicht van de indeling van vrouwelijk sexueel dysfunctioneren. De huidige stand van zaken van kennis van de neurobiologie van sexueel functioneren en dysfunctioneren in vrouwen, zowel in de mens als in diermodellen, kwam aan bod. Sexueel gedrag wordt centraal gereguleerd door zowel excitatoire en inhibitoire factoren, o.a. steroïd hormonen, neuropeptiden en neurotransmitters. Aan de excitatoire kant van de balans spelen oestrogenen, testosteron, oxytocine, melanocortine, dopamine en norepinephrine een belangrijke rol, terwijl aan de inhibitoire kant opioïden, cannabinoïden en serotonine een onderdrukkend effect hebben. Het centrale serotonine neurotransmitter systeem werd in meer detail besproken en de stimulerende en remmende werking van de verschillende serotonine receptor subtypes werd benadrukt. De werking

van flibanserin en 8-OH-DPAT op vrouwelijk sexueel gedrag in knaagdieren (8-OH-DPAT) en in de mens (flibanserin, 8-OH-DPAT) werd beschreven. In Hoofdstuk 1 kwam ook aan bod waarom het penseelaapje een zeer geschikt diermodel is voor het bestuderen van vrouwelijke sexualiteit en voor het testen van de chronische effecten van flibanserin en 8-OH-DPAT m.b.t. gedrag en de onderliggende neurobiologische substraten.

De doelstelling van **Hoofdstuk 2** was om de effecten te bestuderen van chronische behandeling met flibanserin en 8-OH-DPAT op sexuele en sociale interacties tussen behandelde vrouwtjes en hun onbehandelde partners. Er waren opvallende verschillen en tegengestelde effecten waarneembaar van flibanserin enerzijds en 8-OH-DPAT anderzijds op de interacties tussen het vrouwtje en mannetje van een koppel bij hun weerzien na een scheidingsperiode van 90 minuten, ondanks het feit dat beide stoffen 5-HT_{1A} agonist eigenschappen gemeen hebben. Terwijl koppels met daarin flibanserin-behandelde vrouwtjes vaker sexueel-gerelateerde en toenaderende, pro-sociale interacties vertoonden, was de band tussen 8-OH-DPAT-behandelde vrouwtjes en hun partner verstoord. Dit was zichtbaar als een toename van afwijzing van sexuele avances door het vrouwtje, verminderde aantrekkelijkheid van het vrouwtje, minder toenaderende interacties en een toename van agonistische interacties. Dit suggereert dat beide stoffen (flibanserin en 8-OH-DPAT) een belangrijke rol spelen in het bepalen van vrouwelijk sexueel gedrag van het penseelaapje. Verder werd in Hoofdstuk 2 de farmacokinetische 24-uurs profielen beschreven van flibanserin en 8-OH-DPAT spiegels in de circulatie tijdens chronische toediening. Dit toonde aan dat flibanserin en 8-OH-DPAT spiegels in het bloed laag of afwezig waren op het moment dat de gedragstesten werden uitgevoerd, en ook tijdens de endocriene studies, hersen imaging en gen expressie experimenten die hieronder worden beschreven.

In **Hoofdstuk 3** werd de impact van chronische flibanserin en 8-OH-DPAT behandeling op HPA-as functie onderzocht en gekeken of de HPA-as een rol speelde in de serotonerge regulatie van vrouwelijk sexueel gedrag. Zowel chronische flibanserin als 8-OH-DPAT behandeling gaven geen verandering in basale ochtend cortisol spiegels, en hadden ook geen effect op de ACTH en cortisol respons in reactie op een acute 5-HT_{1A} agonist challenge. Activatie van hypothalamische 5-HT_{1A} receptoren stimuleerden de HPA-as, en een neuroendocriene 5-HT_{1A} challenge kan dus dienst doen als een perifere indicator van centrale 5-HT_{1A} receptor functie. Blootstelling aan 30 minuten immobilisatie stress resulteerde in een verhoogd ACTH respons na flibanserin en 8-OH-DPAT behandeling, wat wijst op een sensitisatie van HPA-as reactiviteit op stress na chronische flibanserin en 8-OH-DPAT

behandeling. Cortisol was verhoogd na 8-OH-DPAT, maar niet na flibanserin behandeling. Er bleek een correlatie te zijn tussen toename van de ACTH respons op immobilisatie en toegenomen aggressie en verminderde sexuele ontvankelijkheid in 8-OH-DPAT behandelde vrouwtjes, wat consistent is met de hypothese dat toegenomen stress reactiviteit bijdraagt aan de inhibitie van sexueel gedrag. Dergelijke correlaties waren afwezig in flibanserin-behandelde vrouwtjes, wat suggereert dat er geen remmend effect is van HPA-as reactiviteit op vrouwelijk sexueel gedrag na flibanserin behandeling.

In **Hoofdstuk 4** werden de resultaten beschreven van een PET/MRI functionele imaging experiment gericht op het meten van cerebrale glucose metabolisme, een indicator van neurale activiteit, in chronische 8-OH-DPAT of vehicle-behandelde vrouwelijke penseelaapjes tijdens sexuele en sociale interacties met hun mannelijke partners. Radioactief gelabeled [^{18} F]fluorodeoxyglucose (FDG) werd aan de vrouwtjes toegediend direkt voorafgaand aan een 30 minuten durende interactie na hereniging met hun partner, waarna de vrouwtjes onder isofluorane narcose een PET scan ondergingen. Structurele MRI scans werden met de PET beelden gecombineerd om de visualisatie van anatomische structuren te verbeteren. In vooraf bepaalde hersengebieden, gekozen op basis van hun rol in vrouwelijk sexueel gedrag en een hoge 5-HT$_{1A}$ dichtheid (mPFC, mPOA, VMH, CA1 en DRN), bleek chronische 8-OH-DPAT geen effect te hebben op FDG opname. Echter, voxel-wise mapping van de gehele hersenen liet een significante afname van neurale activiteit zien in een cluster gelegen in de mediale occipitale cortex (mOCC). Deze cluster vertoonde overlap met een cluster verkregen uit correlaties van scores van vrouwelijk afwijzingsgedrag en FDG signalen. Dit resultaat wijst erop dat verminderde neurale activiteit in de mOCC mogelijk ten grondslag ligt aan de 8-OH-DPAT geïnduceerde afname in vrouwelijke sexuele ontvankelijkheid.

In **Hoofdstuk 5** werden de resultaten van een grootschalig genexpressie experiment beschreven waarbij gebruik gemaakt werd van een microarray met specifieke sequenties afkomstig van het penseelaapje (marmoset-specific microarray EUMAMA). Daarnaast werd de expressie van diverse kandidaat-genen van het serotonine systeem gemeten m.b.v. real-time kwantitatieve PCR, namelijk 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{7}$ en 5-HTT. Dezelfde hersengebieden als in de PET imaging studie (Hoofdstuk 4) werden onderzocht, met uitzondering van de VMH.

Clustering van microarray data op basis van functionele annotatie liet zien dat chronische 8-OH-DPAT in de mPOA specifiek de expressie van genen geassocieerd met neurotransmissie beïnvloedde, terwijl de expressie van gen clusters geassocieerd met ion kanaal activiteit en leren en geheugen in

het CA1 gebied veranderd was. Expressie van genen die een rol spelen in intracellulaire signaal overdracht was met name in de DRN veranderd. Gen clusters betrokken bij neurale ontwikkeling waren veranderd in de mPFC, mPOA and DRN, gen clusters betrokken bij energie productie in de mPFC en mPOA, gen clusters voor mitochondriele functie in de CA1 en DRN, en tenslotte gen clusters voor eiwit transport in de mPOA en DRN. Op basis hiervan postuleerden wij dat transcriptoom veranderingen gekoppeld aan neurale plasticiteit, energie productie en leren en geheugen processen in corticale, hippocampale en hypothalamische hersengebieden mogelijk bijgedragen hebben aan de sexuele remming door chronische 8-OH-DPAT toediening. De microarray analyse detecteerde ook een meer dan 10-voudige toename in oxytocine (OXT) expressie in de mPOA van 8-OH-DPAT-behandelde vrouwtjes. Deze vondst is van belang, aangezien hypothalamische OXT functie in nauw verband gebracht is met sociaal en sexueel gedrag in knaagdieren, primaten en mensen. Serotonerge regulatie van sexueel en sociaal gedrag van het penseelaapje wordt dus mogelijk gemedieerd door hypothalamische OXT. Echter, doorgaans wordt aan een toename van hypothalamische oxytocine een pro-sexuele en pro-sociale werking toegeschreven, wat in tegenstelling is met de waarneming in Hoofdstuk 5. Verder onderzoek naar de expressie van oxytocine receptoren en signalering is essentieel om meer inzicht te verschaffen in deze ogenschijnlijke paradox.

De kandidaat-gen aanpak liet een sterkte toename zien van expressie van het serotonine transporter gen (5-HTT) in de DRN. De expressie van 5-HT_{1A} autoreceptoren in de DRN was niet veranderd door chronische 8-OH-DPAT toediening, maar er was wel een trend in de richting van toegenomen HT_{1A} expressie in de mPFC. Expressie van 5-HT_{2A} werd niet beïnvloed door 8-OH-DPAT in alle onderzochte hersengebieden, maar er was wel een trend waarneembaar richting een afname van 5-HT_{7} expressie in het CA1 gebied van de hippocampus. Mogelijk resulteert activatie van 5-HT_{1A} autoreceptoren in de DRN door 8-OH-DPAT in een onderdrukking van serotonerge activiteit, wat een compensatoire upregulatie induceert van 5-HTT in de DRN en van 5-HT_{1A} in de mPFC om de serotonerge tonus te herstellen.

In **Hoofdstuk 6** werd een synthese gegeven van de experimentele bevindingen beschreven in Hoofdstukken 2-5. Contrasterende effecten van flibanserin en 8-OH-DPAT op vrouwelijk sexueel en sociaal gedrag (Hoofdstuk 2) werden besproken in de context van toegenomen HPA-as responsiviteit op stress (Hoofdstuk 3), en hersengebied-specifieke veranderingen in glucose metabolisme (Hoofdstuk 4) en genexpressie (Hoofdstuk 5). In het licht van de discipline-overstijgende data set beschreven in dit proefschrift en de multifactoriele etiologie van sexuele dysfunctie in vrouwen (Hoofdstuk

1), postuleren wij dat flibanserin en 8-OH-DPAT vrouwelijk sexueel gedrag reguleren via een viertal individuele maar interactieve modules.

In de monoamine regulatoire module (i), veranderen flibanserin en 8-OH-DPAT serotonerge, dopaminerge en noradrenerge neurotransmissie, resulterend in een verandering in excitatoir of inhibitoir input op vrouwelijk sexueel gedrag. Via deze module zou flibanserin zowel een excitatoire en dis-inhibitoire invloed hebben op vrouwelijk sexueel gedrag. De HPA-as module (ii) is verantwoordelijk voor sexueel remmende effecten vanwege verhoogde activatie van het endocriene stress systeem. Deze module speelt mogelijk een rol in de toegenomen sexuele afwijzing van de partner door chronisch 8-OH-DPAT behandelde vrouwelijke penseelaapjes. De paarvorming, ervaring en geheugen module (iii) werd voorgesteld als mechanisme via welk flibanserin en 8-OH-DPAT mogelijk hun pro-sexuele and pro-sociale (flibanserin), of anti-sexuele en anti-sociale (8-OH-DPAT) werking uitoefenen. Oxytocine speelt mogelijk een sleutelrol bij het verbinden van deze module aan een regulatoire module voor vrouwelijk sexueel en sociaal gedrag (iv), die input ontvangt van modules (i)-(iii), deze integreert en uiteindelijk vertaalt naar vrouwelijk sexueel en sociaal gedrag.

Concluderend, uit de experimenten beschreven in dit proefschrift komt het belang van de kwaliteit van de band tussen de twee partners van een koppel voor de expressie van vrouwelijk sexueel gedrag sterk naar voren. De rol van oxytocine in de serotonerge regulatie van vrouwelijk sexueel gedrag moet verder onderzocht worden. Het therapeutisch effect van flibanserin voor vrouwen met HSDD zou kunnen berusten op verbeteringen in de sexuele, sociale en emotionele band tussen partners. Bij het extrapoleren van de bevindingen in het penseelaapje, beschreven in dit proefschrift, naar de mens zouden toekomstige klinische studies op het terrein van HSDD er goed aan doen als klinische uitleesparameters maten voor de intimiteit van een relatie en de rol van de partner mee te nemen.

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ACKNOWLEDGEMENTS

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CURRICULUM VITAE

CURRICULUM VITAE

Yves Aubert, from Longirod and Le Chenit (Vaud) in Switzerland, was born May 25, 1980. He obtained his Gymnasium diploma in 1999 from the Realgymnasium Rämibühl in Zurich, Switzerland. In 2000, following military service with the Swiss Air Force, he enrolled at the Department of Biology at the Swiss Federal Institute of Technology Zurich (ETHZ) and graduated in 2004, with specializations in neuroscience, ethology, pharmacology and immunology. During his undergraduate study, he assisted on botanical excursions in the Swiss Alps and participated in a research project at the Laboratory of Behavioural Neurobiology under supervision of Dr. Holger Russig and PD Dr. Christopher Pryce, to study anhedonic behavior in early deprived rats. His Master's research project, awarded with the highest grade, was conducted at the same laboratory under supervision of PD Dr. Christopher Pryce and Prof. Dr. Joram Feldon, investigating the effects of early life stress on EEG-defined sleep patterns in the common marmoset monkey. This project developed into a PhD program at the ETHZ in 2005, to investigate the genomic actions of cortisol after early-life stress in common marmosets. The project was designed in collaboration with Prof. Dr. Ron de Kloet and Dr. Nicole Datson from the division of Medical Pharmacology of the Leiden/Amsterdam Center for Drug Research (LACDR) at Leiden University, The Netherlands. In 2006, after a year of research including a 4-month visit to Leiden University, both the project and the PhD program were terminated due to the mentor's resignation.

The research described in this thesis was performed at the Wisconsin National Primate Research Center (WNPRC), University of Wisconsin-Madison, U.S.A., and at the division of Medical Pharmacology of the LACDR and Leiden University Medical Center (LUMC), The Netherlands. From 2006 to 2009, Yves was appointed by the WNPRC as a research intern under supervision of Prof. Dr. David Abbott. From 2009 to 2012, he was enrolled as PhD student at the LUMC and Leiden University to continue the Boehringer Ingelheim-sponsored project under supervision of Prof. Dr. Ron de Kloet and Dr. Nicole Datson. In November 2012, he has started a postdoctoral research project on the perception of music with the Bergen fMRI Group, Department of Biological and Medical Psychology, University of Bergen, Norway, under mentorship of Prof. Dr. Karsten Specht.

LIST OF PUBLICATIONS

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Aubert Y, Allers KA, Sommer B, de Kloet ER, Abbott DH and Datson NA. Brain region-specific transcriptomic markers of serotonin-1a receptor agonist mediated sexual rejection and aggression in female marmoset monkeys.
Submitted

Zalachoras I, Grootaers G, van Weert L, **Aubert Y**, de Kreij S, Datson NA, van Roon-Mom WMC, Aartsma-Rus AM and Meijer OC. Antisense-mediated SRC-1 isoform switching in the central nucleus of the amygdala of the mouse brain.
Submitted

Aubert Y, Bohl MA, Lange JR, Diol NR, Allers KA, Sommer B, Datson NA and Abbott DH. (2012) Chronic systemic administration of serotonergic ligands flibanserin and 8-OH-DPAT enhance HPA axis responses to restraint in female marmosets.
Psychoneuroendocrinology, DOI 10.1016/j.psyneuen.2012.05.011

Converse AK, **Aubert Y**, Farhoud M, Weichert JP, Rowland IJ, Ingrisano NM, Allers KA, Sommer B and Abbott DH. (2012) 8-OH-DPAT-mediated changes in cerebral glucose metabolism in female marmosets assessed by positron emission tomography (PET).
Neuroimage 60(1):447-455

Aubert Y, Gustison ML, Gardner LA, Bohl MA, Lange JR, Allers KA, Sommer B, Datson NA, Abbott DH. (2012) Flibanserin and 8-OH-DPAT implicate serotonin in association between female marmoset monkey sexual behavior and changes in pair-bond quality.
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Pryce CR, **Aubert Y**, Maier C, Pearce PC and Fuchs E. (2011) The developmental impact of prenatal stress, prenatal dexamethasone and postnatal social stress on physiology, behavior and neuroanatomy of primate offspring: studies in rhesus macaque and common marmoset.
Psychopharmacology (Berl) 214(1):33-53

Datson NA, Morsink MC, Steenbergen PJ, **Aubert Y**, Schlumbohm C, Fuchs E and de Kloet ER. (2009) A molecular blueprint of gene expression in hippocampal subregions CA1, CA3, and DG is conserved in the brain of the common marmoset.
Hippocampus 19(8):739-752