FACULTY OF HEALTH SCIENCES
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PhD thesis

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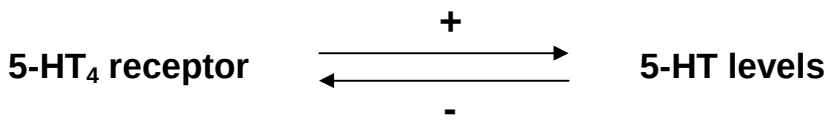
Changes in the 5-HT_4 receptor in animal models of depression and antidepressant treatment



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Cecilie Löe Licht

Summary

The serotonin 4 (5-HT_4) receptor may be implicated in depression and is a new target for antidepressant treatment. This PhD project is focused on the 5-HT_4 receptor in depression related states, and on the interaction between the 5-HT_4 receptor and the 5-HT system. The brain 5-HT_4 receptor binding was studied by receptor autoradiography in genetic and environmental rodent depression models associated with endophenotypes of 5-HT system disturbances, Flinders Sensitive Line (FSL) and Olfactory Bulbectomy (OBX); and hypothalamic-pituitary-adrenal (HPA) axis modulations, Glucocorticoid Receptor Hetero-zygous Mice ($GR^{+/-}$) and Chronic Mild Stress (CMS). Brain 5-HT_4 receptor binding was related to 5-HTT binding and model-specific behavior. Regulation of 5-HT_4 receptor binding was studied in vivo by sub-chronic 5-HT depletion, and acute and chronic administration of a selective serotonin reuptake inhibitor (SSRI) in rats, and was compared to changes in 5-HT_{2A} receptor binding. The effect of acute and sub-chronic 5-HT_4 receptor agonism on 5-HT levels in the ventral hippocampus was investigated by in vivo microdialysis.

In both the FSL and OBX depression models, hippocampal 5-HT_4 receptor binding was changed but in opposite directions. These models differ in 5-HT system disturbances and show opposite behavioral responses to stress and novelty, which may explain the difference in 5-HT_4 receptor binding. In the models based on HPA axis modulation, the 5-HT_4 receptor binding was increased in the caudate putamen of $GR^{+/-}$, while no effect of CMS was found. The 5-HT_4 receptor binding was increased by sub-chronic 5-HT depletion, and decreased by chronic administration of the SSRI paroxetine. Acute 5-HT_4 receptor activation in the presence of paroxetine, and sub-chronic 5-HT_4 receptor agonism alone, increased 5-HT levels in the ventral hippocampus.

The present findings implicate the 5-HT_4 receptor in 5-HT and HPA system associated endophenotypes of depression, modeled by three rodent depression models. As chronic manipulations of 5-HT levels affected 5-HT_4 receptor binding with an inverse relationship between 5-HT levels and 5-HT_4 receptor binding, the 5-HT system disturbances in the FSL and OBX models may underlie the changes in 5-HT_4 receptor binding. This hypothesis is supported by changes in 5-HTT binding in several of the brain regions, where changes in 5-HT_4 receptor binding was found. Given that sub-chronic 5-HT_4 receptor stimulation increases 5-HT levels in the hippocampus, the regulation of 5-HT_4 receptor binding by chronic changes in 5-HT levels may represent a feedback regulation of the 5-HT_4 receptor.

Dansk resumé

Serotonin 4 (5-HT_4) receptoren kan være impliceret i depression og er et nyt mål for antidepressiv behandling. Dette ph.d.-projekt omhandler 5-HT_4 receptoren i depressions-relaterede tilstande og interaktionen mellem 5-HT_4 receptoren og 5-HT systemet. 5-HT_4 receptor binding i hjernen blev studeret med receptor autoradiografi i genetiske og miljøinducerede dyremodeller for depression associeret med 5-HT system forstyrrelser, Flinders Sensitive Line (FSL) og Olfactory Bulbectomy (OBX); og hypothalamus-hypofyse-binyre (HPA) akse modulering, Glucocorticoid Receptor Heterozygous Mice ($GR^{+/-}$) og Chronic Mild Stress (CMS). 5-HT_4 receptor binding blev relateret til 5-HTT binding og model-specifik adfærd. Regulering af 5-HT_4 receptor binding blev undersøgt in vivo ved hjælp af sub-kronisk 5-HT depletering, og akut og kronisk administration af en selektiv serotonin reuptake inhibitor (SSRI) i rotter, og blev sammenholdt med ændringer i 5-HT_{2A} receptor binding. Effekten af akut og sub-kronisk 5-HT_4 receptor agonisme på 5-HT niveauer i ventral hippocampus blev bestemt med in vivo mikrodialyse.

Den hippocampale 5-HT_4 receptor binding var ændret i både FSL og OBX depressionsmodellerne, men i modsat retning. Disse dyremodeller adskiller sig i 5-HT system ændringer og udviser modsatrettede adfærdsmæssige reaktioner på stress og nye omgivelser, hvilket muligvis kan forklare forskellene i 5-HT_4 receptor binding. I modellerne baseret på HPA akse modulering var 5-HT_4 receptor bindingen øget i caudate putamen i $GR^{+/-}$ mus, mens CMS ikke havde nogen effekt. Sub-kronisk 5-HT depletering medførte øget 5-HT_4 receptor binding, mens kronisk administration af SSRIet paroxetin inducerede en nedregulering. Akut 5-HT_4 receptor aktivering ved tilstedeværelse af paroxetin, samt sub-kronisk 5-HT_4 receptor agonisme alene, medførte et øget 5-HT niveau i ventral hippocampus.

Disse resultater tyder på, at 5-HT_4 receptoren har en rolle i 5-HT og HPA system relaterede depressions endofænotyper, som de er repræsenteret i tre depressionsmodeller. Da kronisk manipulering af 5-HT niveau influerede 5-HT_4 receptor binding med en invers sammenhæng mellem 5-HT niveau og 5-HT_4 receptor binding, kan ændringerne i 5-HT systemet i FSL og OBX modellerne være årsag til ændringer i 5-HT_4 receptoren. Denne hypotese understøttes af, at ændringer i både 5-HT_4 receptor og 5-HTT binding blev påvist i flere hjerneregioner. Idet sub-kronisk 5-HT_4 receptor aktivering øger 5-HT niveauet i hippocampus, er det muligt, at ændringer i 5-HT_4 receptor binding som følge af kroniske ændringer i 5-HT niveau i hjernen udgør en feedback regulering af 5-HT_4 receptoren.

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List of abbreviations

5,7-DHT	5,7-dihydroxytryptamine
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HTT	5-HT transporter
ACC	anterior cingulate cortex
aCSF	artificial CSF
ACTH	adrenocorticotropic hormone
B_{max}	maximal binding capacity
CA1,-2,-3	cornu ammoni 1, -2, -3
CMS	Chronic Mild Stress
CNS	central nervous system
CRH	corticotropin-releasing hormone
CSF	cerebrospinal fluid
DFP	diisopropyl fluorophosphate
DRN	dorsal raphe nucleus
DSM-IV	Diagnostic and Statistical Manual, 4^{th} edition
ECS	electroconvulsive shock
ECT	electroconvulsive therapy
FRL	Flinders Resistant Line
FSL	Flinders Sensitive Line
FST	Forced swim test
GABA	gamma aminobutyric acid
GR	glucocorticoid receptor
$GR^{+/-}$	GR heterozygous mouse
GTPγS	guanosine-5'-(γ-thio) triphosphate
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance liquid chromatography
ICD-10	International Classification of Diseases, 10^{th} Revision
K_d	equilibrium dissociation constant
LGP	lateral globus pallidus
MAO	monoamine oxidase
MDD	Major Depressive Disorder
MRN	median raphe nucleus
OBX	Olfactory bulbectomy
pCPA	*p*-chlorophenylalanine
PET	positron emission tomography
PFC	prefrontal cortex
PVN	paraventricular nucleus
REM	rapid eye movement
SNRI	serotonin and noradrenaline reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic antidepressant

List of manuscripts

This thesis is based on the following manuscripts, which in the text are referred to by their Roman numerals.

I Licht CL, Kirkegaard L, Zueger M, Chourbaji S, Gass P, Aznar S, Knudsen GM. The 5-HT_4 receptor and 5-HTT binding are altered in two murine depression models. *Submitted (J Neurosci Res)*

II Licht CL, Marcussen AB, Wegener G, Wiborg O, Overstreet DH, Aznar S, Knudsen GM. Changes in brain 5-HT_4 receptor binding in rat depression models and in response to paroxetine administration. *Submitted (J Neurochem)*

III Licht CL, Knudsen GM, Sharp T. Effects of 5-HT_4 receptor agonism and paroxetine administration on hippocampal extracellular 5-HT levels. *Manuscript*

Introduction

Depression is a debilitating and potentially life-threatening disease, which in 2020 is estimated to become the second most important cause of years of life lost or lived with disability in the World, according to the World Health Organization (Murray and Lopez 1997). Antidepressant treatment is available but drug treatment requires several weeks to have effect and is only effective in a subset of patients. The impetus to develop more effective and faster acting treatments for depression is therefore strong.

The first antidepressant drugs developed affect the transport and metabolism of the monoamines serotonin (5-hydroxytryptamine, 5-HT) and noradrenaline in the brain, leading to the monoamine hypothesis of depression, which states that a relative deficiency in monoamine transmission in the brain is associated with the depressed state (Rosenblatt et al. 1960). With the advent of a new class of antidepressants, the selective serotonin reuptake inhibitors (SSRIs), focus within the field of depression research was directed at the 5-HT system, in particular the 5-HT transporter (5-HTT) and 5-HT_1 and 5-HT_2 receptors. Several other theories of depression, implicating other neurotransmitter systems and neurodegenerative changes in the brain, exist and reflect the complexity of the disorder and the multitude of systems and brain functions involved.

The 5-HT receptors studied in depression include 5-HT_{1A} and 5-HT_{1B} autoreceptors, and postsynaptic 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors, which have an inhibitory effect on 5-HT system activity. To augment the effect of SSRIs on 5-HT system activity and reduce the time of treatment required, addition of antagonists of inhibitory 5-HT receptors to SSRI treatment has been pursued. However, a stimulatory effect on 5-HT neuron firing activity by activation of the 5-HT_4 receptor has recently been reported (Lucas and Debonnel 2002), indicating a role for this receptor in depression. Polymorphisms of the human 5-HT_4 receptor gene have been associated with depression (Ohtsuki et al. 2002), and changes in brain 5-HT_4 receptor binding have been detected in depressed suicide victims (Rosel et al. 2004).

This thesis is focused on the role of the 5-HT_4 receptor in the pathophysiology of depression and its potential as a target for antidepressant treatment. The brain 5-HT_4 receptor binding is examined in environmental and genetic animal models of depression and in response to experimental manipulations of central 5-HT levels; and the effects of 5-HT_4 receptor activation alone and in combination with SSRI administration on brain 5-HT levels are analyzed.

Theoretical background

Clinical depression and antidepressant treatment

According to the World Health Organization Global Burden of Disease Study, unipolar major depression was the leading cause of disability in the World in 1990 (Murray and Lopez 1996). Depression is diagnosed according to symptomatic criteria described in the International Classification of Diseases, 10th Revision, Classification of Mental and Behavioral Disorders (ICD-10) or in the Diagnostic and Statistical Manual 4th ed. (DSM-IV) used mainly in the United States (American Psychiatric Association 1994;Lublin et al. 2002;WHO 2004). The diagnosis is syndromal, based on the presence of a subset of possible symptoms, and depression should therefore be viewed as comprised of several diseases with distinct pathophysiologies (Nestler et al. 2002a). The ICD-10 classification system distinguishes between mild, moderate and severe depressive episodes, and the criteria include core symptoms of depressed mood, anhedonia and decreased energy, of which at least two must be present for a minimum of two weeks in combination with a subset of additional symptoms (Table 1).

Core symptoms	Depressed mood most of the day, nearly every day
	Loss of interest or pleasure (anhedonia)
	Decreased energy or increased fatigability
Symptoms	Loss of confidence or self-esteem
	Excessive or inappropriate guilt, or self-reproach
	Recurrent thoughts of death or suicide, or suicidal behavior
	Decreased ability to think or concentrate
	Psychomotor agitation or retardation
	Sleep disturbance (insomnia or hypersomnia)
	Change in appetite and body weight

Table 1. ICD-10 diagnostic criteria for a depressive episode (adapted from (Pedersen et al. 2001)

About 50 to 60% of depressed patients will experience more than one depressive episode (recurrent depressive disorder, or major depressive disorder (MDD)), the average number of lifetime episodes being 4.7 (American Psychiatric Association 1994;Hasin et al. 2005).

The two-week prevalence in the Danish general population of DSM-IV major depressive episode and ICD-10 depressive episode (mild, moderate, severe) is 3.3% and 4.1% (1.5%, 1.2%, 1.4%), respectively (Olsen et al. 2004;Eplov et al. 2005). With respect to the Danish population figures for the year 2006, a 3.3% point prevalence corresponds to 180,000

individuals being affected by depression at any given time (Danmarks Statistik 2006). A recent survey of MDD in the United States found a 12-month and lifetime prevalence of 5.28% and 13.23%, respectively (Hasin et al. 2005). The mean age of onset of depression is 30.4 years in the United States, and corresponding ages are reported worldwide (Wong and Licinio 2001;Hasin et al. 2005).

Based on a meta-analysis of five twin studies, the heritability of liability to MDD has been estimated to be 33%, making depression a heritable disorder to the same degree as hypertension and type II diabetes (Fava and Kendler 2000;Nestler et al. 2002a). However, vulnerability to depression is also influenced by non-genetic environmental risk factors, of which gender (higher among women), stressful life events, adverse childhood experiences, and the personality trait of 'neuroticism' are strongly associated with MDD (Fava and Kendler 2000;Olsen et al. 2004;Wong and Licinio 2001;Hasin et al. 2005). Other environmental adversities associated with MDD include job loss, marital difficulties, having a low income, viral infections, and stochastic processes during brain development (Fava and Kendler 2000;Nestler et al. 2002a;Hasin et al. 2005). Apart from the obvious increase in risk of suicide, depression also carries an increased risk of medical morbidity, being an independent risk factor for myocardial infarction and associated with decreased bone mineral density (Wong and Licinio 2001;Hansen et al. 2003;Hasin et al. 2005).

The treatment approach to depressive episodes depends on depression severity and on the presence or absence of melancholic or psychotic symptoms. Mild depressive episodes can be treated with cognitive behavioral therapy, while moderate and severe episodes require treatment with antidepressant drugs (Stage 2001). Moderate to severe episodes can be treated with SSRIs, serotonin and noradrenaline reuptake inhibitors (SNRI) or tricyclic antidepressants (TCA) (Stage 2001). In case of severe episodes with melancholic symptoms, TCAs are used, and in case of psychotic symptoms, either TCAs or electroconvulsive therapy (ECT) is recommended (Eplov et al. 2005). A given antidepressant drug will be efficient in 60-70% of cases, and it is therefore often necessary to test several drugs of different pharmacological classes in succession (Stage 2001). It takes two to three weeks for an antidepressant drug to have a noticeable effect, and an additional two to three weeks of treatment for the patient to feel well (Stage 2001). However, even after the symptoms of depression disappear, continued drug treatment is recommended for approximately one year to ensure that the underlying depression has been fully treated.

Biological characteristics of depression

In order to identify genes involved in depression, and to understand the pathophysiological mechanisms of depression, two groups of endophenotypes of depression have been proposed: psychopathological endophenotypes based on a dissection of the DSM-IV phenotype, and biological endophenotypes based on biological markers (Table 2) (Hasler et al. 2004). An endophenotype is an internal, not directly visible, phenotype, lying between gene effect and clinical symptom, and should be specific to the disease of interest, state-independent, heritable, and biologically and clinically plausible (Hasler et al. 2004). The proposed endophenotypes show biological consistency and can be investigated in experimental animals.

Psychopathological endophenotypes	**Biological endophenotypes**
Depressed mood (mood bias)	REM sleep abnormalities
Anhedonia (impaired reward function)	Increased amygdala activity
Impaired learning and memory	Decreased subgenual PFC activity
Direction of appetite change	Left ACC volume reduction
Diurnal variation	Hippocampal volume reduction
Executive cognitive function (response speed)	Reduced 5-HT_{1A} receptor binding potential
Psychomotor change	Sensitive to tryptophan depletion
Increased stress sensitivity	Sensitive to catecholamine depletion
	Abnormal dexamethasone/CRH test
	CRH dysfunction

Table 2. Adapted from (Hasler et al. 2004). ACC: anterior cingulate cortex. CRH: corticotropin-releasing hormone. PFC: prefrontal cortex. REM: rapid eye movement.

In patients with primary, familial MDD there is a significant increase in cerebral blood flow in the amygdala (Drevets et al. 1992), which indicates corresponding changes in afferent synaptic transmission (reviewed in (Drevets 2000)). In contrast with the increased activity, amygdala volume is found to be reduced in symptomatic and remitted mood disorders, possibly as a result of amygdala hyperactivity (Drevets et al. 2004;Hasler et al. 2004). The amygdala is a part of the limbic system and is important for organizing emotional and stress responses. Amygdala activation produces anxiety, fear, dysphoria, and increased cortisol secretion (Hasler et al. 2004). Amygdala projects to the locus coeruleus, containing noradrenaline neurons, and excessive amygdala activity may thereby contribute to the agitation and insomnia seen in depressive episodes (Drevets 2000). On the contrary, activity decrease in association with volumetric reduction has been found in the subgenual prefrontal cortex (PFC), which is involved in autonomic responses to emotional stimuli and in evaluating adverse consequences of detrimental social behaviors (Drevets et al. 1997). Cerebral blood flow, glucose metabolism and volume are also decreased in the caudate

nucleus of depressed individuals (Drevets et al. 1992;Drevets 2000;Sheline 2003), and reductions in hippocampal volume have also been detected in some studies (Sheline 2003;Sheline et al. 2003). These findings implicate abnormal structure and function of a limbic (amygdala) - cortical (PFC) - striatal (caudate) - pallidal - thalamic circuit, with an excitatory triangular limbic - thalamic - cortical circuit and a disinhibitory limbic - striatal - pallidal - thalamic side loop (Drevets et al. 1992). Increased activity in the limbic - thalamic - cortical circuit during depression may be associated with a decreased inhibitory effect of dopaminergic projections to striatal regions, and increased dopaminergic and serotonergic activity would both be predicted to inhibit activity in the limbic - thalamic - cortical circuit (Drevets et al. 1992).

In a large proportion of MDD patients excessive activity of the hypothalamic-pituitary-adrenal (HPA) axis, which is normally activated in response to stress, has been found and can be corrected by antidepressant treatment (Nestler et al. 2002a;Hasler et al. 2004). In response to sympathetic, amygdaloid, and serotonergic activation, neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete corticotropin-releasing hormone (CRH), which stimulates the anterior pituitary to synthesize and release adrenocorticotropic hormone (ACTH) to the blood stream (Azmitia 1999;Nestler et al. 2002a). ACTH induces synthesis and release of the glucocorticoid cortisol from the adrenal cortex to the blood, affecting general metabolism and brain function (Nestler et al. 2002a). The release of CRH from the PVN is inhibited by hippocampal afferents and is under negative feedback control by glucocorticoid receptors (GR) on PVN neurons and in the hippocampus (Nestler et al. 2002a;Hasler et al. 2004). This negative feedback mechanism is the physiological basis of the dexamethasone/CRH suppression test, where pretreatment with the synthetic glucocorticoid, dexamethasone, prevents an increase in ACTH and cortisol in plasma after CRH injection in healthy volunteers (Heuser et al. 1994). However, 80% of MDD patients have abnormally increased ACTH and cortisol levels after CRH injection, indicating that the negative feedback mechanism is inhibited (Heuser et al. 1994). A sustained high level of cortisol decreases branching and length of dendrites of hippocampal neurons and inhibits adult neurogenesis in the dentate gyrus through excitotoxicity and inhibition of neurotrophin function (Sapolsky 2000). Damage to the hippocampus may interfere with the normal GR mediated negative feedback regulation of the HPA axis and thereby result in a further rise in cortisol levels (Nestler et al. 2002a). Cortisol induced hippocampal atrophy could contribute to the impaired learning and memory deficits, found in depression (Hasler et al. 2004).

The 5-HT system in depression and antidepressant treatment

The first drugs with therapeutic effect in depression, iproniazid and imipramine, were discovered in the late 1950s (Bloch et al. 1954;Kuhn 1958). Iproniazid inhibits the enzyme monoamine oxidase (MAO), involved in the degradation of the monoamines 5-HT and noradrenaline in the brain, and thereby increases their extracellular concentrations. In combination with reports on the depressive effect of reserpine, which depletes 5-HT and noradrenaline storages, these observations led to the monoamine hypothesis, proposing that a relative deficiency of monoamines is associated with the depressive state (Rosenblatt et al. 1960). In 1969, the monoamine hypothesis was followed by the indoleamine hypothesis of depression, stating that vulnerability to depression is related to decreased serotonergic activity (Mann 1999).

The 5-HT system has an important influence on several biological functions, such as affective states, cognition, motor function, circadian rhythm, sleep, pain, sexual function, and feeding behavior (Mann 1999;Bockaert et al. 2004). Apart from depression, it has been associated with a range of neuropsychiatric conditions, including anxiety, suicidal behavior, obsessive-compulsive disorder, mania, eating disorders, and alcoholism (Mann 1999). Serotonin exerts its effects through up to 14 5-HT receptor subtypes located pre- and postsynaptically, before it is inactivated by diffusion away from the 5-HT receptors or reuptake by the 5-HTT. The majority of 5-HT neuronal cell bodies are located in the median (MRN) and the dorsal raphe nucleus (DRN) (Jacobs and Azmitia 1992), and project through the medial forebrain bundle to the cortex, basal ganglia, limbic system and diencephalon (Tork 1990). As the rate of 5-HT synthesis is dependent on the availability of the precursor of 5-HT, the essential amino acid tryptophan, brain 5-HT levels can be acutely lowered by tryptophan depletion (Bell et al. 2001). Tryptophan depletion induces a lowering of mood, memory impairment and an increase in aggression in healthy volunteers, and causes a relapse in depressed patients successfully treated with serotonergic antidepressants (Bell et al. 2001).

Most antidepressant drugs used today inhibit reuptake of 5-HT by the 5-HTT, in particular the SSRIs (Berton and Nestler 2006). Studies of brain 5-HTT binding in MDD in vivo have shown increased, decreased or unchanged binding potential in various brain regions (Meyer 2007). However, a recent positron emission tomography (PET) study using the highly selective [^{11}C]DASB radioligand found pronounced increases in 5-HTT binding potential in the thalamus, striatum, insular cortex, anterior cingulate cortex (ACC), and periaqueductal

grey of MDD subjects (Cannon et al. 2007). The regulation of the 5-HTT by 5-HT tonus is paradoxical (Fig. 1), as both chronic SSRI administration (Benmansour et al. 1999;Johnson et al. 2008) and 5-HT depletion downregulate 5-HTT binding (Rattray et al. 1996a;Rothman et al. 2003) and decrease 5-HTT function (Rattray et al. 1996a;Benmansour et al. 2002). Apart from the 5-HTT, the 5-HT_1 and 5-HT_2 receptors have been the most extensively studied 5-HT system factors in mood disorders (Mossner et al. 2007).

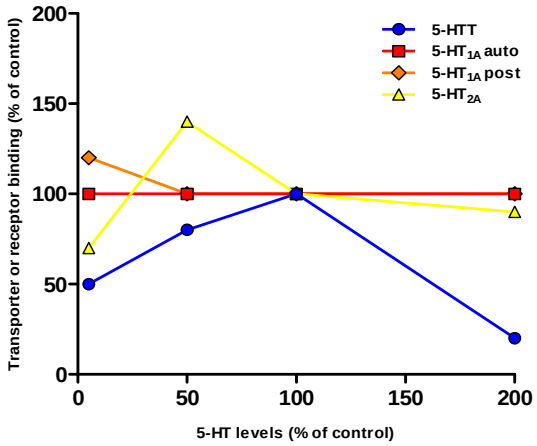

Figure 1. Chronic autoregulation of the rat 5-HT system. Relative changes in 5-HT transporter and receptor binding in response to changes in central 5-HT levels, achieved by chronic 5-HT depletion or SSRI administration. See text for references. 5-HT levels after SSRI administration are estimated from (Kreiss and Lucki 1995;Hajos-Korcsok et al. 2000).

The 5-HT_{1A} receptors are located presynaptically on 5-HT neurons in the raphe nuclei, and postsynaptically in 5-HT terminal projection fields, mainly in cortico-limbic areas (Celada et al. 2004). During depression, the binding potential of the 5-HT_{1A} receptor is reduced in terminal projection fields and the raphe nuclei in vivo (Drevets et al. 1999;Drevets et al. 2000;Sargent et al. 2000). Acute increases in the extracellular concentration of 5-HT inhibits 5-HT neuron firing through 5-HT_{1A} receptors in the raphe nuclei (Gartside et al. 1995;Hajos et al. 1995). However, chronic antidepressant treatment reduces the electrophysiological responsivity of 5-HT_{1A} receptors in the raphe nuclei (Invernizzi et al. 1994;Sprouse and Aghajanian 1987) and thereby disinhibits the 5-HT neuronal firing, increasing extracellular 5-HT levels in the hippocampus and frontal cortex (Hajos-Korcsok et al. 2000). Addition of 5-HT_{1A} receptor antagonists to acute SSRI administration prevents the inhibition of 5-HT neuronal activity (Gartside et al. 1995), which had led to experimental addition of 5-HT_{1A} receptor antagonists to SSRI treatment to accelerate onset and augment the therapeutic response (Celada et al. 2004). In contrast to the desensitizing effect of chronic SSRI administration on the somatodendritic 5-HT_{1A} receptors, chronic SSRI administration does

not affect 5-HT_{1A} autoreceptor binding in the DRN or postsynaptic 5-HT_{1A} receptor binding in the hippocampus (Fig. 1) (Le Poul et al. 1995;Riad et al. 2008). Tryptophan depletion acutely decreases 5-HT_{1A} receptor [^{3}H]WAY100635 binding in the DRN, which may be a compensatory response to low 5-HT levels (Cahir et al. 2007). However, this effect is transient, as chronic tryptophan depletion inducing 29-81% reduction in brain 5-HT does not affect [^{3}H]WAY100635 binding in any region (Cahir et al. 2007). Chronic, partial 5-HT depletion by MDMA exposure also has no effect on 5-HT_{1A} [^{125}I]cyanopindolol binding in cortical and subcortical regions (McGregor et al. 2003), On the other hand, chronic severe (>95%) 5-HT depletion by pCPA but not by 5,7-dihydroxytryptamine (5,7-DHT) increases cortical 5-HT_{1A} receptor [^{3}H]-8-OH-DPAT binding (Compan et al. 1998). In addition to 5-HT_{1A} receptors, 5-HT_{1B} autoreceptors regulate 5-HT system activity by inhibiting terminal 5-HT synthesis and release (Barnes and Sharp 1999).

The postsynaptic 5-HT_{2} receptors have inhibitory effects on 5-HT neuron firing rate, which are mainly mediated by the 5-HT_{2A} receptor subtype (Boothman et al. 2003). The 5-HT_{2A} receptor binding is increased in the frontal cortex and the caudate nucleus of depressed suicide victims postmortem (Stanley and Mann 1983;Rosel et al. 2004), while most PET studies have found decreased cortical binding (Meyer 2007). The effect of chronic 5-HT depletion on 5-HT_{2A} receptor binding may depend on degree of depletion, as partial (29-70%) 5-HT depletion induces an increase in cortical 5-HT_{2A} receptor binding (Cahir et al. 2007;Heal et al. 1985), while severe (>95%) 5-HT depletion by 5,7-DHT causes dramatic decreases in 5-$HT_{2A/2C}$ receptor [^{125}I]DOI binding in the cortex (Compan et al. 1998). However, partial but long lasting 5-HT depletion by chronic MDMA also causes large decreases in 5-$HT_{2A/2C}$ [^{125}I]DOI binding striatum, thalamus, hypothalamus and other regions (McGregor et al. 2003), while severe 5-HT depletion by 5 days of pCPA administration has no effect on [^{125}I]DOI binding in the cingulate, frontal, and parietal cortex (Compan et al. 1998). The cause of these discrepancies may be differences in method of 5-HT depletion and time point of binding analysis. If 5-HT depletion can induce both an increase and a decrease in 5-HT_{2A} receptor binding depending on degree of 5-HT depletion, there may be an intermediary point of substantial 5-HT depletion and no change in 5-HT_{2A} receptor binding. This may be around 50-90% 5-HT depletion as some studies have found no change in [^{3}H]ketanserin (Fischette et al. 1987) or [^{125}I]DOI binding (Compan et al. 1998;Owens et al. 1996) at this degree of 5-HT depletion. Chronic SSRI treatment moderately decreases cortical 5-HT_{2A} receptor binding in depressed subjects in vivo (Meyer et al. 2001) and in some animal studies (Gunther et al. 2008;Nelson et al. 1989;Maj et al. 1996).

Animal models of depression

Animal depression models represent particular symptoms or endophenotypes of depression, allowing investigation of the underlying pathophysiological mechanisms (Nestler et al. 2002b), and can be used to investigate efficacy and mechanisms of action of new approaches to antidepressant treatments. Depression models have been developed by exposing animals to chronic, environmental stressors mimicking natural conditions such as early life stress, Chronic Mild Stress (CMS), and social stress, or by lesioning the olfactory and interconnected systems by olfactory bulbectomy (OBX). Genetic models have been developed by selective breeding for extreme responses to stress or drugs, such as the congenital learned helplessness model and the Flinders Sensitive Line (FSL), or by changed expression of depression related genes. In this project, analyses are conducted in two rat depression models, FSL and CMS; and in two mouse models, OBX and glucocorticoid receptor heterozygous ($GR^{+/-}$) mice.

The Flinders Sensitive Line

The FSL was developed from Sprague-Dawley rats by selective breeding at the Flinders University of South Australia in an attempt to create a strain with resistance to the organophosphate diisopropyl fluorophosphate (DFP), an inhibitor of acetylcholinesterase (Overstreet et al. 1979). Instead a line of rats with increased sensitivity to muscarinic cholinergic agonism was developed (Overstreet et al. 1979;Overstreet and Russell 1982;Overstreet et al. 1984). The FSL breeding program was based on 30 generations of selection for extremes in hypothermic response, changes in water intake, and changes in body weight in response to DFP (Overstreet et al. 2005). Reports of increased sensitivity to muscarinic agonists in MDD patients prompted studies of FSL as a genetic model of depression (Risch et al. 1981;Overstreet et al. 1998), and the FSL was later found to display increased immobility in the forced swim test (FST) and increased 5-HT_{1A} receptor sensitivity compared to the Flinders Resistant Line (FRL) rats (Overstreet et al. 1994). The increase in immobility in the FST was correlated with increased 5-HT_{1A} receptor sensitivity but not with cholinergic sensitivity, indicating that the depression-like phenotype of the FSL rats was associated with changes in the 5-HT system (Overstreet et al. 1994). The FSL model exhibits several depression related endophenotypes, and its behavioral responses to drug treatment in the FST reflect antidepressant effects of TCAs, SSRIs, and new antidepressant drugs (Table 3).

The Chronic Mild Stress Model

The CMS model is a model of chronic stress-induced depression, based on applying a variety of mild, unpredictable, environmental stressors for several weeks (Willner 2005). The CMS model was designed to model anhedonia (Willner et al. 1992), which in biological terms is translated into a generalized decrease in responsiveness to reward, mediated by the mesolimbic dopaminergic pathway (Kornetsky et al. 1991;Willner et al. 1992) and evaluated by reduction in intake of and/or preference for a dilute sucrose solution (Willner 1997). The CMS protocols typically consist of 2-3 weeks of sucrose solution-consumption training, followed by application of CMS for several weeks with weekly sucrose intake monitoring. The stressors are applied for several hours each, and include interchanging periods of food and water deprivation, overnight illumination, paired housing, 45^{o} cage tilt, soiled cage bedding, and stroboscopic illumination (Willner et al. 1992).

	Flinders Sensitive Line	**Chronic Mild Stress**	**Olfactory bulbectomy**	**GR$^{+/-}$ mouse**
Construct	Genetic cholinergic and 5-HT$_{1A}$ hypersensitivity (Overstreet et al. 1994)	CMS-induced anhedonia (Willner et al. 1992)	Neurodegeneration in limbic and 5-HT system (Song and Leonard 2005)	Heterozygous for the glucocorticoid receptor (Ridder et al. 2005)
Validation	5-HT$_{1A}$ response, FST	Sucrose intake	Open field test	Genotype
Endophenotypes				
Behavior	Increased immobility in FST, decreased appetite and weight, reduced psychomotor activity (Overstreet et al. 2005), vulnerable to CMS (Pucilowski et al. 1993)	Anhedonia, increased immobility in FST, decreased sexual behavior, grooming and aggression (Willner 2005), decreased weight (Willner 1997)	Hyperactivity in novel, stressful environments, increased exploration (Zueger et al. 2005), changes in aggression (Mucignat-Caretta et al. 2004)	Deficit in corticosterone suppression in dexamethasone/CRH test, increased helplessness after stress (Ridder et al. 2005)
REM sleep	Increased (Willner and Mitchell 2002)	Increased (Willner 2005)	Decreased in rats (Kelly et al. 1997)	ND
HPA axis	No change (Zangen et al. 2002)	Increased activity (Willner 1997)	Increased activity in rat (Cairncross et al. 1977b)	Deficit in regulation (Ridder et al. 2005)
5-HT levels	Increased content (Zangen et al. 1997), decreased synthesis (Hasegawa et al. 2006)	Decreased (Li et al. 2003;Bekris et al. 2005;Kang et al. 2005)	Decreased turnover (Hellweg et al. 2007) and synthesis (Neckers et al. 1975)	No change (Schulte-Herbruggen et al. 2007)
5-HT$_{1A}$ receptor binding/mRNA	No change in hippocampus (Bjornebekk et al. 2007)	Increased in hippocampus (Papp et al. 1994)	No change (Gurevich et al. 1993)	ND
5-HT$_2$ receptor binding/ mRNA	Decreased in hippocampus (Bjornebekk et al. 2007), cortex and amygdala (Osterlund et al. 1999)	Increased in cortex (Papp et al. 1994)	Increased in frontal cortex (Gurevich et al. 1993)	Decreased in frontal cortex (Kostova et al. unpublished results)
Predictive validity	High (Overstreet et al. 2005)	High (Willner 1997)	Good (Jarosik et al. 2007)	ND

Table 3. Summary of selected depression-related characteristics of the FSL, CMS, OBX, and GR$^{+/-}$ depression models. ND: no data.

The CMS procedure reveals an underlying heterogeneity with respect to stress sensitivity and response, as CMS induces anhedonia in approximately 70% of animals, while the remaining

animals display unchanged or increased sucrose intake (Bergstrom et al. 2007). The CMS induced difference in reward sensitivity is also evident in the conditioned place preference test (Bergstrom et al. 2008), and is reflected in differential regulation of 156 genes in the hippocampus (Bergstrom et al. 2007). The CMS model displays several depression endophenotypes, in particular anhedonia, which is normalized by chronic administration of most antidepressant drugs and by electroconvulsive shock (ECS) (Table 3).

Olfactory bulbectomy

OBX consists of bilateral ablation of the olfactory bulbs, which in rodents induces behavioral and neurobiological changes similar to certain aspects of clinical depression (Table 3) (Kelly et al. 1997;Song and Leonard 2005). The OBX mouse is characterized by decreased 5-HT turnover in the hippocampus, frontal cortex, and hypothalamus, and has been conceptualized as a model of agitated depression (Hellweg et al. 2007). The key behavioral change is hyperactivity in response to novel, stressful environments, such as the open field test performed under bright illumination, but bulbectomized rodents also display increased irritability, anhedonia, learning and memory deficits, and changes in aggression (Zueger et al. 2005;Kelly et al. 1997;Song and Leonard 2005). The OBX behavioral syndrome develops over 14 days after surgery due to anterograde and retrograde neurodegeneration, and resulting structural and functional changes in brain areas (e.g. amygdala, raphe nuclei, and hippocampus) connected to the olfactory bulbs (Kelly et al. 1997;Song and Leonard 2005). The bulbectomy model has high predictive validity, since chronic but not acute treatment with the majority of antidepressants can reverse the biochemical and behavioral deficits (Jarosik et al. 2007;Kelly et al. 1997). Several studies have shown that changes in the 5-HT system are involved in the neurobiological basis of the OBX syndrome. Injection of a serotonergic toxin into the olfactory bulbs is sufficient to induce the OBX syndrome (Cairncross et al. 1977a), and OBX induces neurodegeneration in the DRN of mice (Nesterova et al. 1997), possibly due to retrograde degeneration of the serotonergic fibers innervating the main olfactory bulb (McLean and Shipley 1987).

Glucocorticoid receptor heterozygous mice

Given the importance of HPA axis deregulation in the pathophysiology of depression, mice with genetic alterations of the GR have been developed as models of depression (Chourbaji and Gass 2008). The GR heterozygous mice ($GR^{+/-}$) lack one GR allele and have reduced GR mRNA and protein level (Ridder et al. 2005). The $GR^{+/-}$ mice have ineffective corticosterone

suppression in the dexamethasone/CRH test (Ridder et al. 2005), indicating deficits in the negative feedback control of glucocorticoid release. A comparable deficit is seen in more than 50% of patients with severe depression (Heuser et al. 1994). While exhibiting normal behavior at baseline, the $GR^{+/-}$ mice display depression-like behavior upon stress exposure (Ridder et al. 2005).

Characteristics of the 5-HT_4 receptor

The 5-HT_4 receptor is a post-synaptic G_s protein-coupled 5-HT receptor, which was originally identified as the mediator of 5-HT stimulated increase in adenylate cyclase activity in colliculi neurons and hippocampal membranes (Dumuis et al. 1988;Bockaert et al. 1990). Activation of the 5-HT_4 receptor inhibits neuronal K^+ currents, resulting in increased neuronal excitability (Fagni et al. 1992). The 5-HT_4 receptor mediated stimulation of adenylate cyclase activity is rapidly and strongly desensitized by exposure to 5-HT in mouse colliculi neurons, and requires a recovery period of 72 hours to return to maximal capacity (Ansanay et al. 1992). The 5-HT_4 receptor is alternatively spliced into at least 10 receptor isoforms with distinct expression pattern and functional profiles in vitro (Bockaert et al. 2004;Brattelid et al. 2004). However, as the isoforms differ mainly in the intracellular C-terminal domain, they cannot be discerned by binding of available 5-HT_4 receptor ligands (Bockaert et al. 2004).

The 5-HT_4 receptor binding is high in the olfactory tubercles, substantia nigra, ventral pallidum, nucleus accumbens, interpeduncular nucleus, medial habenula, and locus coeruleus; and intermediary in the caudate putamen, globus pallidus, hippocampus, amygdala, hypothalamus, and lateral septum, while cortical binding is low (Jakeman et al. 1994;Waeber et al. 1994;Manuel-Apolinar et al. 2005). The 5-HT_4 receptors are associated with at least two functional systems, the cortico-striato-nigro-tectal pathway, and the septo-hippocampo-habenulo-interpeduncular pathway, which transmits impulses from the limbic forebrain to the raphe nuclei (Waeber et al. 1994). The 5-HT_4 receptors are expressed on striatal GABAergic neurons and on their projections to the globus pallidus and substantia nigra pars lateralis (Patel et al. 1995;Compan et al. 1996). In the hippocampus, 5-HT_4 receptors are localized on glutamatergic CA1 pyramidal neurons, on granule cells in the dentate gyrus, and on CA3 neurons (Bockaert and Dumuis 1998). In the periphery, 5-HT_4 receptors are present in the gastrointestinal tract, bladder, heart, vasculature, and adrenal gland (Hegde and Eglen 1996).

The 5-HT_4 receptor has stimulatory effects on both the cholinergic (Yamaguchi et al. 1997;Matsumoto et al. 2001) and nigrostriatal dopaminergic system (Lucas et al. 2001).

Acute agonism of the 5-HT_4 receptor increases the mean 5-HT neuron firing rate in the DRN by 96%, and increases extracellular 5-HT levels by 200% in the hippocampus, while decreasing 5-HT levels by 80% in the frontal cortex (Barnes et al. 1992;Ge and Barnes 1996;Lucas and Debonnel 2002). On the other hand, 5-HT_4 receptor antagonism inhibits mean 5-HT neuron firing in the DRN by 80-100% and decreases hippocampal 5-HT levels, indicating the presence of an endogenous tone on the 5-HT_4 receptor (Ge and Barnes 1996;Lucas and Debonnel 2002). The response to 5-HT_4 receptor ligands in the DRN is present in a subset (50%) of 5-HT neurons, which are characterized by a higher mean basal firing rate than the non-responding group (Lucas and Debonnel 2002). The stimulatory effect of acute 5-HT_4 receptor partial agonism is unchanged after 3 and 21 days of agonist administration (Lucas et al. 2005). The 5-HT_4 receptor influence on 5-HT neurons may be mediated by a long feedback loop from the medial PFC, as overexpression of the 5-HT_4 receptor in this region increases mean activity of 5-HT neurons by 70% (Lucas et al. 2005).

The stimulatory effects of 5-HT_4 receptor activation on 5-HT neuron activity suggest a role for the receptor in depression and antidepressant treatment. However, compared to the 5-HT_{1A} and 5-HT_{2A} receptors relatively little is known about the 5-HT_4 receptor's role in depression. According to one study 5-HT_4 receptor binding is increased in the frontal cortex and caudate nucleus and unchanged in the amygdala and hippocampus of depressed violent suicide victims (Rosel et al. 2004). In the same study, total cAMP levels were increased in regions with increased 5-HT_4 receptor binding (Rosel et al. 2004). Also, three polymorphisms in the splice variant region of the *HTR4* gene have been associated with MDD and bipolar disorder (Ohtsuki et al. 2002). Behavioral studies of the 5-HT_4 receptor knockout mouse indicate that the 5-HT_4 receptor is implicated in normal reactions to stress and novelty (Compan et al. 2004). Chronic treatment with the antidepressants paroxetine, citalopram, fluvoxamine, imipramine or ECS attenuates the stimulatory effect of the 5-HT_4 receptor agonist zacopride on hippocampal CA1 neuronal excitability (Bijak et al. 1997;Bijak 1997;Zahorodna et al. 2002). However, chronic citalopram administration does not affect 5-HT_4 receptor binding in the substantia nigra (Gobbi et al. 1997). Though acute 5-HT_4 receptor antagonism with SB204070A does not inhibit the antidepressant effect of fluoxetine in the FST (Cryan and Lucki 2000), the 5-HT_4 receptor partial agonist RS67333 has antidepressant-like effects in the FST, and in the CMS and OBX depression models after only 3 days of administration (Lucas et al. 2007).

Aims of the thesis

The general objective of this thesis was to investigate the 5-HT_4 receptor in animal models of depression, and to determine the regulatory interactions between the 5-HT system and the 5-HT_4 receptor, including the antidepressant potential of 5-HT_4 receptor stimulation.

The specific aims were:

I To investigate brain 5-HT_4 receptors in mouse and rat models of depression

II To determine the effects of chronic changes in central 5-HT levels on 5-HT_4 receptors

III To explore antidepressant-like effects of 5-HT_4 receptor activation, alone and in combination with SSRI administration

Experimental methods

Determination of 5-HT_4 receptor levels

Brain receptor levels can at protein level be determined with anatomical resolution by receptor autoradiography and immunohistochemistry, requiring availability of radioligands or selective antibodies, respectively. The same principles of receptor detection can be applied to brain homogenates in the form of homogenate receptor binding and immunoblot, which are faster techniques but have reduced anatomical resolution. So far protocols and suitable antibodies for immunohistochemistry or immunoblot of the 5-HT_4 receptor are not available. In this project it was important to determine brain 5-HT_4 receptor levels with high anatomical resolution and in vitro receptor autoradiography was chosen, as this technique is semi-quantitative and because a selective radioligand [^{3}H]SB207145 was available.

In vitro receptor autoradiography

In vitro receptor autoradiography employs radioactively labeled ligands, which bind reversibly but with high affinity to specific receptors in their native, properly folded state, allowing the localization and quantitation of receptors in thin brain sections. Quantitative in vitro autoradiography with radiolabeled 5-HT_4 receptor agonists and antagonists has been used to characterize brain 5-HT_4 receptor binding in different species, disease states, and in response to pharmacological manipulations (Bockaert and Dumuis 1998;Bonaventure et al. 2000;Bonhaus et al. 1997;Domenech et al. 1994;Jakeman et al. 1994;Varnas et al. 2003;Vilaro et al. 2005;Waeber et al. 1993;Waeber et al. 1994;Grossman et al. 1993;Compan et al. 1996). The compound SB207145 is a novel 5-HT_4 receptor antagonist with high selectivity (GlaxoSmithKline, unpublished information) and affinity for the 5-HT_4 receptor, yielding K_d values between 0.13 and 1.01 nM for different brain regions in rat (Parker et al. 2003). SB207145 has been labeled to relatively high specific activity (66.7 Ci/mmol) with tritium, which in comparison to labeling with iodine-125 has the advantage of not necessitating changes in the molecular structure of the ligand, and of higher stability due to the long half-life of tritium (Bennett 1978).

Low-energy β^--radiation from tritium decay can generate autoradiograms with high spatial resolution, which normally comes at the cost of long (weeks-months) exposure times on tritium sensitive film. To reduce exposure times and because tritium sensitive films were

out of production, radiolabeled brain sections were exposed to tritium sensitive phosphor imaging plates, which is a recently developed alternative to conventional radiation sensitive film for receptor autoradiography (Amemiya and Miyahara 1988). The imaging plates are based on the temporary storage in special crystals of a latent spatial image of emitted radiation energy, which is later released as photostimulated luminescence in a dedicated scanner and converted to a digital image of the radioactive decay (Amemiya and Miyahara 1988). The high sensitivity of imaging plates allows a marked reduction (4-5 times) in exposure time compared to tritium sensitive film at the cost of lower resolution (Mori K 1993;Pavey et al. 2002).

The receptor autoradiography studies were performed at a single radioligand concentration as the aim was to compare 5-HT_4 receptor binding between groups. While a saturation analysis using a range of at least 6 ligand concentrations is necessary to determine whether changes in binding are due to changes in receptor affinity (K_d) or the total number of receptor binding sites (B_{max}), this would require a 6-fold expansion of each study and was not deemed necessary in this project. When deciding on a concentration for a single concentration study, one can chose between two main approaches: one is to select a low concentration at or below the K_d, where the difference between total binding and non-specific binding (to tissue and other receptors) is large, giving a high signal-to-noise ratio (Herkenham and Pert 1982); another is to choose a concentration approaching saturation of the receptor (>5-6 times K_d) and interpret the binding as a estimate of B_{max} (Chen et al. 1992). The concentration of [^{3}H]SB207145 used in this project was chosen based on the optimal signal-to-noise ratio of autoradiograms, when comparing a range of concentrations around the K_d, and corresponds to 1-7 times the K_d depending on brain region. The 5-HT_4 receptor binding at this concentration is compared between groups but is not assumed to represent B_{max}. The non-specific binding was determined in the presence of an excess (10000 fold) of the 5-HT_4 receptor antagonist RS39604, which was chosen for its high affinity (pK_i of 9.1) (Hegde et al. 1995), and because it belongs to a different structural class, allowing subtraction of potential binding to other receptors from the [^{3}H]SB207145 binding (Burt 1978). The MAO inhibitor pargyline and the antioxidant ascorbic acid were added to the incubation buffer to reduce degradation of [^{3}H]SB207145.

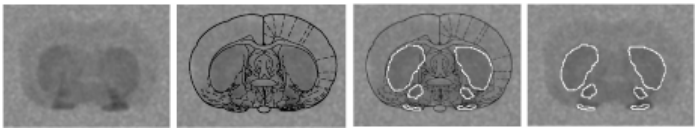

Figure 2. Definition of regions of interest. The same brain section autoradiogram is shown from left to right: without modification, with overlaid line drawing, with super-imposed line drawing and regions of interest, and with regions of interest alone.

Brain regional measurements were performed with regions of interest, defined as shown in figure 2 by aligning autoradiograms of representative brain sections with anatomical line drawings of corresponding section levels from digitized versions of The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson 2005) and the Mouse Brain in Stereotaxic Coordinates (Paxinos and Franklin 2001).

In situ hybridization

To determine potential changes in 5-HT_4 receptor gene expression and to investigate whether 5-HT_4 receptor splice variants were regulated independently, establishment of a protocol for in situ hybridization of the 5-HT_4 receptor was initiated. In situ hybridization is based on the principle of specific annealing of complementary nucleic acid molecules, and can among other things be used to detect specific mRNA strands in tissue sections by hybridization with a probe consisting of the complementary 'antisense' nucleotide sequence (Wilkinson 1999). Among the different types of probes, synthetic oligonucleotide probes allow the design of probes from published sequences, can be readily ordered, and are sufficiently sensitive for many purposes (Wilkinson 1999). The labeled probe is applied to pretreated tissue sections and allowed to hybridize in a special buffer, non-specific binding is removed by washing, and the labeled probe is detected by autoradiography or immunohistochemistry, depending on the label (Rattray and Michael 1999).

In situ hybridization of brain 5-HT_4 receptors has been performed in rats, guinea-pigs, and humans (Vilaro et al. 1996;Mengod et al. 1996;Vilaro et al. 2005;Bonaventure et al. 2000). Two oligonucleotide probes (abe1 and abe2) both recognizing all three rat 5-HT_4 receptor splice variants (a, b, and e) were tested separately and in combination (Vilaro et al. 1996). Signals in the hippocampus and caudate putamen were obtained with both antisense probes, and the specificity of the signals confirmed by their absence after hybridization with the corresponding 'sense' probes or incubation with excess unlabeled probe. However, uneven background staining, which was not reduced by a shorter (less sticky) probe or optimized washing procedures, impaired quantitative applications of the protocol. These experiments were performed during a research visit at the Department of Pharmacology at the University of Oxford and had to be discontinued, when the research visit ended.

Manipulations of the 5-HT system

The 5-HT system can be modulated in experimental animals by pharmacological and genetic means. Brain 5-HT levels can be reduced chronically by 5-HT or tryptophan depletion and increased by chronic SSRI administration. Overexpression or knockout of the 5-HTT gene in mice affects 5-HTT binding and function, and thereby 5-HT system neurotransmission, in opposite directions (Jennings et al. 2006;Bengel et al. 1998;Shen et al. 2004).

Sub-chronic 5-HT depletion

Experimental 5-HT depletion provides a means of studying effects of reduced serotonergic availability on behavior, receptor regulation and gene expression. 5-HT depletion can be achieved with selective neurotoxins and 5-HT synthesis inhibitors, through dietary tryptophan depletion, or by 5-HT neuron lesioning with 5,7-DHT (Moja et al. 1989;Kornum et al. 2006;Lieben et al. 2004;Compan et al. 1998). Various degrees of 5-HT depletion can be achieved by varying the extent of 5-HT lesioning and dose and frequency of pharmacological agents, and depend on recovery time. Compounds used for 5-HT depletion include the substituted amphetamines, such as fenfluramine (Baumann et al. 2000), p-chloroamphetamine (Rudnick and Wall 1992) and MDMA (Garcia-Osta et al. 2004), which release 5-HT from presynaptic storages through the 5-HTT, while simultaneously blocking 5-HT reuptake; and irreversible tryptophan hydroxylase inhibitors, e.g. *p*-chlorophenylalanine (pCPA) (Jequier et al. 1967).

To determine the influence of reduced 5-HT levels on 5-HT_4 receptor binding, a protocol of three days of once daily pCPA followed by one administration of fenfluramine was chosen for this project, as this method gives the largest (95%) and most widespread reduction in 5-HT levels (Kornum et al. 2006). The addition of a final fenfluramine administration to 2-3 pCPA injections was originally employed to attain larger reductions in brain 5-HT content, while avoiding the risk of toxicity associated with high doses of pCPA (Prinssen et al. 2002). The combination treatment reduces 5-HT immunoreactivity in the amygdala, medial forebrain bundle, olfactory tubercles, and substantia nigra to a greater extent than sub-chronic treatment with pCPA alone, while some 5-HT remains in the raphe nuclei and the medial forebrain bundle (Kornum et al. 2006). Though both pCPA and fenfluramine have selective effects on the 5-HT system, they also influence other systems. Two to five days of pCPA treatment reduces brain noradrenaline levels by 21-56% (Koed and Linnet 2000;Jha et al. 2006), and

both fenfluramine and its main metabolite norfenfluramine have affinity for 5-HT_1 and 5-HT_2 receptors (Garattini et al. 1987) and influence noradrenaline and dopamine release (Fuxe et al. 1975;Clineschmidt et al. 1976).

Acute and chronic SSRI administration

Administration of SSRIs to experimental animals can be used to investigate their mechanism of action, as well as to analyze effects of increased 5-HT neurotransmission. Paroxetine has high affinity for the 5-HTT and is the most potent (Ki 1.1 nM) and the second-most selective inhibitor of 5-HT reuptake but also inhibits noradrenaline reuptake (Ki 350 nM) to a smaller degree (Magnussen et al. 1982). Paroxetine has approved indications for depression, obsessive-compulsive disorder, panic disorder, and social phobia, and is also used in the treatment of generalized anxiety disorder and post traumatic stress disorder (reviewed in (Bourin et al. 2001)). Paroxetine is extensively metabolized in rat, mice, monkey, and human but the main metabolites have minimal activity and do not modify the pharmacological profile (Haddock et al. 1989).

In this project, a dose of 10 mg/kg administered orally once daily was chosen because this protocol has maximal effects on 5-HT reuptake, being well above the ED_{50} of 1.9 mg/kg in rats, has minor effects on noradrenaline reuptake (ED_{50} > 30 mg/kg) (Thomas et al. 1987), and uses a route of administration which is similar to the clinical situation. A comparable dosing of 5 mg/kg paroxetine twice daily causes a doubling of basal extracellular 5-HT levels in the hippocampus after 14 days (Hajos-Korcsok et al. 2000). However, small effects on noradrenaline reuptake are not avoided, as chronic administration of 5 mg/kg paroxetine inhibits reuptake by 10% (Thomas et al. 1987). Paroxetine has no affinity for α_1-, α_2-, and β-adrenergic receptors, D_2, H_1, 5-HT_2, or 5-HT_4 receptors but has weak affinity for 5-HT_3 and cholinergic muscarinic receptors (Thomas et al. 1987;Lucchelli et al. 1995). As paroxetine has a half-life of 4 hours in mouse brain (Hirano et al. 2004), a drug washout period of 24 hours as used in this project should be sufficient to ensure that little paroxetine is left in the tissue, when neurochemical analyses are performed. Based on a pilot study indicating an effect of 21 days of paroxetine administration on 5-HT_4 receptor binding, three time points of 1, 14 and 21 days of paroxetine administration were chosen to confirm the pilot study, compare acute and chronic effects, and evaluate the time course of the effect.

5-HTT overexpressing and knockout mice

To further investigate the regulation of 5-HT_4 receptor binding in response to changes in the 5-HT system, investigations of 5-HT_4 receptor binding in 5-HTT knockout, heterozygous and overexpressing mice were initiated. The 5-HTT overexpressing mice have 2-3 fold increases in 5-HTT binding and 50-60% reduced extracellular 5-HT levels (Jennings et al. 2006). In comparison, the homozygous 5-HTT knockout mice have opposite characteristics, displaying no 5-HTT binding (Bengel et al. 1998) and more than 8 fold increased extracellular 5-HT levels (Shen et al. 2004). The heterozygous 5-HTT knockout mice have a 50% reduction in 5-HTT binding and intermediate changes in 5-HT autoreceptor binding (Fabre et al. 2000) but normal 5-HT reuptake (Bengel et al. 1998) and extracellular 5-HT levels (Shen et al. 2004). Both 5-HTT overexpressing and homozygous knockout mice have reduced whole-tissue 5-HT levels (by 15-35% and 40-80%, respectively), which may reflect deficits in 5-HT storage (Jennings et al. 2006) and impaired recycling of released 5-HT due to inhibited 5-HT reuptake (Bengel et al. 1998;Kim et al. 2005), respectively.

Based on extracellular 5-HT levels, opposite directionality of 5-HT_4 receptor binding changes are expected in 5-HTT overexpressing and homozygous knockout mice and the directionality of changes are expected to correspond to the effects of sub-chronic 5-HT depletion and chronic SSRI administration, respectively. Female mice were used in this study, as limited numbers of male mice were available. This project was initiated during a research visit at the Department of Pharmacology, University of Oxford, and for this purpose [^{3}H]SB207145 autoradiography was established in the laboratory of T. Sharp. The study was not completed before the end of the visit, and is now continued in collaboration with T. Sharp.

Functional analyses of the 5-HT_4 receptor

While 5-HT_4 receptor function has been studied in neuronal and tissue slice cultures, few functional assays exist for in vitro analysis of native brain tissue. To be able to study brain 5-HT_4 receptor function with high anatomical resolution, development of a protocol for 5-HT_4 receptor stimulated functional autoradiography was initiated.

Functional GTPγS autoradiography

Binding of agonists to G protein-coupled receptors promote and stabilize the formation of a complex between agonist, receptor and heterotrimeric G protein, activating the G protein and

inducing exchange of bound GDP for GTP in the G_α subunit (Sovago et al. 2001). This guanine nucleotide exchange can be detected by incorporation of metabolically stable, radioactively labeled GTP analogues, including [^{35}S]GTPγS (Fig. 3). Agonist stimulated [^{35}S]GTPγS binding is unique in providing information on receptor functionality at the initial G protein activation step, which compared to measures of second messenger levels and signal cascade enzymatic activity is subject to less signal amplification and convergence with downstream effects of other receptors (Sovago et al. 2001). The [^{35}S]GTPγS assay was originally performed in membranes but was adapted for in vitro autoradiography by Sim et al. (Sim et al. 1995), enabling functional biochemical information to be gained in the context of high anatomical resolution. As in receptor autoradiography, the specificity of [^{35}S]GTPγS autoradiography derives from a selective receptor ligand but the radioactive signal pertains to the level of [^{35}S]GTPγS bound by activated G proteins, making it a measure of catalytic activity. Brain tissue also has a basal [^{35}S]GTPγS binding capacity due to constitutive receptor activity, endogenous agonists in the tissue, and to GDP-GTP exchange of non-heterotrimeric G proteins (Milligan 2003). Non-specific [^{35}S]GTPγS binding is determined by excess unlabeled GTPγS, and receptor specificity can be checked by incubation with excess of an antagonist. Functional autoradiography has been performed mainly for $G_{i/o}$ coupled receptors and for a few G_q coupled receptors but no successful application for G_s coupled receptors has been reported (Sim et al. 1997;Adlersberg et al. 2000;Sovago et al. 2001).

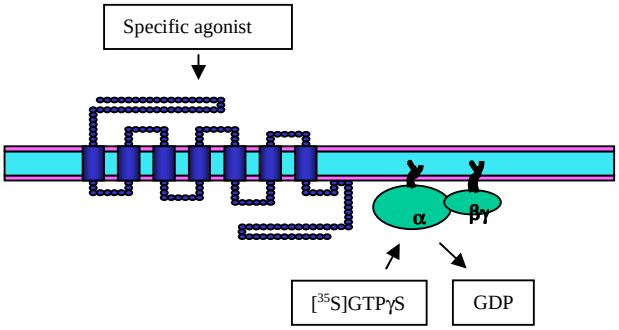

Figure 3. The principle of agonist-stimulated [^{35}S]GTPγS binding. Stimulation of the receptor activates G protein, inducing exchange of bound GDP for labeled GTP by the G_α subunit.

The difference in applicability of this technique for G_i versus G_s coupled receptors may be due to the higher level of G_i proteins in brain, higher guanine nucleotide exchange rate in G_i proteins compared to G_s, and differences in receptor to G protein-coupling efficiency (Wieland T 1994;Sim et al. 1997;Milligan 2003). In accordance with the 5-HT_4 receptor being G_s coupled, stimulation with the 5-HT_4 agonist SC53116 does not increase [^{35}S]GTPγS binding in brain sections (Waeber and Moskowitz 1997). However, 5-HT_4 receptor stimulated [^{35}S]GTPγS binding has been performed in homogenate of cell lines transfected with 5-HT_4

receptors (Pindon et al. 2002). The lack of detectable response to stimulation of G_s coupled receptors in [^{35}S]GTPγS autoradiography is most likely due to a low agonist-stimulated signal in the context of high basal [^{35}S]GTPγS binding (Wieland T 1994;Milligan 2003;Cussac et al. 2004).

In this project the protocol for 5-HT_4 receptor functional autoradiography used by Waeber et al., 1997 was chosen as the starting point. As full, selective 5-HT_4 receptor agonists are not available (Bockaert et al. 2004), the more selective and highly potent partial agonist RS67333 was chosen (Eglen et al. 1995). In a course of experiments several parameters were varied to increase agonist stimulated binding and decrease basal binding. However, no RS67333 stimulated [^{35}S]GTPγS binding was detected, and stimulation with the full 5-HT_4 receptor agonist, zacopride, had no effect either.

In vivo microdialysis

As in vitro methods proved difficult to establish, in vivo approaches to evaluate central 5-HT_4 receptor function and interactions with the 5-HT system came into focus. Among the assays of 5-HT_4 receptor function in vivo, clear effects of 5-HT_4 receptor activation and inhibition on extracellular 5-HT levels in the ventral hippocampus (Ge and Barnes 1996) and on basal 5-HT neuron firing rate in the DRN (Lucas and Debonnel 2002;Lucas et al. 2005) have been reported. In vivo microdialysis of 5-HT in the ventral hippocampus was chosen as the primary method to investigate interactions between the 5-HT_4 receptor and the SSRI paroxetine.

In vivo microdialysis is a well-documented technique for measuring extracellular neurotransmitter concentrations in the brain (reviewed in (Sharp and Zetterström 1992)). A microdialysis probe with a semi-permeable dialysis membrane is stereotaxically implanted in a discrete region of the brain and perfused with artificial cerebrospinal fluid (aCSF) (Fig. 4). Perfusates are collected every 20-30 minutes and analyzed for neurotransmitter content by high performance liquid chromatography (HPLC) with electrochemical detection. The microdialysis approach has been used to measure extracellular 5-HT levels in different brain regions in both anaesthetized and awake, freely moving animals in response to different pharmacological and behavioral manipulations (Sharp et al. 1990;Sharp and Hjorth 1990;Kreiss and Lucki 1995;Gartside et al. 1995;Sharp et al. 1997;Hajos-Korcsok et al. 2000). Despite the small size of the microdialysis probes, implantation causes neuronal damage and breaches in the blood-brain barrier (Sharp and Zetterström 1992) but after a 2 hour stabilization period, the dialysate 5-HT levels reflect 5-HT release in response to 5-HT

neuron firing (Sharp et al. 1990). The 5-HT levels obtained in the dialysates are influenced by the 5-HT recovery and dialysis membrane area of the probe, and by aCSF perfusion speed and sample collection time (Sharp and Zetterström 1992).

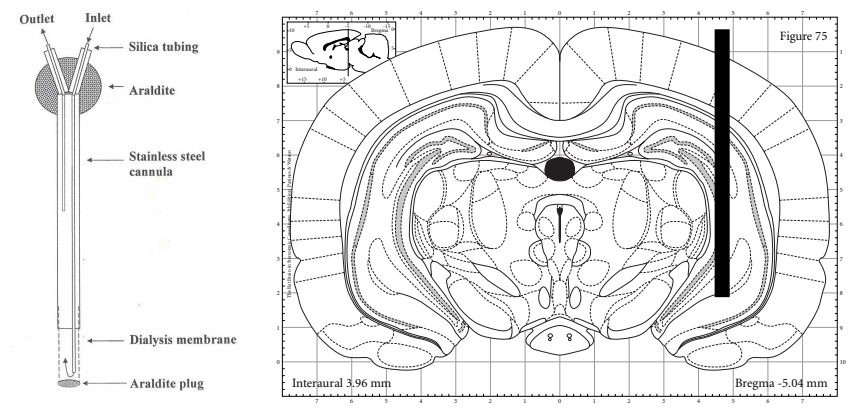

Figure 4. Single cannula probe for microdialysis (left, adapted from (Sharp and Zetterström 1992)), and placement of probe in ventral hippocampus (right, adapted from (Paxinos and Watson 2005)).

The HPLC method of molecule separation is based on forcing molecules by high pressure through a thin column packed with small beads, which retain molecules for different times depending on their physicochemical properties and the composition of the mobile phase used. Monoamines can be separated by reversed-phase ion-pair chromatography, where changes in pH, concentration of ion-pairing agents (e.g. sodium octanyl sulphonate), concentration of organic solvent (e.g. methanol), and ionic strength of the mobile phase influence elution times of monoamines and their acid metabolites (e.g. 5-HIAA) (Sharp and Zetterström 1992). In HPLC with electrochemical detection, eluted molecules pass over a carbon-based electrode, where they are oxidized at the ring hydroxyl group, creating an electric current proportional to the number of molecules oxidized and reflected on a chromatogram as variations in peak height (Marsden and Joseph 1986). Monoamine peaks are identified and calibrated by comparison with elution times and peak heights of external reference compounds, respectively.

The experiments were performed in chloral hydrate anaesthetized animals, and drugs were administered by the intravenous administration route to reduce variability due to differences in uptake of drug from tissue. The selective and potent partial agonist RS67333 was used (Eglen et al. 1995) at a dose of 1.5 mg/kg i.v., which increases 5-HT neuron firing rate under chloral hydrate anesthesia (Lucas et al. 2005). Among SSRIs, paroxetine was chosen as it had shown effects on 5-HT_4 receptor binding in previous experiments within the PhD-project. For combination experiments, RS67333 was administered 80 minutes after

paroxetine, when the effect of paroxetine on 5-HT levels was reaching a plateau. Combinations of fluoxetine and the 5-HT_{1A} antagonist WAY100635 have shown that the effect on 5-HT levels depends on sequence of administration, being maximal when WAY100635 is administered 80 min after fluoxetine (Taber et al. 2000).

Expression of the immediate early gene Arc

The effector immediate early gene Arc/Arg3.1 (activity-regulated cytoskeleton-associated protein) is rapidly expressed in neuronal dendrites in response to robust synaptic activity, making it a useful marker of neuronal activity (Tzingounis and Nicoll 2006). Arc transcription is induced by NMDA receptor activation, calcium influx and cAMP (Tzingounis and Nicoll 2006), and Arc mRNA levels are increased in cortical regions and the striatum in response to elevated brain 5-HT levels (Pei et al. 2000). The sensitivity to increased 5-HT levels is reflected in increased Arc mRNA expression after chronic antidepressant drug treatment (Pei et al. 2003) and in response to combined acute SSRI and 5-HT_{1A} receptor antagonism (Castro et al. 2003).

The effect of acute 5-HT_4 receptor activation by RS67333 administration on Arc mRNA expression was examined by in situ hybridization. An initial dose-response (0.1-10 mg/kg) experiment showed downregulation of Arc expression in cortical regions after 2 hours. However, as this effect was present at all doses and the change in expression was in the opposite direction of what was expected, the experiment was repeated with a single RS67333 concentration (1.5 mg/kg) and Arc expression determined 2 hours after a single injection (acute) and 24 hours after 3 days of RS67333 administration. These experiments were due to partial degradation of the mRNA and high non-specific binding inconclusive and were not included in manuscript III.

Results and discussion

Changes in the 5-HT_4 receptor in animal models of depression (Aim I)

To investigate the involvement of the 5-HT_4 receptor in depression, the 5-HT_4 receptor binding was analyzed in four rodent models of depression (Manuscript I and II). Several models were included to evaluate the 5-HT_4 receptor in different depression endophenotypes, induced by genetic or environmental manipulations. The implication of the 5-HT_4 receptor in depression endophenotypes related to 5-HT system changes was investigated in a genetic model with increased 5-HT system neurotransmission, Flinders Sensitive Line, and in a surgically induced model of 5-HT system disturbances, Olfactory Bulbectomy; and the relationship between the 5-HT_4 receptor and depression endophenotypes of HPA axis modulation was analyzed in a genetic model of impaired HPA axis regulation, the glucocorticoid receptor heterozygous mouse, and in a model of environmental stress-induced depression, Chronic Mild Stress. The 5-HT_4 receptor binding was investigated in the hippocampus and caudate putamen of all models, and additional brain regions of relevance to each model were analyzed post-hoc. In each model, significant changes in 5-HT_4 receptor binding were related to 5-HTT binding as a common presynaptic marker of 5-HT system change. Each model was validated by behavioral testing or genotyping (Table 3).

Animal models of 5-HT system disturbances

In the FSL model, the 5-HT_4 receptor binding was decreased in the dorsal (23%, $p = 0.014$) and ventral (11%, $p = 0.045$) hippocampus, while no change was detected in the caudate putamen (Table 4). This may be related to the higher hippocampal but similar striatal 5-HT levels of FSL rats (Zangen 1997).

	FRL	FSL	Percent change[a]	P value
Caudate putamen, medial	19.1 ± 0.6	18.6 ± 0.9	-2.6	0.654
Caudate putamen, caudal	32.2 ± 0.9	31.8 ± 0.8	-1.4	0.733
Hippocampus, dorsal	10.7 ± 0.7	8.3 ± 0.5	-22.6	0.014*
Hippocampus, ventral	20.1 ± 0.6	17.9 ± 0.8	-11.0	0.045*
Hypothalamus	16.8 ± 0.6	17.1 ± 0.6	1.8	0.732
Lateral globus pallidus	33.2 ± 0.6	30.9 ± 0.8	-7.0	0.027*

Table 4. 5-HT_4 receptor binding in Flinders Line . Values are mean ± SEM in fmol/mg tissue. [a]FSL compared to FRL.

Given our present finding that chronic paroxetine administration downregulates hippocampal 5-HT_4 receptor binding, and is known to increase extracellular 5-HT levels (Owen and Whitton 2005;Hajos-Korcsok et al. 2000), we find it likely that the 5-HT_4 receptor binding is decreased in the FSL hippocampus in response to elevated 5-HT levels. Similar to the 5-HT_4 receptor binding changes, the 5-HTT binding was decreased in both dorsal (12%, $p < 0.0001$) and ventral (10%, $p = 0.020$) hippocampus with no change in the caudate putamen of FSL rats (Manuscript II). Furthermore, the changes in 5-HT_4 receptor and 5-HTT binding were directly correlated in the dorsal hippocampus ($r = 0.48$, $p = 0.034$) but not in the ventral hippocampus (Fig. 5). As chronic SSRI administration (Benmansour et al. 1999;Johnson et al. 2008) and 5-HT depletion (Rattray et al. 1996a;Rothman et al. 2003) have been shown to downregulate hippocampal 5-HTT binding, the decrease in 5-HTT binding may be a response to changes in 5-HT levels.

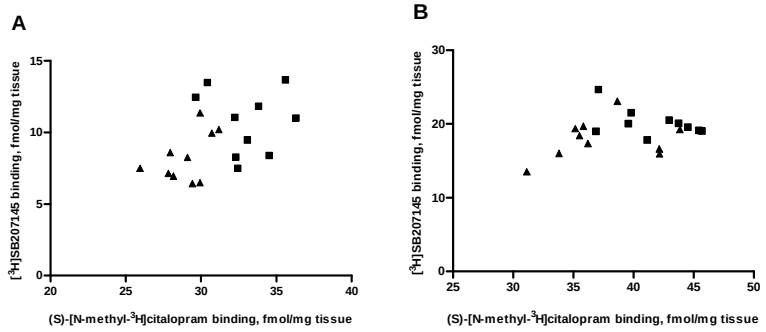

Figure 5. Correlation between 5-HT_4 receptor and 5-HT transporter binding in Flinders Line rats. [^{3}H]SB207145 versus (S)-[N-methyl-^{3}H]citalopram binding in A) dorsal hippocampus ($r = 0.48$, $p = 0.034$), and B) ventral hippocampus ($r = 0.24$, $p = 0.307$) of FSL (triangles, $n = 10$) and FRL (squares, $n = 10$). Values are fmol/mg tissue.

Post-hoc analyses of additional brain regions in the FSL and FRL rats showed a downregulation of 5-HT_4 receptor binding in the LGP (7%, $p = 0.027$) (Table 4) but this was not paralleled by a change in 5-HTT binding (Manuscript II). Chronic dopamine depletion upregulates 5-HT_4 receptor binding in the LGP (Compan et al. 1996), and conversely, one might expect an increase in dopamine to downregulate pallidal 5-HT_4 receptor binding. The FSL have increased dopamine tissue levels in the caudate putamen (Zangen et al. 1999), which may affect the 5-HT_4 receptors in the LGP, as they are expressed axonally on GABA neurons projecting from the caudate putamen (Compan et al. 1996).

As the hippocampal 5-HT system in the FSL is affected by ageing (Husum et al. 2006), 5-HT_4 receptor binding was also determined in aged (12-13 months) male FSL and FRL rats and compared to data from adult (4-8 months) FSL and FRL. The aged FSL data are not part of the manuscripts included in this thesis, and are therefore presented here in greater detail.

While adult FSL have a tendency towards longer 5-HT fibers in the dorsal hippocampus compared to adult FRL (3 months), aged 11 months-old FSL rats have shorter 5-HT fiber lengths in the CA1 and CA3 compared to adult FSL, and in the CA1 compared to aged FRL (Husum et al. 2006), suggesting an enhanced age-related decrease in serotonergic innervation in the FSL dorsal hippocampus. Two-way ANOVA of 5-HT_4 receptor binding by Strain (FRL vs. FSL) and Age (Adult vs. Aged) showed a significant effect of Age ($F(1,32) = 9.289$, $p = 0.0046$) and an interaction between Age and Strain ($F(1,32) = 6.976$, $p = 0.0127$) in the dorsal hippocampus. Bonferroni posttests showed that a strain difference in 5-HT_4 receptor binding is present in adult animals only (Fig. 6).

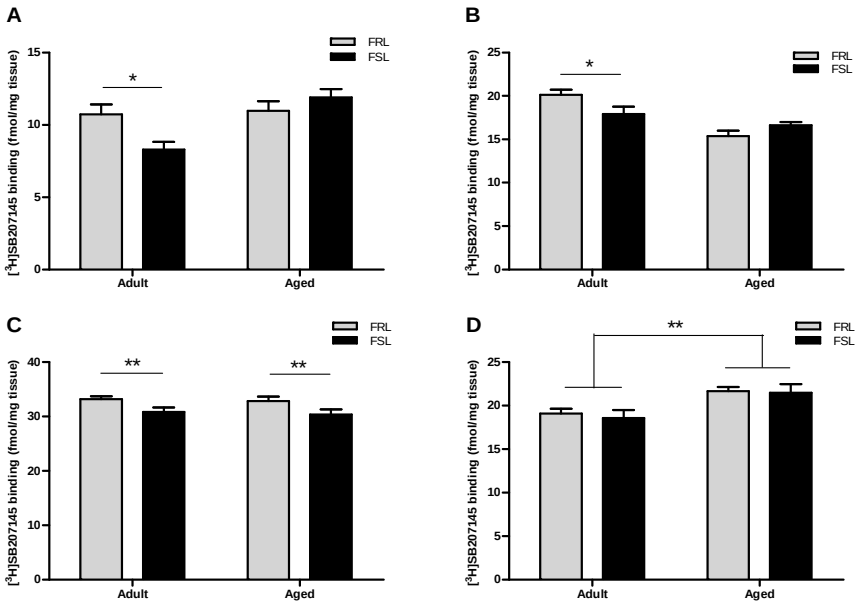

Figure 6. 5-HT_4 receptor binding in the adult and aged Flinders Line. Comparison of [^{3}H]SB207145 binding (fmol/mg tissue) in adult FRL (n = 10) and adult FSL (n = 10) with aged FRL (n = 8) and FSL (n = 8) rats in A) dorsal hippocampus, B) ventral hippocampus, C) LGP, and D) medial caudate putamen. Columns are mean ± SEM. * $p < 0.05$, ** $p < 0.01$

There was also an effect of Age ($F(1,32) = 20.96$, $p < 0.0001$) and an interaction between Age and Strain ($F(1,32) = 6.881$, $p = 0.0132$) in the ventral hippocampus, and posttests showed that a difference in 5-HT_4 receptor binding between the FSL and FRL was present in the adult animals only (Fig. 6). In the LGP, there was an effect of Strain ($F(1,32) = 9.184$, $p = 0.0048$) and no effect of Age or interaction on the 5-HT_4 receptor binding but Bonferroni posttests did not show significant differences between FSL and FRL. In the medial caudate putamen there was an effect of Age ($F(1,32) = 12.26$, $p = 0.0014$) but no effect of Strain or interaction between the two. This suggests differential effects of ageing on 5-HT_4 receptor binding in the FSL and FRL hippocampus only, which in the dorsal hippocampus may relate to the

shortening of 5-HT fibers with ageing in the FSL (Husum et al. 2006), possibly counteracting the increased 5-HT levels in the adult FSL. The difference between FSL and FRL in 5-HT_4 receptor binding in the LGP is present in both adult and aged animals, supporting the notion that the 5-HT_4 receptor binding in this region is regulated differently. The strain-independent age-related increase in 5-HT_4 receptor binding in the medial caudate putamen is in line with an increase in 5-HT_4 receptor binding in the fundus striatum (and a non-significant increase in the caudate putamen) of 9 months-old rats (Manuel-Apolinar et al. 2005).

In the OBX mice, we found an increase in 5-HT_4 receptor binding in the ventral hippocampus (12%, $p = 0.041$) but no change in the dorsal hippocampus or caudate putamen (Table 5). The ventral hippocampal upregulation may be due to reduced serotonergic tonus, as chronic 5-HT depletion increases the 5-HT_4 receptor binding in the hippocampus (Compan et al. 1996), and 5-HIAA tissue levels are decreased in the hippocampus but unchanged in the caudate putamen after bulbectomy (Hellweg et al. 2007). The cause of the regional specificity of the 5-HT_4 receptor change within the hippocampus is unknown but may be dorso-ventral differences in connectivity (Bannerman et al. 2004), and is in accordance with differential changes in 5-HTT binding (Manuscript I) between the dorsal and ventral hippocampus in a pattern complementary to the 5-HT_4 receptor changes.

	Sham	OBX	Percent change[1]	p value
Caudate putamen, frontal	10.5 ± 0.8	9.8 ± 0.5	-7.3	0.399
Caudate putamen, caudal	28.6 ± 1.3	28.3 ± 0.5	-1.0	0.845
Hippocampus, dorsal	14.4 ± 0.8	15.2 ± 0.8	5.5	0.487
Hippocampus, ventral	24.1 ± 0.6	26.9 ± 0.9	11.5	0.041*
Frontal cortex	4.4 ± 0.8	5.7 ± 0.6	28.4	0.221
Hypothalamus	20.8 ± 0.7	21.4 ± 0.7	2.8	0.576
Lateral globus pallidus	32.2 ± 1.2	29.9 ± 0.9	-6.9	0.165
Lateral septum	17.3 ± 0.7	18.1 ± 0.3	4.6	0.307
Medial amygdala	18.6 ± 0.7	19.9 ± 0.6	6.9	0.168
N. accumbens shell	26.2 ± 0.7[2]	24.5 ± 1.2	-6.3	0.331
Olfactory tubercles	31.9 ± 1.6	27.4 ± 1.1	-14.1	0.037*

Table 5. 5-HT_4 receptor binding after olfactory bulbectomy. Values are mean ± SEM fmol/mg tissue. [1] OBX compared to Sham. [2] n = 4. *p < 0.05.

Changes in the 5-HT system are involved in the neurobiological basis of the OBX syndrome, as the syndrome can be induced by injection of serotonergic toxins into the olfactory bulbs (Cairncross et al. 1977a). OBX in mice induces neurodegeneration in the dorsal raphe nucleus (Nesterova et al. 1997), possibly due to degeneration of serotonergic fibers innervating the main olfactory bulb (McLean and Shipley 1987), and a decrease in brain tryptophan hydroxylase activity and 5-HT synthesis rate (Neckers et al. 1975). The pathophysiological

relevance of the increase in 5-HT_4 receptor binding after OBX is supported by the antidepressant effect of chronic 5-HT_4 receptor agonism in the OBX rat (Lucas et al. 2007).

Post-hoc analysis showed a downregulation of the 5-HT_4 receptor binding in the olfactory tubercles (14%, $p = 0.037$) (Table 5). The olfactory tubercles are implicated in the rewarding effects of morphine, cocaine, and brain-stimulation reward (Ikemoto 2007;Kornetsky et al. 1991;Ikemoto 2003), and have high 5-HT_4 receptor (Jakeman et al. 1994;Waeber et al. 1994) and 5-HTT binding (Scheffel and Hartig 1989), indicating dense innervation by serotonergic fibers. After bulbectomy, a marked (qualitative) increase in indoleamine content is present in the olfactory tubercles, indicating an increase in 5-HT content in this region (Garris et al. 1984). As the 5-HT_4 receptor binding is downregulated in response to chronic paroxetine administration, a possible explanation of the decrease in 5-HT_4 receptor binding in the olfactory tubercles could be a downregulation of the receptor in response to increased 5-HT levels. The 5-HTT binding in the olfactory tubercles was also reduced after OBX, and both 5-HTT (Manuscript I) and 5-HT_4 receptor binding in the olfactory tubercles were inversely correlated with activity in the open field test, indicating a relation to the behavioral phenotype (Fig. 7).

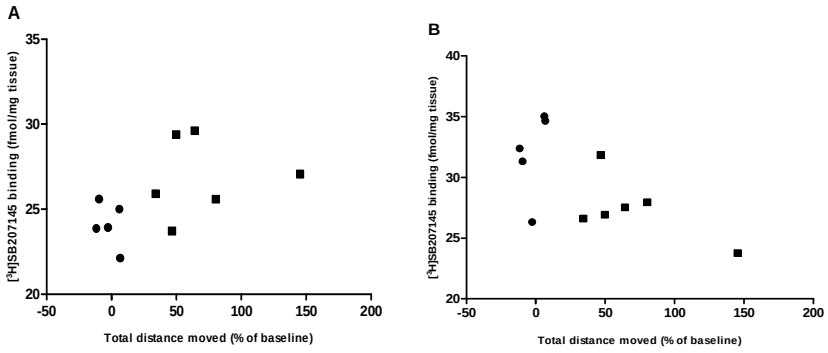

Figure 7. Relationship between 5-HT_4 receptor binding and activity in the open field test of bulbectomized and sham-operated mice. [^{3}H]SB207145 binding in A) ventral hippocampus ($r = 0.51$, $p = 0.105$) and B) olfactory tubercles ($r = -0.66$, $p = 0.027$) versus total distance moved in the open field test (expressed as percentage of baseline activity). Sham-operated animals are indicated by circles and bulbectomized animals by squares.

While both FSL and OBX are models of depression, they exhibit opposite behavioral responses to stress and novelty. The FSL model is characterized by increased immobility in the FST (Willner and Mitchell 2002), while the OBX model displays increased activity (Mucignat-Caretta et al. 2004). Similarly, the OBX model shows increased activity in the aversive open field test (Zueger et al. 2005), while the FSL rats are less active in this test (Overstreet et al. 1986). Differences in both psychomotor response to stress and 5-HT system changes may underlie these behavioral differences. In both models, we found changes in 5-

HT_4 receptor binding in the hippocampus but not in the caudate putamen. Importantly, the 5-HT_4 receptor binding was changed in opposite directions in the two models; decreased in FSL and increased after OBX. This may be caused by opposite directionality of 5-HT system changes in these models, increased hippocampal 5-HT levels in FSL rats and decreased 5-HT turnover in the OBX model. The directionality of the hippocampal 5-HT_4 receptor changes in the two models are in accordance with decreased activity of 5-HT_4 receptor knockout mice in the novel open field test (Compan et al. 2004). We also found changes in hippocampal 5-HTT binding in FSL and after OBX but in this case the binding was decreased in both models. However, this does not contradict the opposite changes in 5-HT_4 receptor binding, as both increasing (Benmansour et al. 1999;Johnson et al. 2008) and decreasing (Rattray et al. 1996b;Rothman et al. 2003) 5-HT levels can downregulate 5-HTT binding. The post-hoc analysis of 5-HT_4 receptor binding implicated specific brain regions for each model, the LGP in FSL and the olfactory tubercles after OBX, explainable by model-specific characteristics. Of potential importance for the differences in reactivity to novelty and stress, post-hoc analysis of 5-HTT binding also showed opposite changes in 5-HTT binding in the basolateral amygdala, being increased in FSL (Manuscript II) and decreased after OBX (Manuscript I).

Animal models of HPA axis change

The $GR^{+/-}$ mice exhibited an 11% increase ($p = 0.036$) in 5-HT_4 receptor binding in the caudate putamen (Table 6), which is in agreement with the finding of increased postmortem 5-HT_4 receptor binding in the caudate nucleus of depressed suicide victims (Rosel et al. 2004). Notably, 5-HT_4 receptor binding was increased in the caudal but not in the frontal caudate putamen (Table 6). While serotonergic denervation leads to an increase in 5-HT_4 receptor binding in the frontal but not in the caudal caudate putamen, lesions of the nigrostriatal dopaminergic pathway induce an increase in 5-HT_4 receptor binding in the caudal but not the frontal caudate putamen after 21 days (Compan et al. 1996). Our finding of increased 5-HT_4 receptor binding in the caudal caudate putamen may therefore reflect decreased dopaminergic activity in $GR^{+/-}$ mice. The increased 5-HT_4 receptor binding is not related to changes in tissue dopamine levels, as these are similar in $GR^{+/+}$ and $GR^{+/-}$ mice (Schulte-Herbruggen et al. 2007), but could potentially reflect changes in dopaminergic neuronal activity. In the $GR^{+/-}$ mice, there was also a small (6%, $p = 0.038$) decrease in 5-HTT binding in the frontal caudate putamen but no change in the dorsal hippocampus or in the post-hoc analyzed regions (Manuscript I). The decrease in 5-HTT binding in the frontal caudate putamen was unexpected given the absence of 5-HT system change in the $GR^{+/-}$

model (Schulte-Herbruggen et al. 2007). Post-hoc analysis showed a 10% increase in 5-HT_4 receptor binding in the olfactory tubercles of the $GR^{+/-}$ mice (Table 6).

	$GR^{+/+}$	$GR^{+/-}$	Percent change[1]	p value
Caudate putamen, frontal	13.8 ± 1.1	14.6 ± 0.7	6.4	0.500
Caudate putamen, caudal	41.0 ± 1.1	45.5 ± 1.5	10.9	0.036*
Hippocampus, dorsal	15.7 ± 1.0	18.4 ± 0.7	17.0	0.052
Hippocampus, ventral	28.0 ± 0.9[3]	29.8 ± 1.4[2]	6.3	0.303
Hypothalamus	24.3 ± 0.9	26.3 ± 0.8	8.1	0.115
Lateral globus pallidus	40.2 ± 0.7	42.1 ± 1.2	4.9	0.180
Lateral septum	22.3 ± 1.3	23.7 ± 0.7	6.5	0.353
Medial amygdala	21.6 ± 0.8	22.9 ± 0.9	6.0	0.305
N. accumbens shell	33.4 ± 2.1[2]	38.4 ± 1.1[2]	14.8	0.070
Olfactory tubercles	40.9 ± 1.1	44.8 ± 1.2	9.6	0.032*

Table 6. 5-HT_4 receptor binding in $GR^{+/-}$ mice. Values are mean ± SEM in fmol/mg tissue. [1] $GR^{+/-}$ compared to $GR^{+/+}$. [2] n = 5, [3] n = 4. *p < 0.05.

The 5-HT_4 receptor binding in the caudate putamen and hippocampus did not correlate with total sucrose intake during CMS (Manuscript II). However, the 5-HTT binding in the frontal caudate putamen was inversely correlated with total sucrose intake (r = -0.41, p = 0.044), associating increased 5-HTT binding with the anhedonic response to CMS (Manuscript II). The absence of a change in hippocampal 5-HT_4 receptor and 5-HTT binding after CMS is in accordance with anhedonic and CMS resilient animals not differing in hippocampal expression of genes related to the 5-HT system (Bergstrom et al. 2007).

	5-HT system disturbance		HPA axis modulation	
	FSL	OBX	GR	CMS
Hippocampus	**Decrease**	**Increase**	**No change**	**No change**
Caudate putamen	**No change**	No change	**Increase**	No change
Model-specific	LGP	**Olfactory tubercles**	Olfactory tubercles	**None**

Table 7. Comparison of 5-HT_4 receptor binding changes in animal models of depression. Changes marked with bold: supported by effects on 5-HTT binding in the same region (no directionality)

The $GR^{+/-}$ and CMS models are both based on HPA axis modulation, representing a genetic disposition to inhibited feedback of HPA axis activity and continuous HPA axis activation by chronic, environmental stressors, respectively. The 5-HT_4 receptor binding was increased in the caudal caudate putamen of the $GR^{+/-}$ model but was unchanged after CMS. However in both models, 5-HTT binding was changed in the frontal caudate putamen, though in opposite directions.

Animal models of depression display distinct changes in 5-HT system and behavior, and cross-model consistency is found in few if any biological marker (Table 3). From the comparison of 5-HTT binding, a 5-HT system factor implicated in the pathophysiology and

treatment of depression, in four depression models of different constructs within this project it is clear that the 5-HT system is affected in model-specific ways, and that differences between models are not explained by methodological differences between studies (Manuscript I and II). Similarly, the changes in 5-HT_4 receptor binding differ between the four models (Table 7). The majority of 5-HT_4 receptor changes may be explainable by region-specific changes in endogenous agonism due to changes in 5-HT neuron firing and release, while a few may have their origin in changes in other neurotransmitter systems, e.g. the dopaminergic system.

Regulation of the 5-HT_4 receptor by 5-HT system changes (Aim II)

In order to understand the mechanisms behind the 5-HT_4 receptor changes observed in the animal depression models, we investigated regulation of 5-HT_4 receptor binding in response to alterations in cerebral 5-HT levels (Manuscript II). To alter 5-HT levels, normal rats were subjected to different treatment paradigms: 1, 14, and 21 days of paroxetine administration, and sub-chronic 5-HT depletion. While there was no effect after one day of paroxetine administration, a global and substantial (11-47%) downregulation of 5-HT_4 receptor binding was present after 14 and 21 days of treatment (Fig. 8, and Manuscript II). The absence of change in 5-HT_4 receptor binding after one day of paroxetine administration shows that the changes in 5-HT_4 receptor binding after 14 and 21 days are not due to acute effects of paroxetine but require chronic administration.

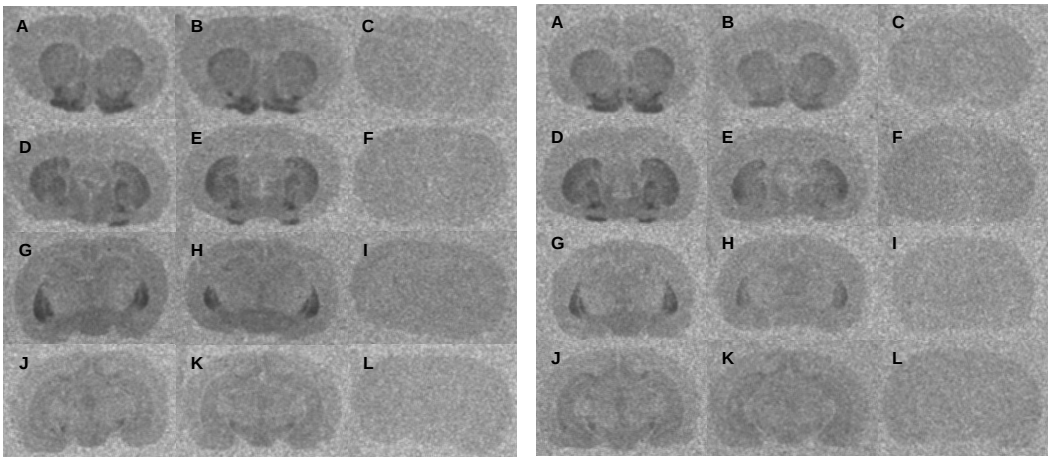

Figure 8. 5-HT_4 receptor binding after acute and chronic paroxetine administration. Autoradiograms of specific [^{3}H]SB207145 binding (1 nM) at four section levels after 1 (left panel) and 21 (right panel) days of vehicle (n = 7-8; A, D, G, J) or paroxetine (10 mg/kg, n = 7-8; B, E, H, K) administration. C, F, I, L) Non-specific binding in the presence of 10 μM RS39604.

One study has previously investigated the effect of chronic antidepressant treatment on 5-HT_4 receptor binding and reported no effect of citalopram on 5-HT_4 receptor binding in the

substantia nigra, which was the only region investigated (Gobbi et al. 1997). Though we have not quantified 5-HT_4 receptor binding in this region (due to its small size) after chronic paroxetine administration, the global nature of the 5-HT_4 receptor downregulation and visual inspection of the autoradiograms suggest that the substantia nigra is affected too (Fig. 8, right panel). Our findings are in accordance with electrophysiological studies in hippocampal slices, showing a decrease in 5-HT_4 receptor sensitivity after chronic but not acute treatment with paroxetine (Bijak et al. 1997). The same group found a similar decrease in sensitivity after chronic administration of other antidepressant drugs (imipramine, citalopram, fluvoxamine) and after repeated ECS, indicating that this is a general effect of antidepressant treatment (Bijak 1997;Bijak et al. 2001;Bijak et al. 1997). While paroxetine by acute blockade of the 5-HTT increases synaptic 5-HT in certain regions, chronic administration strongly downregulates 5-HTT binding globally, leading to a larger decline in 5-HT clearance from the synapse (Benmansour et al. 1999). The decreased 5-HT_4 receptor binding after 14 and 21 days of paroxetine administration may be due to increased agonism-induced downregulation of the 5-HT_4 receptor, as exposure to a saturating concentration of 5-HT decreases 5-HT_4 receptor binding in colliculi neurons (Ansanay et al. 1996) through endocytosis of plasma membrane receptors (Barthet et al. 2005).

Sub-chronic 5-HT depletion induced a 13-41% increase in 5-HT_4 receptor binding in the dorsal hippocampus, hypothalamus, and LGP (Table 8, Manuscript II). As previously published, our four day combined pCPA and fenfluramine 5-HT depletion paradigm results in a 95% reduction in brain 5-HT on day five (Kornum et al. 2006).

	Saline	pCPA + FEN	Percent change[a]	p value
Caudate putamen, medial	17.6 ± 0.7	17.8 ± 0.7	1.0	0.871
Caudate putamen, caudal	27.4 ± 0.7	28.9 ± 0.6	5.7	0.102
Hippocampus, dorsal	7.8 ± 0.4	11.0 ± 0.7	40.7	0.0007***
Hippocampus, ventral	15.1 ± 0.9[b]	16.4 ± 0.4	8.6	0.163
Hypothalamus	14.8 ± 0.2	18.1 ± 0.6	22.2	0.0002***
Lateral globus pallidus	26.6 ± 0.7	30.0 ± 1.0	12.8	0.014*
Ventral pallidum	26.9 ± 0.6	29.3 ± 1.1	8.8	0.087

Table 8. 5-HT_4 receptor binding after sub-chronic 5-HT depletion. Values are mean ± SEM in fmol/mg tissue. [a]pCPA + FEN compared to saline. [b]n = 7.

Our results support the previous report by Compan et al. (1996) of increased (28-83%) 5-HT_4 receptor binding in the rostral caudate putamen, nucleus accumbens, substantia nigra, globus pallidus, and hippocampus 21 days after 5,7-DHT lesions of the raphe nuclei, and shows that this effect is also present after sub-chronic pharmacological 5-HT depletion. After 5-HT depletion, the hippocampal 5-HT_4 receptor binding was increased in the dorsal subfield only, which is similar to the pattern of change in 5-HT_1 receptor binding after 5,7-DHT induced 5-

HT depletion, where binding is decreased in the anterior (dorsal) but not the posterior (ventral) hippocampus (Fischette et al. 1987).

To investigate whether the regulation of 5-HT_4 receptor binding by changes in 5-HT levels was specific to this receptor, we compared it with that of an extensively described 5-HT receptor, the 5-HT_{2A} receptor. In agreement with previous studies using [^{3}H]ketanserin (Maj et al. 1996;Nelson et al. 1989), we found a decrease in cortical 5-HT_{2A} receptor [^{3}H]MDL100607 binding after 21 days of paroxetine administration (Manuscript II). Whereas chronic paroxetine administration was associated with a profound and global reduction in the 5-HT_4 receptor binding, the effect on the 5-HT_{2A} receptor was smaller and confined to frontal and cingulate cortices (Fig. 9, Manuscript II).

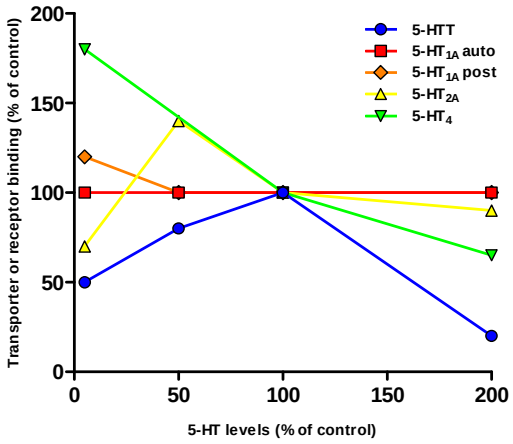

Figure 9. Chronic autoregulation of the rat 5-HT system. Relative changes in 5-HT transporter and receptor binding in response to changes in central 5-HT levels, achieved by chronic 5-HT depletion or SSRI administration. See text (p.13-14 and 37-39) for references.

In contrast to the limited effects of chronic paroxetine administration, the 5-HT_{2A} receptor binding was globally and markedly decreased after sub-chronic 5-HT depletion (Manuscript II). While this is the first report to use [^{3}H]MDL100907 binding to evaluate 5-HT_{2A} receptor binding after 5-HT depletion, it has been reported that severe 5-HT depletion by 5,7-DHT (but not by pCPA) causes a 16-26% decrease in 5-$HT_{2A/2C}$ receptor [^{125}I]DOI binding in the cingulate, frontal, and parietal cortex (Compan et al. 1998). Generally the effects of 5-HT depletion on 5-HT_{2A} receptor binding are complex, and may depend on 5-HT depletion protocol, degree of 5-HT depletion, radioligands used and time point of receptor binding analysis (see p.16).

Based on the experiments in Manuscript II and the study by Compan et al. (1996), the model of effects of chronic changes in 5-HT levels on 5-HT system markers (Fig. 1) can be

expanded to include regulation of 5-HT_4 receptor binding (Fig. 9). In this model, a chronic decrease in 5-HT levels will increase 5-HT_4 receptor binding in several regions, while a chronic increase in 5-HT levels will decrease 5-HT_4 receptor binding globally. The influence of chronic changes in brain 5-HT levels on 5-HT_4 receptor binding is unique in its plasticity and unidirectional relationship to 5-HT levels compared to the 5-HTT and other 5-HT receptors investigated so far (Fig. 9). This suggests that brain 5-HT_4 receptor binding may be used as a marker of chronic changes in central 5-HT levels in animals, and possibly also in humans in vivo by [^{11}C]SB207145-PET studies.

Effects of 5-HT_4 receptor agonism on the 5-HT system (Aim III)

Given that paroxetine administration strongly affects 5-HT_4 receptor binding with a time-course similar to the clinical effect of paroxetine, the data from Manuscript II implicate regulation of the 5-HT_4 receptor in the effects of antidepressant treatment. Since 5-HT_4 receptor agonism increases 5-HT neuron firing rates in the DRN (Lucas et al. 2005), the decrease in 5-HT_4 receptor binding in response to increased 5-HT levels may be a compensatory downregulation of a positive regulator of 5-HT system function. This suggests that activation of the 5-HT_4 receptor may augment the response of the 5-HT system to paroxetine. To explore this possibility and to further characterize the effects of the 5-HT_4 receptor on 5-HT system activity, the effects of 5-HT_4 receptor stimulation on extracellular 5-HT levels in the ventral hippocampus alone and in combination with paroxetine were investigated by in vivo microdialysis (Manuscript III).

Acute administration of the 5-HT_4 receptor agonist RS67333 (1.5 mg/kg, i.v.) had no effect on extracellular 5-HT or 5-HIAA levels in the ventral hippocampus of the anaesthetized rat, while acute paroxetine administration (0.5 mg/kg i.v.) caused the expected increase in 5-HT (276% of baseline) and decrease in 5-HIAA levels (Fig. 10, Manuscript III). When administering RS67333 after the effect of paroxetine had stabilized, an additional increase in 5-HT levels (to 398% of baseline) was observed (Fig. 10). The absence of an acute effect of RS67333 alone was unexpected as a previous microdialysis study has shown markedly increased 5-HT levels in the ventral hippocampus after systemic administration of the 5-HT_4 receptor agonist renzapride (Ge and Barnes 1996). The reason for this discrepancy could be that RS67333 is a partial agonist (Eglen et al. 1995), while renzapride is a full agonist. Another difference between the two studies is that Ge et al. (1996) performed their

experiments in wake rats, while we used chloral hydrate anaesthetized animals. However, administration of RS67333 at the dose used in our study increases 5-HT neuron activity in the DRN of the chloral hydrate anaesthetized rat (Lucas et al. 2005), and stimulation of the DRN is normally reflected in increased 5-HT release in the ventral hippocampus (Sharp and Hjorth 1990). The absence of a detectable increase in 5-HT levels in response to acute RS67333 administration could be due to rapid reuptake of 5-HT by the 5-HTT.

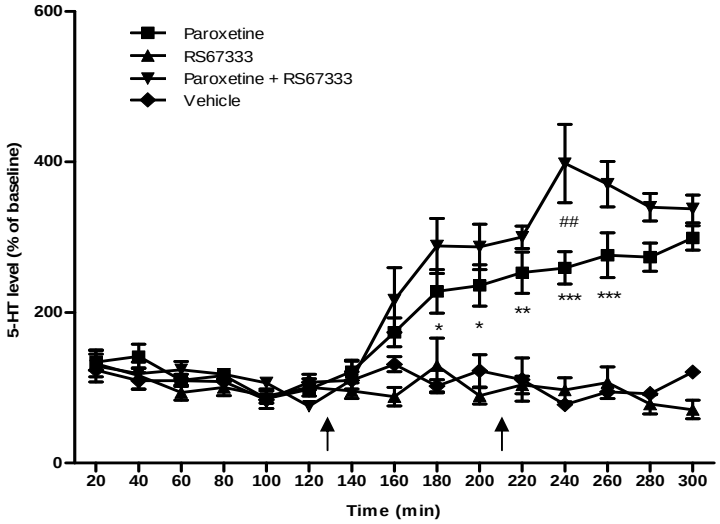

Figure 10. Extracellular 5-HT levels in the ventral hippocampus, expressed as percentage of baseline, after paroxetine (0.5 mg/kg, n = 6), RS67333 (1.5 mg/kg, n = 5), or vehicle (n = 3) administration, and in response to combined paroxetine and RS67333 (n = 7) administration. Arrows indicate injection times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: paroxetine relative to vehicle (0-260 min); $^{\#\#}p < 0.01$: paroxetine + RS67333 compared to paroxetine (140-300 min).

The increase in extracellular 5-HT levels in the ventral hippocampus in response to acute systemic paroxetine administration is in agreement with previous studies (e.g. (Hajos-Korcsok et al. 2000)). The increased 5-HT levels are a result of inhibited 5-HT reuptake in combination with basal 5-HT release due to 5-HT neuron firing (Sharp et al. 1990), the latter of which is regulated by changes in extracellular 5-HT levels among other factors. Increased synaptic 5-HT levels inhibit 5-HT neuron firing through 5-HT_{1A} autoreceptors in particular (Sprouse and Aghajanian 1987) and postsynaptic 5-HT_{2A} and $5\text{-HT}_{2B/C}$ receptors (Boothman et al. 2003). However, increased synaptic 5-HT levels may also have stimulatory effects on 5-HT neuron firing through postsynaptic 5-HT_4 receptors in a long feedback loop from the medial PFC (Lucas et al. 2005). Addition of a 5-HT neuron activating compound, RS67333, to acute systemic paroxetine administration may shift the balance between inhibitory and stimulatory 5-HT receptor effects on 5-HT neuron activity, and thereby increase the 5-HT release in the ventral hippocampus (Fig. 10). The transient nature of the additional increase in

5-HT levels after RS67333 administration may be due to negative feedback regulation of 5-HT release, as the increase in 5-HT levels in response to RS67333 will increase the activation of inhibitory 5-HT receptors. Our results are in line with the ability of renzapride to increase 5-HT levels in the ventral hippocampus in the presence of paroxetine after local administration of both compounds via the microdialysis probe (Ge and Barnes 1996), though the latter implies a direct, local effect on 5-HT release.

	Control	3 days RS67333	p value
5-HT	0.008 ± 0.001	0.014 ± 0.001	0.0001***
5-HIAA	7.536 ± 0.459	5.478 ± 0.834	0.0474*
DOPAC	0.424 ± 0.034	0.352 ± 0.055	0.3209

Table 9. Basal 5-HT and metabolite levels after 3 days of RS67333 (1.5 mg/kg). Values are mean ± SEM in pmol.

Three days of RS67333 (1.5 mg/kg, i.p.) administration increased 5-HT (by 73%) and decreased 5-HIAA baseline levels in the ventral hippocampus (Table 9). Given that acute RS67333 administration did not have a similar effect, the sub-chronic effect is most likely due to a combination of 5-HT_4 receptor stimulatory effects on 5-HT neuron activity (Lucas et al. 2005) and desensitization of 5-HT_{1A} autoreceptors in the raphe nuclei (Lucas et al. 2007). Our finding is in agreement with the observed increased tonus on hippocampal postsynaptic 5-HT_{1A} receptors after sub-chronic RS67333 administration (Lucas et al. 2007).

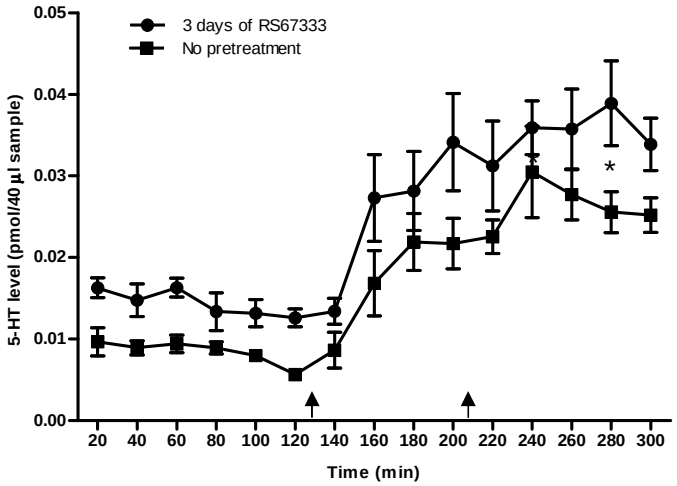

Figure 11. Effect of paroxetine and RS67333 administration on 5-HT levels after sub-chronic RS67333 pretreatment. Changes in extracellular 5-HT levels (pmol) in the ventral hippocampus after paroxetine (0.5 mg/kg) and RS67333 (1.5 mg/kg) administration in animals receiving 3 days of RS67333 (1.5 mg/kg, n = 5) pretreatment or no pretreatment (n = 7). Arrows indicate injection times. * $p < 0.05$.

The increase in 5-HT levels after 3 days of RS67333 administration is smaller than the 2-3-fold elevation, which has been observed after 14 days of SSRI treatment (Hajos-Korcsok et al. 2000;Kreiss and Lucki 1995). Together with the paroxetine augmenting effect of acute

RS67333 administration this suggests that a combination of SSRI administration and 5-HT_4 receptor partial agonism enhances 5-HT system activity. This is supported by the observation that a paroxetine challenge can still elevate 5-HT levels after 3 days of RS67333 administration and to a higher absolute level than reached with paroxetine alone (Fig. 11).

The regulation of 5-HT_4 receptor binding by changes in 5-HT levels, and in particular by chronic SSRI administration (Fig. 8), in combination with the stimulatory influence of sub-chronic 5-HT_4 receptor agonism on 5-HT levels in the ventral hippocampus suggests, a role for the 5-HT_4 receptor in antidepressant treatment. While acute 5-HT_4 receptor antagonism does not inhibit the antidepressant-like effect of fluoxetine in the FST (Cryan and Lucki 2000), a recent study has provided convincing data supporting a fast antidepressant-like effect of 5-HT_4 receptor partial agonists (RS67333 and prucalopride) in the FST, CMS and OBX models of depression (Lucas et al. 2007). The antidepressant-like effects of RS67333 and prucalopride may relate to their ability to stimulate 5-HT neuron firing rate both after acute and chronic administration (Lucas et al. 2005), increasing 5-HT release in 5-HT projection areas after sub-chronic administration (Table 9). The absence of desensitization of the 5-HT_4 receptor effect on 5-HT neuron activity after chronic partial agonist administration is surprising given the present finding of chronic paroxetine induced downregulation of 5-HT_4 receptor binding. However, the resistance to desensitization may be due to the partial agonist profile of RS67333 and prucalopride (Duman 2007), as sub-chronic RS67333 administration (1.5 mg/kg i.p.) does not affect brain 5-HT_4 receptor binding (unpublished observations).

Conclusions

This thesis is focused on the 5-HT_4 receptor in depression and antidepressant treatment, in particular the regulatory interactions between the 5-HT_4 receptor and the 5-HT system. These interactions have been explored in experimental animals, which have been used as models of depression, antidepressant treatment, chronic changes of brain 5-HT levels, and antidepressant-like responses of the 5-HT system.

The 5-HT_4 receptor binding was changed in three rodent models of depression, the FSL, OBX and $GR^{+/-}$, which are based on distinct constructs and exhibit different endophenotypes of depression. In the FSL model, the 5-HT_4 receptor binding was decreased in the dorsal and ventral hippocampus, and in the dorsal hippocampus the change was directly correlated to 5-HTT binding. Differential effects of ageing on hippocampal 5-HT_4 receptor binding was found in the FSL and FRL rats, while aged FSL rats exhibited a similar decrease in 5-HT_4 receptor binding in the LGP as found in post-hoc analysis of adult FSL rats. While the FSL is characterized by increased immobility in response to forced swim stress, the OBX mice are hyperactive in the aversive open field test. The opposite effects of stress on locomotor activity in the two models were reflected in opposite changes in hippocampal 5-HT_4 receptor binding and 5-HTT binding in the basolateral amygdala. The OBX mice display increased 5-HT_4 receptor binding in the ventral hippocampus, and decreased 5-HTT binding in several brain regions, including the basolateral amygdala. The FSL and OBX models are characterized by changes in the 5-HT system, which may affect endogenous agonism on the 5-HT_4 receptor, and thereby influence 5-HT_4 receptor binding. In comparison, the $GR^{+/-}$ model is based on a genetically induced deficit in negative feedback control of the HPA axis in response to stress. The $GR^{+/-}$ model showed increased 5-HT_4 receptor binding in the caudal caudate putamen, which was not readily explainable by changes in the 5-HT system. However, the 5-HT_4 receptor binding was not affected by chronic exposure to environmental stress (CMS).

Manipulation of central 5-HT levels indicated an inverse relationship between 5-HT levels and 5-HT_4 receptor binding. Effects of sub-chronic 5-HT depletion on 5-HT_4 receptor binding were detected in the dorsal hippocampus, hypothalamus, and LGP, while chronic SSRI (paroxetine) administration downregulated 5-HT_4 receptor binding in all brain regions evaluated. The regionally confined pattern of changes induced by 5-HT depletion may relate to the relatively short duration (5 days) of decreased 5-HT levels, as others have reported larger and more widespread changes after 21 days. The regulation of 5-HT_4 receptor binding

by these pharmacological manipulations of brain 5-HT levels differed from that of another postsynaptic 5-HT receptor, the 5-HT_{2A} receptor, which was moderately downregulated by chronic paroxetine treatment in cortical regions only but globally downregulated by sub-chronic 5-HT depletion. The inverse relationship between 5-HT_4 receptor binding and central 5-HT levels is unique among 5-HT receptors analyzed so far, and suggests the potential use of 5-HT_4 receptor binding as a marker of chronic changes in endogenous 5-HT levels. It also indicates that regulation of 5-HT_4 receptor binding may be a compensatory feedback mechanism in the 5-HT system of relevance to antidepressant treatment and 5-HT system control.

In support of a central role of the 5-HT_4 receptor in 5-HT system control, 5-HT_4 receptor activation increased the effect of acute SSRI (paroxetine) administration on 5-HT levels in the ventral hippocampus, both acutely and after three days of administration with the 5-HT_4 receptor partial agonist RS67333. This effect is most likely due to the acute stimulation of 5-HT neuron firing rate by 5-HT_4 receptor partial agonism, which has been reported by others. While acute administration of RS67333 did not affect extracellular 5-HT levels in the ventral hippocampus, three days of RS67333 administration sufficed to elevate 5-HT levels. As an increase in extracellular 5-HT levels is associated with antidepressant effect of drug treatment, these data suggest that 5-HT_4 receptor partial agonism have antidepressant potential, in particular in combination with SSRI administration.

Collectively the results of this thesis suggest a model of reciprocal regulatory interactions between the 5-HT_4 receptor and central 5-HT levels: 5-HT_4 receptor activation stimulates 5-HT release, leading to increased 5-HT levels, which by increased receptor agonism downregulates 5-HT_4 receptor binding. The regulatory effects require sub-chronic 5-HT system stimulation, possibly to overcome 5-HT_{1A} autoreceptor mediated inhibition through desensitization. This model provides a framework for interpretation of changes in 5-HT_4 receptor binding found in three animal depression models and their relation to changes in 5-HTT binding. Given that 5-HT system changes are among the biological endophenotypes of depression, the reciprocal regulation of 5-HT_4 receptor binding and 5-HT levels supports a role for the 5-HT_4 receptor in the pathophysiology of depression.

Research Perspectives

While this thesis has addressed important questions regarding the role of the 5-HT_4 receptor in the pathophysiology of depression and its potential as a target for antidepressant treatment, these lines of research have potential for further extension.

The importance of changes in brain regional 5-HT_4 receptor binding for the depression-like behavior of the three animal models should be confirmed by reversal of the changes by chronic antidepressant treatment, and potential functional implications of the changes could be examined by behavioral tests or in vivo pharmacological assays. The changes in 5-HT_4 receptor binding could also be studied at the cellular level in the affected brain regions to examine potential relations to neurodegenerative changes and determine sub-cellular localization of affected receptors, requiring the development of suitable antibodies.

The mechanism and time course of the effects of changes in brain 5-HT levels on 5-HT_4 receptor binding should be further explored. The hypothesis of the 5-HT_4 receptor binding changes being mediated by changes in 5-HT_4 receptor stimulation should be tested by studying the effects of acute, sub-chronic and chronic 5-HT_4 receptor antagonism, partial agonism and full agonism on 5-HT_4 receptor binding. Of relevance to the potential use of 5-HT_4 receptor binding as a marker of chronic changes in 5-HT levels, the effects of intermediate degrees of 5-HT depletion on 5-HT_4 receptor binding should be explored. It would also be relevant to determine the detailed time course of the effects of paroxetine between one and 14 days of administration, and to evaluate the time course of reversal of the decrease in 5-HT_4 receptor binding, when discontinuing administration. To determine if the effect of paroxetine can be extended to other antidepressant treatments, 5-HT_4 receptor binding should be evaluated after administration of other antidepressant drugs and ECS. It is also important to determine whether the changes in 5-HT_4 receptor binding in response to changes in 5-HT levels are reflected in differences in 5-HT_4 receptor function.

The effects of 5-HT_4 receptor stimulation on 5-HT levels in the ventral hippocampus should be confirmed with another 5-HT_4 receptor agonist and by blocking the response with a 5-HT_4 receptor antagonist. The antidepressant potential of combined 5-HT_4 receptor agonism and SSRI administration should be further explored in animal models of antidepressant-like effects. In addition, it would be interesting to test the effects of combined 5-HT_4 receptor agonism and 5-HT_{1A} receptor antagonism as a possible means to achieve antidepressant-like effects acutely.

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Appendices

I. The 5-HT_4 receptor and 5-HTT binding are altered in two murine depression models.

II. Changes in brain 5-HT_4 receptor binding in rat depression models and in response to paroxetine administration.

III. Effects of 5-HT_4 receptor agonism and paroxetine administration on hippocampal extracellular 5-HT levels.

Manuscript I

The 5-HT$_4$ receptor and 5-HT transporter binding are altered in two murine depression models

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Abstract

The 5-HT_4 receptor is a new target for antidepressant treatment and may be implicated in the pathogenesis of depression. This study investigated differences in 5-HT_4 receptor and 5-HT transporter (5-HTT) levels by quantitative autoradiography of [^{3}H]SB207145 and (S)-[N-methyl-^{3}H]citalopram in two murine depression models, olfactory bulbectomy and glucocorticoid receptor heterozygous ($GR^{+/-}$) mice, respectively. The olfactory bulbectomy model is characterized by 5-HT system changes, while the $GR^{+/-}$ mice have a deficit in hypothalamic-pituitary-adrenal (HPA) system negative feedback control. The olfactory bulbectomized mice displayed increased activity in the aversive open field test, a characteristic depression-like feature of this model. After bulbectomy, 5-HT_4 receptor binding was increased in the ventral hippocampus (12%) and decreased in the olfactory tubercles (14%). The bulbectomized mice had a small but significant decrease in 5-HTT binding in the dorsal hippocampus, hypothalamus, basolateral amygdala, lateral septum, olfactory tubercles, and lateral globus pallidus. Both 5-HT_4 receptor and 5-HT transporter binding in the olfactory tubercles were inversely correlated with activity in the aversive open field test. In comparison, $GR^{+/-}$ mice had increased 5-HT_4 receptor (11%) and decreased 5-HTT binding in the caudate putamen, and increased 5-HT_4 receptor binding in the olfactory tubercles (10%). In conclusion, we have found brain regional changes in 5-HT_4 receptor and 5-HTT transporter binding in two murine depression models, supporting a role for 5-HT_4 receptors in serotonergic and HPA system related endophenotypes of depression.

Key words

Serotonin receptors; depression; hippocampus; striatum; mouse

INTRODUCTION

In 2020, depression is projected to be the second most important contributor to the worldwide burden of disease (Murray and Lopez 1997). Among the biological endophenotypes of depression, increased sensitivity to serotonin (5-hydroxytryptamine, 5-HT) system depletion and hypothalamic-pituitary-adrenal (HPA) system sensitivity to manipulation are the most consistent (Hasler et al. 2004). In the 5-HT system, the 5-HT_4 receptor is special in its ability to stimulate 5-HT neuron activity in the dorsal raphe nucleus (Lucas and Debonnel 2002;Lucas et al. 2005). The 5-HT_4 receptor binding is increased in the caudate and frontal cortex of depressed suicide victims (Rosel et al. 2004), and two single nucleotide polymorphisms of the 5-HT_4 receptor gene are associated with depression (Ohtsuki et al. 2002). The 5-HT_4 receptor is regulated by both 5-HT system changes and corticosterone levels: 5-HT depletion upregulates 5-HT_4 receptor binding in the hippocampus and basal ganglia (Compan et al. 1996), and chronic corticosterone administration increases 5-HT_4 receptor effects on hippocampal neurons (Bijak et al. 2001).

Olfactory bulbectomy (OBX) in rodents induces behavioral and neurobiological changes similar to certain aspects of clinical depression, (Kelly et al. 1997;Song and Leonard 2005). The OBX mouse is characterized by decreased 5-HT turnover in several brain regions and has been conceptualized as a model of agitated depression (Hellweg et al. 2007). The key behavioral effect of OBX is hyperactivity in response to novel, stressful environments, such as the aversive open field test (Zueger et al. 2005;Kelly et al. 1997;Song and Leonard 2005). The OBX syndrome develops over 14 days after bilateral ablation of the olfactory bulbs, and is caused by anterograde and retrograde neurodegeneration, and resulting structural and functional changes in brain areas (e.g. amygdala, raphe nuclei, and hippocampus) connected

to the olfactory bulbs (Kelly et al. 1997;Song and Leonard 2005). The bulbectomy model has high predictive validity, since chronic but not acute treatment with the majority of antidepressants can reverse the biochemical and behavioral deficits (Jarosik et al. 2007;Kelly et al. 1997).

Given the importance of HPA system deregulation in the pathophysiology of depression, mice with genetic alterations of the glucocorticoid receptor (GR) have been developed as models of depression (Chourbaji and Gass 2008). The GR heterozygous mice ($GR^{+/-}$) lack one GR allele and have reduced GR mRNA and protein levels (Ridder et al. 2005). The $GR^{+/-}$ mice have ineffective corticosterone suppression in the dexamethasone/corticotropin-releasing hormone test (Ridder et al. 2005), indicating deficits in the negative feedback control of glucocorticoid release. A comparable deficit is seen in >50% of patients with major depression (Heuser et al. 1994). While exhibiting normal behavior at baseline, the $GR^{+/-}$ mice display depression-like behavior upon stress exposure (Ridder et al. 2005).

We have examined the 5-HT_4 receptor [^{3}H]SB207145 binding in olfactory bulbectomized and unstressed $GR^{+/-}$ mice, and related this to 5-HT transporter (5-HTT) (S)-[N-methyl-^{3}H]citalopram binding and behavior. We hypothesized that the 5-HT_4 receptor binding would be increased in the caudate putamen and hippocampus, and performed a post-hoc analysis of additional brain regions with high 5-HT_4 receptor binding and relevance for depression.

MATERIALS AND METHODS

Olfactory bulbectomy

Adult male 3-month-old C57BL/6N mice (Charles River, Germany) were allowed to acclimatize for 2 weeks to a reversed 12: 12h dark/light cycle. Animals were individually housed with free access to food and water. OBX was performed as described previously (Zueger et al. 2005). Briefly, mice (n = 9) were anaesthetized with Xylazin 80 mg/kg i.p. (Bayer, Germany) and Ketamin 90 mg/kg i.p. (Aventis Pharma, Germany) diluted in saline. The skull covering the olfactory bulbs was exposed by skin incision, a burr hole drilled, and the olfactory bulbs removed by suction with a hypodermic needle attached to a water pump. The burr hole was closed with bone wax, Neomycin powder (Sigma, Germany) applied, and the skin closed with Histoacryl (Aesculap AG, Germany). Sham operations (n = 6) were performed in the same way but with the bulbs left intact. The mice were decapitated on days 20-21 after the operation. The brains were removed, frozen on dry ice, and stored at -80 °C. All animal experiments were approved by the German animal welfare authorities (Regierungspräsidium Karlsruhe, Germany).

Open field test

Locomotor hyperactivity in response to a novel, stressful environment is a key behavioural feature of the olfactory bulbectomy model (Song and Leonard 2005), and was analyzed in the open field test on the day before (baseline) and 2 weeks after surgery, as described previously (Zueger et al. 2005). The open field test behaviour was used as an indicator of successful bulbectomy, and data from animals with unchanged activity was not included in the autoradiography analysis (3 out of 9 mice tested). The open field test was performed during the first hours of the dark (active) phase, in a circular arena (90 cm diameter) with white plastic floor and a reflecting aluminium wall under 320 Lux light intensity. Mice were placed near the sidewall and locomotion was recorded with a video camera for 5 min. The xy-

coordinates were analyzed with Noldus Etho Vision 2.3, and the parameter 'total distance moved (cm)' was determined.

Glucocorticoid receptor heterozygous mice

A non-functional GR allele (*Grl1-*) was constructed by homologous recombination of mouse embryonic stem cells with a modified *Grl1* allele carrying loxP sites on either side of the *Grl1* exon 3, followed by transient transfection with Cre recombinase leading to excision of exon 3 (Tronche et al. 1999). Embryonic stem cells carrying the *Grl1-* allele were used to generate *Grl1-* chimaeras, which were then crossed with C57BL/6N mice to generate *Grl1-* heterozygous mice. GR heterozygous ($GR^{+/-}$) mice were generated by crossing *Grl1-* heterozygous C57BL/6N males (backcrossed with the mutation for >10 generations) with wild-type FVB/N females (Ridder et al. 2005). For all experiments, 5-7 months old male $GR^{+/-}$ mice and their male wild-type littermates were used. Animals were supplied with food and water ad libitum and were single-housed on a reversed 12h: 12h dark-light cycle (lights on at 6:00 in the evening), similarly as for the behavioral study described previously (Ridder et al. 2005). The animals were decapitated, and the brains were removed, frozen on dry ice, and stored at -80 °C.

Tissue preparation

The brains were sectioned on a cryostat in 10 µm coronal sections at -25 °C, thaw-mounted onto gelatinated glass slides, allowed to dry at room temperature (RT), and stored at -80 °C until processed. Sections were collected between Bregma 2.58 and 2.34 mm; 1.10 and 0.86 mm; -1.34 and -1.58 mm; and -3.16 and -3.40 mm (Paxinos and Franklin 2001). Tissue

sections were collected in 4 parallel series so that every fourth section was thaw-mounted onto the same glass slide with a total of three sections on each glass slide.

5-HT$_4$ receptor autoradiography

The protocol for [^{3}H]SB207145 autoradiography was adapted from Parker *et al*., 2003 (Parker et al. 2003). Frozen tissue sections were brought to RT and allowed to dry for 60 min. Sections were preincubated at RT for 15 min in 50 mM Tris-HCl, pH 7.4, containing 0.01% ascorbic acid and 10 μM pargyline. Sections were incubated in the same buffer containing 1 nM [^{3}H]SB207145 (1-5 times K_d) for 60 min. Sections used for determination of non-specific binding were preincubated and incubated in the presence of 10 μM RS39604. Sections were washed for 2 x 20 seconds in ice-cold 50 mM Tris-HCl followed by 20 seconds in ice-cold dH_2O. Slides were dried at RT in a gentle air stream for 1 hour. They were then fixed in paraformaldehyde vapour overnight at 4 °C. The sections were then dried for 3 hours in a dessicator at RT, followed by exposure to a tritium-sensitive BAS TR2040 phosphor imaging plate (Fuji, Science Imaging Scandinavia AB) along with 4 autoradiographic [^{3}H]microscales (8 and 80 nCi, Amersham Biosciences) for 2 weeks at 4 °C. The imaging plate was scanned on a BAS-2500 bioimaging analyzer (Fuji Film Photo Co. LTD., Japan). Sections from each model and the respective control group were for each section level processed and exposed to imaging plates together.

5-HT transporter autoradiography

The protocol for (S)-[N-methyl-^{3}H]citalopram autoradiography was adapted from Thomsen *et al*., 2003 (Thomsen and Helboe 2003). Frozen tissue sections were brought to RT and allowed

to dry for 60 min. Sections were preincubated at RT for 20 min in 50 mM Tris-HCl with 120 mM NaCl and 5 mM KCl, pH 7.4; and incubated in the same buffer with 2 nM (S)-[N-methyl-^{3}H]citalopram for 60 min. Sections used for determination of non-specific binding were preincubated and incubated in the presence of 10 μM paroxetine. Sections were washed for 3 x 2 min in buffer, followed by a quick dip in ice-cold dH_2O. The sections were then dried and processed for imaging plate exposure as described for 5-HT_4 receptor autoradiography. Sections were exposed for 7 days to tritium sensitive imaging plates.

Drugs

RS39604 HCl (Tocris Cookson Ltd, UK), [^{3}H]SB207145 (66.7 Ci/mmol, donated by GlaxoSmithKline, UK), (S)-[N-methyl-^{3}H]citalopram (79.0 Ci/mmol, donated by H. Lundbeck A/S, Copenhagen, Denmark), paroxetine HCl (donated by GlaxoSmithKline, UK).

Image analysis of autoradiograms

Autoradiograms were analysed with ImageJ V.1.32j (htttp://rsb.info.nih.gov/ij/). Image optical density was converted to activity density in nCi/mg tissue equivalent (referred to as tissue) using the linear range of [^{3}H]microscale standards. Regions of interest (ROI) for each section level were defined by aligning representative autoradiograms with anatomical line drawings from the digital versions of The Mouse Brain in Stereotaxic Coordinates, Second Edition by Paxinos and Franklin (2001). The alignment was performed with Adobe Illustrator CS2 (v12.0.0). The ROIs were drawn on the atlas-superimposed autoradiograms in ImageJ, and were then used to measure region-specific radioactive density on all sections from the same section level. The activity density in nCi/mg tissue was converted to radioligand binding

in fmol/mg tissue using the specific activity. The specific receptor binding was determined by subtracting the non-specific binding from the total radioligand binding for each animal. The dorsal hippocampus was defined as 50% of the hippocampal volume starting at the septal pole, and the ventral hippocampus as 50% starting at the temporal pole (Bannerman et al. 2004). The dorsal hippocampus was evaluated on sections collected between -1.34 and -1.58 mm from Bregma, and the ventral hippocampus on sections collected between -3.16 and -3.40 mm from Bregma (Paxinos and Franklin 2001). As a fronto-caudal gradient in 5-HT_4 receptor binding in the caudate putamen has been described in rats (Vilaro et al. 2005), and the 5-HT_4 receptor binding pattern in mouse is identical to that in the rat (Waeber et al. 1994), the 5-HT_4 receptor binding was measured in both the frontal and caudal caudate putamen on sections collected between 1.10 and 0.86 mm, and -1.34 and -1.58 mm from Bregma, respectively (Paxinos and Franklin 2001).

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software Inc.). The open field test was analyzed by two-way ANOVA with repeated measures, and Surgery (OBX or Sham) and Time (Basal or Post-op) as independent factors. Group differences after significant ANOVAs were analyzed by Bonferroni post-tests. The brain regional changes were analyzed separately for each hypothesis region by two-tailed unpaired student's *t*-test. For each radioligand and model a post-hoc analysis of additional brain regions was performed. The regional binding data was not corrected for multiple comparisons. Outliers were detected by Grubb's test. If an outlier was detected in one brain region, binding values from other brain regions obtained at the same section level in the same animal were removed from the group. For evaluation of correlations between behaviour and regional binding,

activity in the open field test was expressed as percentage change in activity after surgery. Pearson's correlation analysis was applied. The level of significance was $p < 0.05$.

RESULTS

Olfactory bulbectomy

Olfactory bulbectomy increased the total distance moved (cm) in the open field test (Fig. 1). There was a significant interaction between Surgery and Time ($F(1,9) = 13.54$, $p = 0.0051$), and Bonferroni posttests showed that the increase in activity in the open field test at Post-op versus Basal was present after OBX only ($p < 0.01$).

Representative autoradiograms of quantitative *in vitro* autoradiography with [^{3}H]SB207145 are shown in figure 2. In the sham-operated controls, specific [^{3}H]SB207145 binding was detected in high levels in the lateral globus pallidus (LGP), olfactory tubercles, caudal caudate putamen, nucleus accumbens shell, ventral hippocampus, and hypothalamus (24.1 ± 0.6 to 32.2 ± 1.2 fmol/mg tissue), at intermediate levels in the medial amygdala, lateral septum, dorsal hippocampus, and frontal caudate putamen (10.5 ± 0.8 to 18.6 ± 0.7 fmol/mg tissue), and at low levels in the frontal cortex (4.4 ± 0.8 fmol/mg tissue). The 5-HT$_4$ receptor binding was increased by 11.5% ($p = 0.041$) in the ventral hippocampus of olfactory bulbectomized mice (Table 1). No change in binding was detected in the dorsal hippocampus, frontal or caudal caudate putamen (Table 1). A post-hoc analysis of additional brain regions showed a 14.1% ($p = 0.037$) decrease in 5-HT$_4$ receptor binding in the olfactory tubercles but no change in the frontal cortex, hypothalamus, LGP, lateral septum, medial amygdala, or nucleus accumbens shell (Table 1). There was no correlation ($r = 0.51$, $p = 0.105$) between 5-HT$_4$

receptor binding in the ventral hippocampus and total distance moved in open field test (Fig. 3A). In the olfactory tubercles, the 5-HT_4 receptor binding was negatively correlated (r = -0.66, p = 0.027) with activity in the open field test (Fig. 3B).

Representative autoradiograms of quantitative *in vitro* autoradiography with (S)-[N-methyl-^{3}H]citalopram are shown in figure 4. In the sham-operated mice, (S)-[N-methyl-^{3}H]citalopram binding was detected at high levels in the olfactory tubercles, nucleus accumbens shell, lateral septum, LGP, and hypothalamus (69.9 ± 2.1 to 85.1 ± 0.9 fmol/mg tissue), and at intermediate levels in the basolateral amygdala (BLA), frontal caudate putamen, ventral and dorsal hippocampus (36.6 ± 0.8 to 61.0 ± 1.4 fmol/mg tissue). Olfactory bulbectomy lead to a 12.5% downregulation of the 5-HTT binding in the dorsal hippocampus but had no effect on binding in the ventral hippocampus or frontal caudate putamen (Table 2). The post-hoc analysis showed significant downregulation in additionally 5 brain regions: the hypothalamus, BLA, lateral septum, LGP, and olfactory tubercles (Table 2). The decrease in binding was in the range of 10-12% in the hypothalamus, BLA and LGP, while a 4% decrease was detected in the lateral septum and olfactory tubercles. In the lateral septum (r = -0.64, p = 0.048) and the olfactory tubercles (r = -0.72, p = 0.018), the 5-HTT binding was inversely correlated with the distance moved in the open field test, expressed as percentage of baseline activity (Table 2). In the remaining regions with significant change in 5-HTT binding after OBX the correlations were also negative but not significant (Table 2). The 5-HTT binding was not significantly changed in the frontal cortex and nucleus accumbens shell. While both the 5-HT_4 receptor and the 5-HTT binding were decreased in the olfactory tubercles, the changes were not correlated (r = 0.46, p = 0.18) (Fig. 5).

Glucocorticoid receptor heterozygous mice

Representative autoradiograms of quantitative *in vitro* autoradiography with [^{3}H]SB207145 are shown in figure 6. The specific [^{3}H]SB207145 binding was similar to the pattern found in the sham-operated mice. In the $GR^{+/-}$ mice, the 5-HT_4 receptor binding was increased in the caudal caudate putamen ($p = 0.036$) by 10.9%, and in the dorsal hippocampus there was a tendency ($p = 0.052$) towards a 17.0% increase (Table 3). There was no change in the frontal caudate putamen and ventral hippocampus (Table 3). A post-hoc analysis of the hypothalamus, nucleus accumbens shell, medial amygdala, olfactory tubercles, LGP, and lateral septum revealed a 9.6% increase in 5-HT_4 receptor binding in the olfactory tubercles ($p = 0.032$) in the $GR^{+/-}$ mice (Table 3).

Representative autoradiograms of quantitative *in vitro* autoradiography with (S)-[N-methyl-^{3}H]citalopram are shown in figure 7. The pattern of specific (S)-[N-methyl-^{3}H]citalopram binding was similar to the distribution found in the sham-operated mice. There was a 6% decrease in 5-HTT binding in the frontal caudate putamen ($p = 0.038$) but no change in the dorsal hippocampus (Table 4). The 5-HTT binding was not evaluated in the caudal part of the caudate putamen due to the very low signal in this area, and could not be evaluated in the ventral hippocampus due to methodological noise (data not shown). The 5-HTT binding was unchanged in the post-hoc analyzed regions: BLA, hypothalamus, lateral septum, LGP, nucleus accumbens shell, and olfactory tubercles (Table 4).

DISCUSSION

To the best of our knowledge, this study is the first to investigate 5-HT_4 receptor and 5-HTT binding in OBX and $GR^{+/-}$ mice, and is also the first report of [^{3}H]SB207145 and (S)-[N-methyl-^{3}H]citalopram autoradiography in mice.

Effects of olfactory bulbectomy

The olfactory bulbectomized mice showed an increase in total activity in the open field test, which is in agreement with previous studies (Hellweg et al. 2007;Zueger et al. 2005). Among the hypothesis regions, we found an increase in 5-HT_4 receptor binding in the ventral hippocampus. This upregulation may be due to reduced serotonergic tonus, as chronic 5-HT depletion increases the 5-HT_4 receptor binding in the hippocampus (Compan et al. 1996), and 5-HIAA tissue levels are decreased in the hippocampus after bulbectomy (Hellweg et al. 2007). Changes in the 5-HT system are involved in the neurobiological basis of the OBX syndrome, as the syndrome can be induced by injection of serotonergic toxins into the olfactory bulbs (Cairncross et al. 1977a). OBX in mice induces neurodegeneration in the dorsal raphe nucleus (Nesterova et al. 1997), possibly due to degeneration of serotonergic fibers innervating the main olfactory bulb (McLean and Shipley 1987), and a decrease in brain tryptophan hydroxylase activity and 5-HT synthesis rate (Neckers et al. 1975). Another explanation of the increased 5-HT_4 receptor levels may be glucocorticoid mediated upregulation of the receptor, since chronic corticosterone administration increases the hippocampal neuronal response to 5-HT_4 receptor activation (Bijak et al. 2001), and OBX mice may have disturbances in HPA system regulation. While plasma corticosterone levels of bulbectomized mice have not been reported, OBX rats display increased basal plasma corticosterone levels in some studies and an exaggerated corticosterone response to stress (Cairncross et al. 1977b). The pathophysiological relevance of the increase in 5-HT_4 receptor

binding after OBX is supported by the antidepressant effect of chronic 5-HT_4 receptor agonism in the OBX rat (Lucas et al. 2007). In contrast to the increase in 5-HT_4 receptor binding in the ventral hippocampus, we found no change in the dorsal hippocampus, possibly due to regional differences in connectivity (Bannerman et al. 2004). We also detected differential changes in 5-HTT binding between the dorsal and ventral hippocampus in a pattern complementary to the 5-HT_4 receptor changes. While 5-HTT binding has not been investigated in OBX mice before, [^{3}H]paroxetine homogenate binding in the hippocampus is unchanged 8 weeks after bulbectomy in rats (Zhou et al. 1998). The discrepancy between the study by Zhou *et al*. (1998) and our study may be due to differences in post-surgery time, as tendencies towards decreases in [^{3}H]imipramine binding in rat hippocampal and cortical synaptosomes have been detected four weeks after bulbectomy (Stockert et al. 1988). In the caudate putamen, both 5-HTT and 5-HT_4 receptor binding were not affected by bulbectomy. This is in contrast to the postmortem finding of increased 5-HT_4 receptor binding in the caudate nucleus of depressed suicide victims (Rosel et al. 2004), possibly due to neurochemical differences between depressed violent suicide victims and the depression endophenotype modeled by OBX. In OBX mice, the 5-HT and 5-HIAA levels in the caudate putamen are unchanged (Hellweg et al. 2007), and while OBX reduces caudate nucleus size (Wrynn et al. 2000), and increases caudate 5-HT synthesis (Watanabe et al. 2003) in rats, it has no effect on caudate 5-HT_{1A} and 5-HT_2 receptor binding (Slotkin et al. 2005).

Given the unexplored role of 5-HT_4 receptors in depression, we chose to do a post-hoc analysis of brain regions with high 5-HT_4 receptor expression. Out of 7 regions analyzed, we found a downregulation of the 5-HT_4 receptor binding in the olfactory tubercles. The olfactory tubercles is part of the ventral striatum and have been implicated in the rewarding effects of morphine, cocaine, and brain-stimulation reward (Ikemoto 2007;Kornetsky et al.

1991;Ikemoto 2003). The tubercles receive projections from the main olfactory bulb (Shipley and Adamek 1984), and are therefore likely to be affected by bulbectomy. The tubercles have high 5-HT_4 receptor binding (Jakeman et al. 1994;Waeber et al. 1994), and high 5-HTT binding (Scheffel and Hartig 1989), the latter being indicative of dense innervation by serotonergic fibers. The decrease in 5-HT_4 receptor binding in the olfactory tubercles may be a compensatory downregulation of the 5-HT_4 receptor in response to changes in serotonergic activity, as a marked (qualitative) increase in indoleamine content is present in the olfactory tubercles at 21 days after bulbectomy, indicating an increase in 5-HT content in serotonergic fibers in this region (Garris et al. 1984). As the 5-HT_4 receptor binding in cultured neurons is reduced after 30 min. of exposure to 5-HT (Ansanay et al. 1996), a possible explanation of the decrease in 5-HT_4 receptor binding could be a downregulation of the receptor in response to increased activation. The 5-HTT binding in the olfactory tubercles was also reduced after OBX, and both 5-HTT and 5-HT_4 receptor binding in the olfactory tubercles were inversely correlated with activity in the open field test, indicating that their regulation is related to the behavioral phenotype. The pattern of 5-HT_4 receptor and 5-HTT changes after OBX is similar to a recent report of 5-HT_6 receptor expression being increased in the posterior dentate gyrus and decreased in the anterior dentate gyrus and the olfactory tubercles of rats with a high activity response to a novel and inescapable environment (Ballaz et al. 2007), a response which is comparable to OBX hyperactivity in the open field test. The two studies combined suggest opposing 5-HT system changes in the ventral/posterior hippocampus and olfactory tubercles as a biological marker of novelty-induced hyperactivity. A role for the 5-HT_4 receptor in modulating reactivity to novel environments is supported by decreased novelty-induced locomotion in 5-HT_4 receptor knockout mice (Compan et al. 2004).

While the primary result for 5-HTT binding after OBX was a decrease in the dorsal hippocampus, post-hoc analysis showed downregulation of 5-HTT binding across several brain regions: the BLA, hypothalamus, LGP, lateral septum, and olfactory tubercles. The extensive distribution of changes in 5-HTT binding could indicate degeneration of serotonergic projections to several brain regions, possibly due to degeneration of 5-HT neurons. However, degeneration of 5-HT fibers would be expected to cause a decrease in regional 5-HT levels but these are unchanged after OBX in mice (Hellweg et al. 2007). Alternatively, the decrease in 5-HTT binding can be due to decreased expression or increased local degradation of the 5-HTT. In contrast to previous studies in rats, we did not see an effect of bulbectomy on 5-HTT binding in the frontal cortex (Grecksch et al. 1997;Zhou et al. 1998;Huether et al. 1997). This difference may be related to the longer post-surgery time (12 weeks) applied compared to our study (3 weeks).

Glucocorticoid receptor heterozygous mice

In contrast to the OBX model of depression, the $GR^{+/-}$ mouse is a model of predisposition to depression, where a depression-related behavioural phenotype is evident after stress only (Ridder et al. 2005). The animals used in the present study were not subjected to stress before analysis of 5-HT_4 receptor and 5-HTT binding, and the present findings are therefore related to the endophenotype of decreased HPA system control. $GR^{+/-}$ mice exhibited a modest increase in 5-HT_4 receptor binding in the caudate putamen, which is in agreement with the finding of increased postmortem 5-HT_4 receptor binding in the caudate nucleus of depressed suicide victims (Rosel et al. 2004). Notably, 5-HT_4 receptor binding was increased in the caudal but not in the frontal caudate putamen. While serotonergic denervation leads to an increase in 5-HT_4 receptor binding in the frontal but not in the caudal caudate putamen,

lesions of the nigrostriatal dopaminergic pathway induce an increase in 5-HT_4 receptor binding in the caudal but not the frontal caudate putamen after 3 weeks (Compan et al. 1996). Our finding of increased 5-HT_4 receptor binding in the caudal caudate putamen may therefore reflect decreased dopaminergic activity in $GR^{+/-}$ mice. The increased 5-HT_4 receptor binding is not related to changes in brain regional tissue dopamine levels, as these are similar in $GR^{+/+}$ and $GR^{+/-}$ mice (Schulte-Herbruggen et al. 2007), but could potentially reflect changes in dopaminergic neuronal activity. In the $GR^{+/-}$ mice, there was also a small decrease in the 5-HTT binding in the frontal caudate putamen but no change in the dorsal hippocampus or in the post-hoc analyzed regions. The decrease in 5-HTT binding in the frontal caudate putamen was unexpected given the absence of 5-HT system change in this region in the $GR^{+/-}$ model (Schulte-Herbruggen et al. 2007). Post-hoc analysis showed an increase in 5-HT_4 receptor binding in the olfactory tubercles of the $GR^{+/-}$ mice, which is interesting in the light of the decrease in 5-HT_4 receptor binding in the same region after OBX.

Overall, we have found generally increased 5-HT_4 receptor and decreased 5-HTT binding in two murine models of depression. The changes in binding are small but we know from other biological systems that small changes can have clear effects.

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FIGURE LEGENDS

Figure 1. Activity in open field test after olfactory bulbectomy. Total distance (cm) moved before (basal) and 20 days after (post-op) olfactory bulbectomy (OBX, n = 6) or sham-operated (n = 5) in male mice. Columns are mean ± SEM. Two-way ANOVA with repeated measures and Bonferroni posttests, **p < 0.01 versus OBX basal.

Figure 2. 5-HT_4 receptor binding after olfactory bulbectomy. Autoradiograms of [^{3}H]SB207145 binding (1 nM) at 3 section levels of sham-operated (A, E, I) and olfactory bulbectomized mice (B, F, J). C, G, K) Non-specific binding in the presence of 10 μM RS39604. D, H, L) Section overlay with a digital version of the Mouse Brain in Stereotaxic Coordinates 2nd edition (reproduced with permission from Paxinos and Franklin 2001). Regions of interest are shown in white.

Figure 3. Relationship between 5-HT_4 receptor binding and activity in the open field test of bulbectomized and sham-operated mice. [^{3}H]SB207145 binding (1 nM) in A) ventral hippocampus (r = 0.51, p = 0.105) and B) olfactory tubercles (r = -0.66, p = 0.027) versus total distance moved in the open field test (expressed as percentage of baseline activity). Sham-operated animals are indicated by circles and bulbectomized animals by squares.

Figure 4. 5-HT transporter binding after olfactory bulbectomy. Autoradiograms of (S)-[N-methyl-^{3}H]citalopram binding (2 nM) at 3 section levels of sham-operated (A, E, I) and olfactory bulbectomized mice (B, F, J). C, G, K) Non-specific binding in the presence of 10 μM paroxetine. D, H, L) Section overlay with a digital version of the Mouse Brain in Stereotaxic Coordinates 2nd edition (reproduced with permission from Paxinos and Franklin 2001). Regions of interest are shown in white.

Figure 5. Relationship between 5-HT_4 receptor and 5-HT transporter binding in the olfactory tubercles of bulbectomized and sham-operated mice. [^{3}H]SB207145 binding (1 nM) versus (S)-[N-methyl-^{3}H]citalopram binding in olfactory tubercles ($r = 0.46$, $p = 0.18$). Sham-operated animals are indicated by circles and bulbectomized animals by squares.

Figure 6. 5-HT_4 receptor binding in GR heterozygous mice. Autoradiograms of [^{3}H]SB207145 binding (1 nM) at 3 section levels of $GR^{+/+}$ (A, E, I) and $GR^{+/-}$ mice (B, F, J). C, G, K) Non-specific binding in the presence of 10 μM RS39604. D, H, L) Section overlay with a digital version of the Mouse Brain in Stereotaxic Coordinates 2nd edition (reproduced with permission from Paxinos and Franklin 2001). Regions of interest are shown in white.

Figure 7. 5-HT transporter binding in GR heterozygous mice. Autoradiograms of (S)-[N-methyl-^{3}H]citalopram binding (2 nM) at 2 section levels of $GR^{+/+}$ (A, E) and $GR^{+/-}$ heterozygous mice (B, F). C, G) Non-specific binding in the presence of 10 μM paroxetine. D, H) Section overlay with a digital version of the Mouse Brain in Stereotaxic Coordinates 2nd edition (reproduced with permission from Paxinos and Franklin 2001). Regions of interest are shown in white.

Table 1. 5-HT_4 receptor binding after olfactory bulbectomy

	Sham	OBX	Percent change[1]	p value
Caudate putamen, frontal	10.5 ± 0.8	9.8 ± 0.5	-7.3	0.399
Caudate putamen, caudal	28.6 ± 1.3	28.3 ± 0.5	-1.0	0.845
Hippocampus, dorsal	14.4 ± 0.8	15.2 ± 0.8	5.5	0.487
Hippocampus, ventral	24.1 ± 0.6	26.9 ± 0.9	11.5	0.041*
Frontal cortex	4.4 ± 0.8	5.7 ± 0.6	28.4	0.221
Hypothalamus	20.8 ± 0.7	21.4 ± 0.7	2.8	0.576
Lateral globus pallidus	32.2 ± 1.2	29.9 ± 0.9	-6.9	0.165
Lateral septum	17.3 ± 0.7	18.1 ± 0.3	4.6	0.307
Medial amygdala	18.6 ± 0.7	19.9 ± 0.6	6.9	0.168
N. accumbens shell	26.2 ± 0.7[2]	24.5 ± 1.2	-6.3	0.331
Olfactory tubercles	31.9 ± 1.6	27.4 ± 1.1	-14.1	0.037*

Values are mean ± SEM fmol/mg tissue. [1] OBX compared to Sham. [2] n = 4. *p < 0.05.

Table 2. 5-HT transporter binding after olfactory bulbectomy

	Sham	OBX	Percent change[1]	p value	Correlation[2]
Caudate putamen, frontal	53.1 ± 1.1	51.7 ± 0.6	-2.6	0.302	nd
Hippocampus, dorsal	36.6 ± 0.8	32.0 ± 1.3	-12.5	0.019*	r = -0.52
Hippocampus, ventral	43.6 ± 1.0	43.6 ± 2.0[3]	-0.1	0.991	nd
Basolateral amygdala	61.0 ± 1.4	54.9 ± 0.9	-10.0	0.006**	r = -0.56
Frontal cortex	50.0 ± 1.0	46.5 ± 1.6[3]	-7.0	0.107	nd
Hypothalamus	69.9 ± 2.1	61.6 ± 2.6	-12.0	0.036*	r = -0.39
Lateral globus pallidus	71.0 ± 1.2	63.6 ± 1.2	-10.4	0.003**	r = -0.56
Lateral septum	73.8 ± 0.8	71.0 ± 0.4	-3.8	0.016*	r = -0.64*
N. accumbens shell	75.1 ± 0.7	72.9 ± 0.9	-3.0	0.073	nd
Olfactory tubercles	85.1 ± 0.9	81.8 ± 1.1	-4.0	0.048*	r = -0.72*

Values are mean ± SEM in fmol/mg tissue. [1] OBX compared to sham. [2] 5-HTT binding versus activity in open field test (percentage of baseline activity). [3] n = 6. *$p < 0.05$, **$p < 0.01$

Table 3. 5-HT_4 receptor binding in $GR^{+/-}$ mice

	$GR^{+/+}$	$GR^{+/-}$	Percent change[1]	p value
Caudate putamen, frontal	13.8 ± 1.1	14.6 ± 0.7	6.4	0.500
Caudate putamen, caudal	41.0 ± 1.1	45.5 ± 1.5	10.9	0.036*
Hippocampus, dorsal	15.7 ± 1.0	18.4 ± 0.7	17.0	0.052
Hippocampus, ventral	28.0 ± 0.9[3]	29.8 ± 1.4[2]	6.3	0.303
Hypothalamus	24.3 ± 0.9	26.3 ± 0.8	8.1	0.115
Lateral globus pallidus	40.2 ± 0.7	42.1 ± 1.2	4.9	0.180
Lateral septum	22.3 ± 1.3	23.7 ± 0.7	6.5	0.353
Medial amygdala	21.6 ± 0.8	22.9 ± 0.9	6.0	0.305
N. accumbens shell	33.4 ± 2.1[2]	38.4 ± 1.1[2]	14.8	0.070
Olfactory tubercles	40.9 ± 1.1	44.8 ± 1.2	9.6	0.032*

Values are mean ± SEM in fmol/mg tissue. [1] $GR^{+/-}$ compared to $GR^{+/+}$. [2] n = 5, [3] n = 4. *$p < 0.05$.

Table 4. 5-HT transporter binding in $GR^{+/-}$ mice

	$GR^{+/+}$	$GR^{+/-}$	Percent change[1]	p value
Caudate putamen, frontal	35.7 ± 0.8	33.6 ± 0.5	-6.0	0.038*
Hippocampus, dorsal	39.9 ± 0.7	39.3 ± 0.6	-1.5	0.548
Hypothalamus	65.2 ± 0.8	64.1 ± 0.7	-1.7	0.321
Lateral globus pallidus	57.1 ± 1.1	55.8 ± 0.9	-2.3	0.853
Lateral septum	57.1 ± 1.1	55.8 ± 0.9	-2.3	0.394
Basolateral amygdala	63.3 ± 1.1	60.9 ± 1.2	-3.9	0.158
N. accumbens shell	47.8 ± 2.7[2]	44.5 ± 1.3	-6.9	0.131
Olfactory tubercles	61.3 ± 2.5	61.1 ± 2.6	-0.3	0.955

Values are mean ± SEM in fmol/mg tissue. [1] $GR^{+/-}$ compared to $GR^{+/+}$. [2] n = 5. *$p < 0.05$.

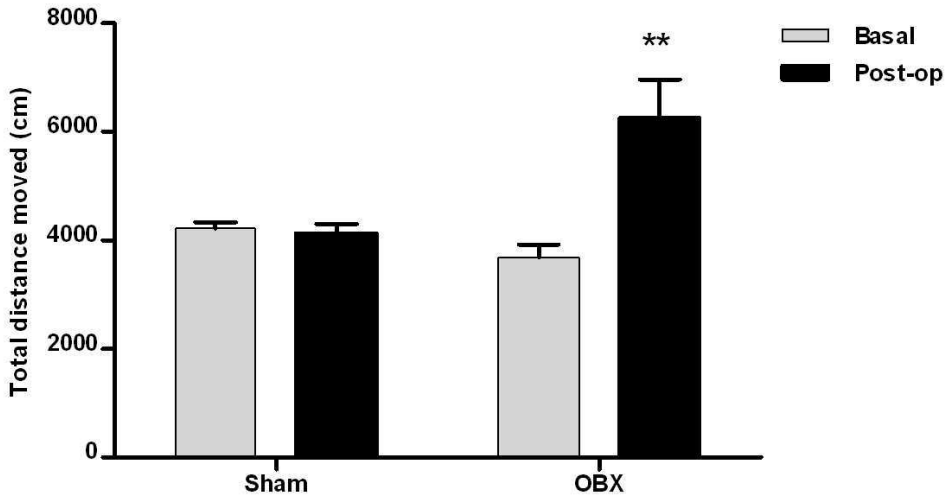

Figure 1. Open field test of olfactory bulbectomized mice
49x28mm (600 x 600 DPI)

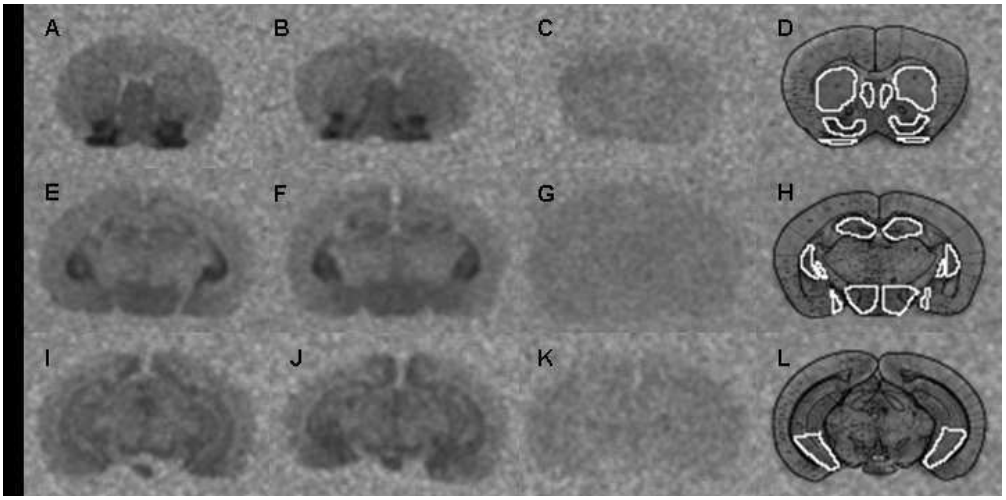

Figure 2. 5-HT_4 receptor binding after olfactory bulbectomy
121x59mm (150 x 150 DPI)

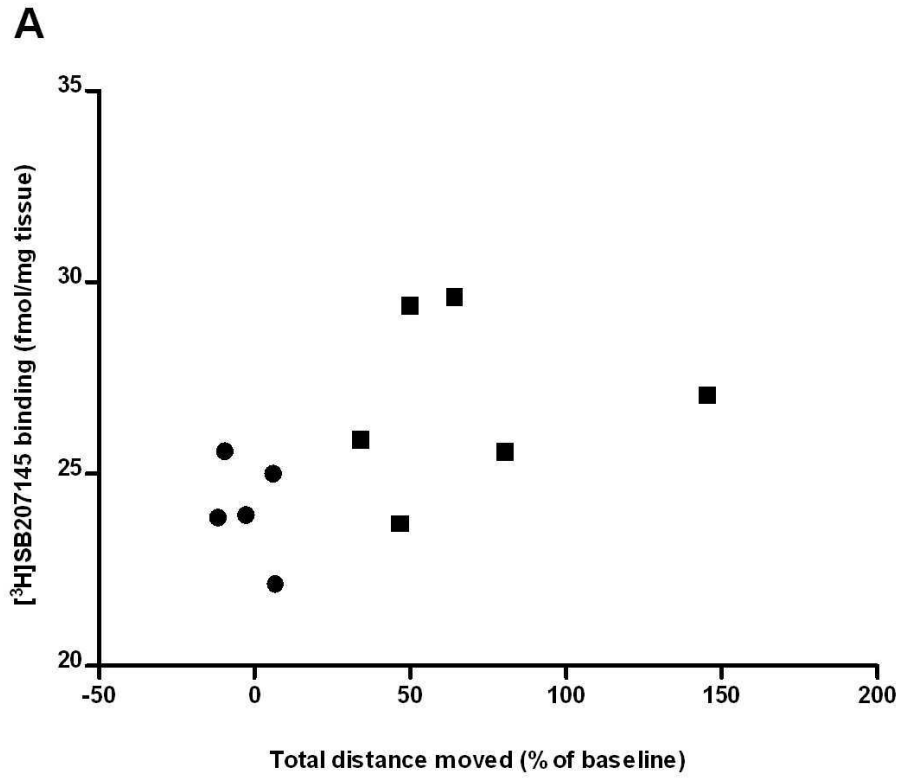

Figure 3A. Relationship between 5-HT_4 receptor binding in the ventral hippocampus and activity in the open field test of after olfactory bulbectomy
49x41mm (600 x 600 DPI)

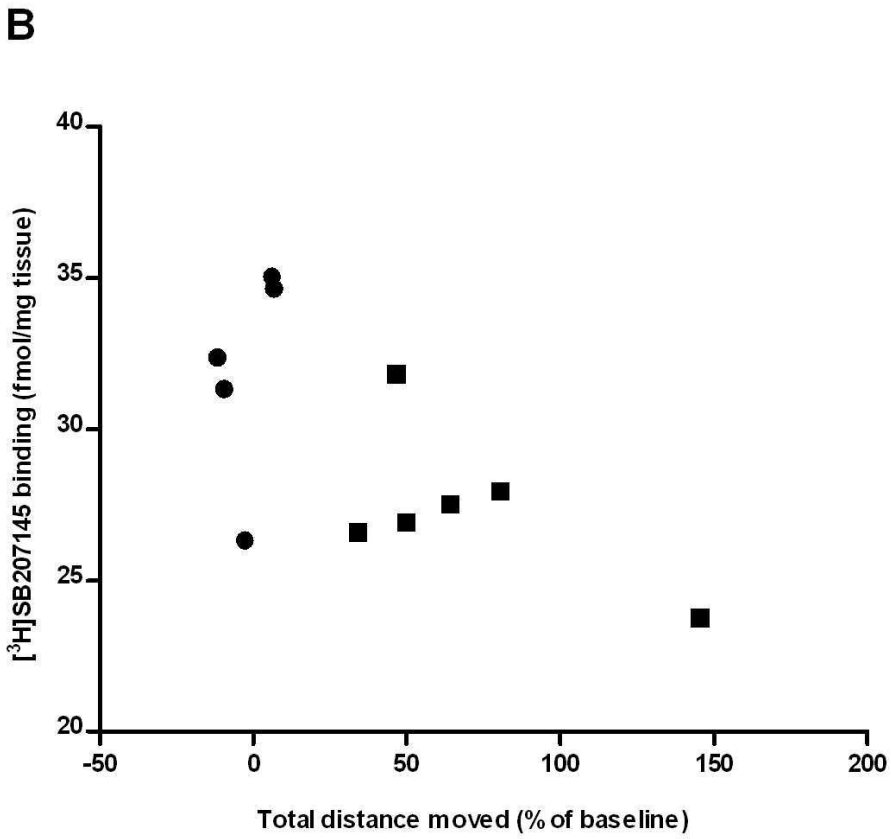

Figure 3B. Relationship between 5-HT_4 receptor binding in the olfactory tubercles and activity in the open field test after olfactory bulbectomy
49x44mm (600 x 600 DPI)

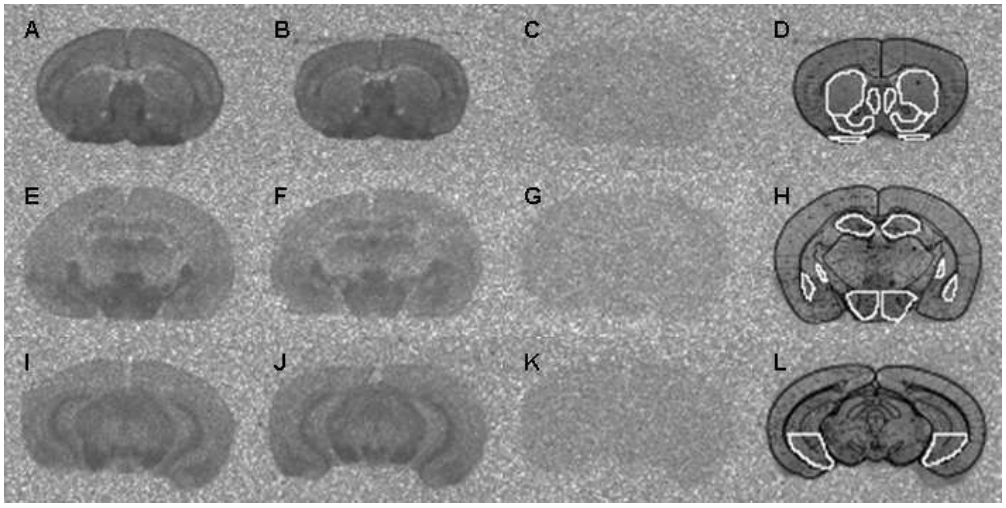

Figure 4. 5-HT transporter binding after olfactory bulbectomy
119x59mm (150 x 150 DPI)

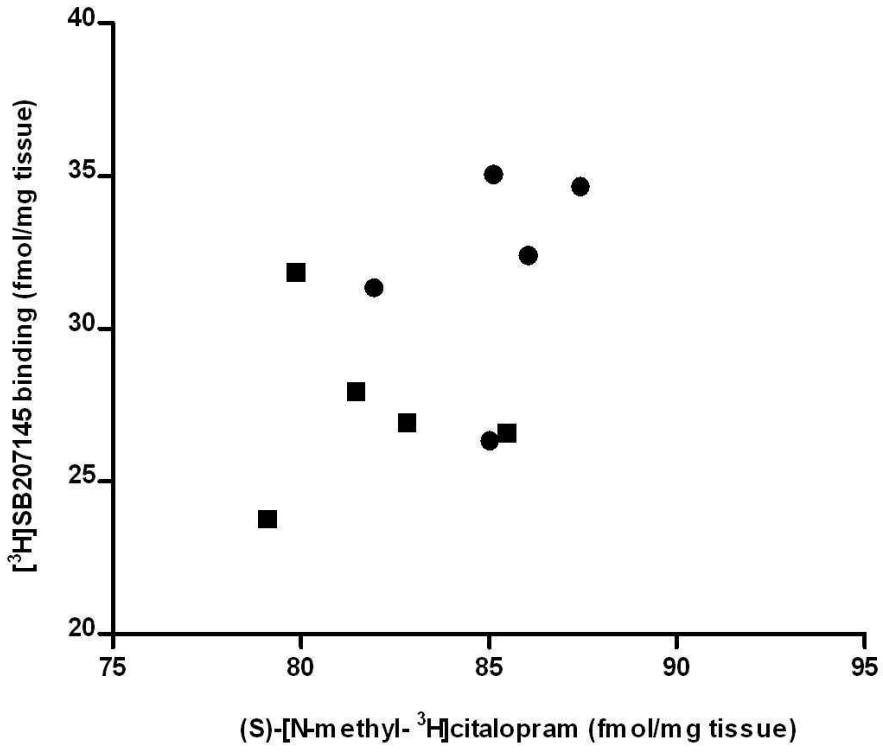

Figure 5. Relationship between 5-HT_4 receptor and 5-HT transporter binding in the olfactory tubercles after olfactory bulbectomy
49x42mm (600 x 600 DPI)

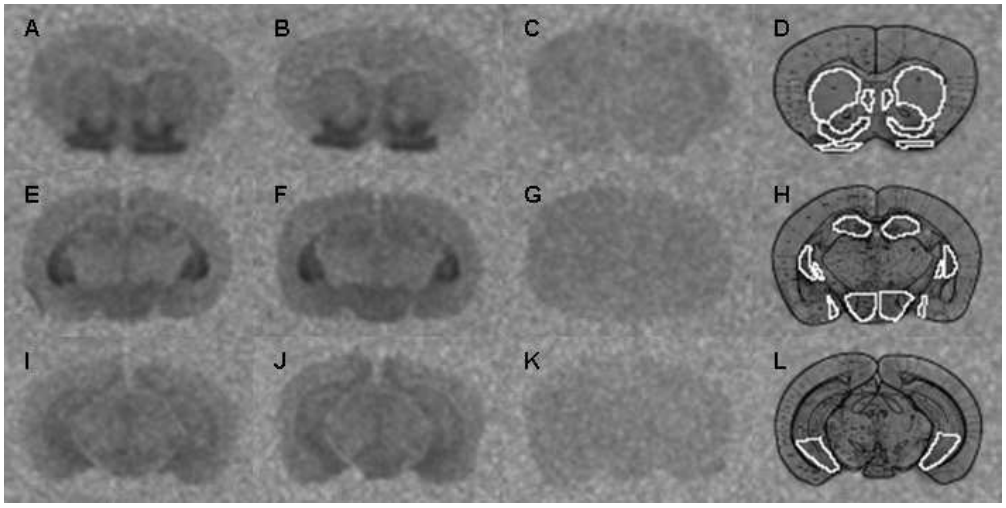

Figure 6. 5-HT_4 receptor binding in GR heterozygous mice
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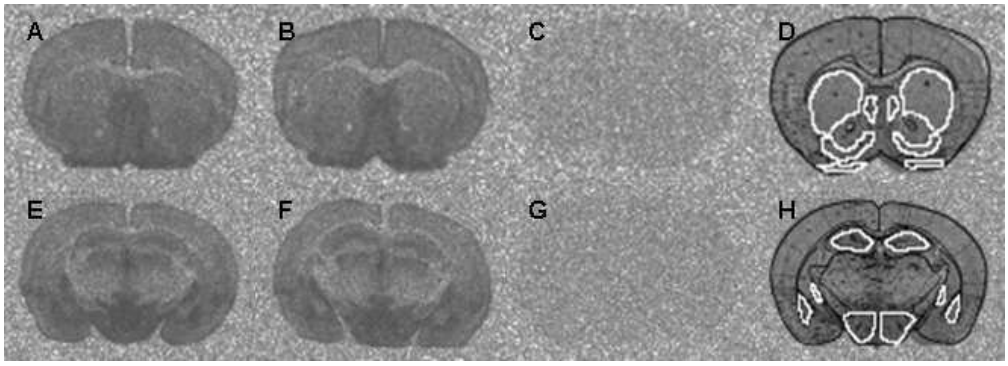

Figure 7. 5-HT transporter binding in GR heterozygous mice
111x39mm (150 x 150 DPI)

Manuscript II

Changes in brain 5-HT$_4$ receptor binding in rat depression models and in response to paroxetine administration

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Abbreviations

5,7-DHT, 5,7-dihydroxytryptamine; 5-HT, 5-hydroxytryptamine (serotonin); 5-HTT, 5-HT transporter; AUC, area under the curve; BLA, basolateral amygdala; CMS, Chronic Mild Stress; DRN, dorsal raphe nucleus; FSL, Flinders Sensitive Line; FST, forced swim test; FRL, Flinders Resistant Line; LGP, lateral globus pallidus; pCPA, para-chlorophenylalanine; ROI, region of interest; SD, Sprague-Dawley; SSRI, selective serotonin reuptake inhibitor.

ABSTRACT

The 5-HT_4 receptor may be implicated in depression and is a new target for antidepressant treatment. We have investigated the brain 5-HT_4 receptor [^{3}H]SB207145 binding in the Flinders Sensitive Line and the Chronic Mild Stress rat depression models by quantitative receptor autoradiography, and related this to 5-HT transporter (S)-[N-methyl-^{3}H]citalopram binding. We also determined the regulation of 5-HT_4 receptor binding by 1, 14 and 21 days of paroxetine administration and sub-chronic 5-HT depletion, and compared this to changes in 5-HT_{2A} receptor [^{3}H]MDL100907 binding. In the Flinders Sensitive Line, the 5-HT_4 receptor and 5-HT transporter binding were decreased in the dorsal and ventral hippocampus. In the Chronic Mild Stress model, the 5-HT transporter binding in the caudate putamen was inversely correlated with sucrose intake during stress. Chronic but not acute paroxetine administration caused a global 16-47% downregulation of 5-HT_4 receptor binding, while 5-HT depletion increased the 5-HT_4 receptor binding in the dorsal hippocampus, hypothalamus, and lateral globus pallidus. In comparison, the 5-HT_{2A} receptor binding was decreased in the frontal and cingulate cortex after chronic paroxetine administration, and markedly reduced in several regions after 5-HT depletion. Thus, the 5-HT_4 receptor binding is decreased in the Flinders Sensitive Line and after chronic paroxetine administration.

Keywords: 5-HT_4 receptor, Flinders Sensitive Line, Chronic Mild Stress, selective serotonin reuptake inhibitor, 5-HT transporter, 5-HT_{2A} receptor

Running title: 5-HT_4 receptor in depression and after paroxetine

INTRODUCTION

The serotonin (5-hydroxytryptamine, 5-HT) system is a focus of research in depression and antidepressant treatment, as most antidepressant drugs used today block synaptic reuptake of 5-HT by the 5-HT transporter (5-HTT) (Berton and Nestler 2006). Apart from the 5-HTT, the 5-HT_1 and 5-HT_2 receptors have been the most extensively studied 5-HT system factors in mood disorders (Mossner et al. 2007). However, the 5-HT_4 receptor may also be implicated in depression and is a new target for antidepressant treatment.

The 5-HT_4 receptor was originally identified as the mediator of 5-HT stimulated increase in adenylate cyclase activity in mouse colliculi neurons (Dumuis et al. 1988). Activation of the 5-HT_4 receptor inhibits K^+ currents in colliculi neurons, resulting in increased neuronal excitability (Fagni et al. 1992). In rodents as in humans, the 5-HT_4 receptor is expressed mainly in basal ganglia and subcortical nuclei pertaining to the limbic system including the hippocampus and amygdala, while the cortical expression is low (Jakeman et al. 1994;Waeber et al. 1994). The 5-HT_4 receptor has been identified as a constitutive positive regulator of 5-HT neuron firing rate in the dorsal raphe nucleus (DRN), and also has phasic stimulatory effects, increasing the firing rate of 50% of the neurons (Lucas and Debonnel 2002;Lucas et al. 2005). Two studies have looked into the 5-HT_4 receptor in clinical depression, reporting increased 5-HT_4 receptor level in the caudate nucleus and frontal cortex of depressed suicide victims (Rosel et al. 2004), and association of two single nucleotide polymorphisms of the 5-HT_4 receptor gene with unipolar depression (Ohtsuki et al. 2002). It has recently been reported that activation of the 5-HT_4 receptor in rodents has an antidepressant potential comparable to or stronger than the selective serotonin reuptake inhibitor (SSRI) citalopram, and with a faster onset of action (Lucas et al. 2007). The 5-HT_4 receptor is regulated by 5-HT

system changes, as chronic antidepressant treatment attenuates the stimulatory effect of 5-HT_4 receptor agonism on hippocampal neuron excitability (Bijak 1997;Bijak et al. 1997;Zahorodna et al. 2002), while chronic 5,7-dihydroxytryptamine (5,7-DHT) mediated 5-HT depletion strongly upregulates 5-HT_4 receptor binding in the hippocampus and basal ganglia (Compan et al. 1996). The 5-HT_{2A} receptor is interesting in comparison to the 5-HT_4 receptor, being a well-described postsynaptic 5-HT receptor with opposite effects on 5-HT neuron firing rate in the DRN (Sharp et al. 2007).

Among the available rat models of depression, the Flinders Sensitive Line (FSL) and the Chronic Mild Stress (CMS) model show good face, construct, and predictive validity (for reviews see (Overstreet et al. 2005) and (Willner 2005)). The FSL model was originally developed by selective breading for increased cholinergic sensitivity but was later found to display increased immobility in the forced swim test (FST), a depression related behavior, and increased 5-HT_{1A} receptor sensitivity compared to the Flinders Resistant Line (FRL) rats (Overstreet et al. 1994). The increase in immobility in the FST is correlated with increased 5-HT_{1A} receptor sensitivity but not with cholinergic sensitivity, indicating that the depression-like phenotype of the FSL rats is associated with changes in the 5-HT system (Overstreet et al. 1994). The CMS model is a model of chronic stress-induced depression, induced by sequentially applying a variety of mild, unpredictable, environmental stressors for several weeks (Willner 2005). The CMS model was designed to model anhedonia, a decreased pleasure in normally pleasurable activities, which is a core symptom of depression (Willner et al. 1992). The anhedonic effect of CMS is evaluated as a reduced intake of a dilute sucrose solution, or by related reward paradigms (Willner 1997). While CMS induces anhedonia in approximately 70% of animals, the remaining animals display unchanged or increased sucrose intake, which is interpreted as stress resistance (Bergstrom et al. 2007).

To further investigate the 5-HT_4 receptor in depression, we have analyzed 5-HT_4 receptor [^{3}H]SB207145 binding in the caudate putamen and hippocampus in two rat models of depression, FSL and CMS, and related this to 5-HTT (S)-[N-methyl-^{3}H]citalopram binding as a presynaptic 5-HT system marker. We also analyzed the regulation of 5-HT_4 receptor binding by acute and chronic treatment with the SSRI paroxetine, and by pharmacologically induced 5-HT depletion, and compared this to effects on 5-HT_{2A} receptor [^{3}H]MDL100907 binding. We hypothesized that the 5-HT_4 receptor binding would be decreased after chronic but not acute paroxetine administration, and increased after sub-chronic 5-HT depletion.

EXPERIMENTAL METHODS

Flinders Sensitive Line

A colony of FSL and FRL rats, originally derived from the colony at the University of North Carolina, was maintained at Centre for Psychiatric Research, Denmark (Overstreet et al. 2005). All breeding pairs were screened in the FST prior to breeding. Animals used for autoradiography experiments were not themselves tested in the FST. Male FSL (n = 10) and FRL (n = 10) rats (325-495 g.) were housed two per cage on a 12 hr light/dark cycle with free access to food and water. All animal procedures were accepted by the Danish National Committee for Ethics in Animal Experimentation (2002/561-585). The FST was performed using a transparent cylinder (diameter 24 cm; height 60 cm, filled with 40 cm of water (25 ± 0.5 °C) (Porsolt et al. 1977). On the first of two test days, the rats were placed in the cylinder for 15 min. The following day, the rats were gently placed in the cylinder for 5 min. The

behavior was video-recorded, and the immobility time in seconds was assessed using NOLDUS Ethovision XT.

Chronic Mild Stress protocol

Male Wistar rats (350 g. at CMS start, Taconic, Denmark) were singly housed under a standard 12 hr light/dark cycle, and food and water were freely available. All procedures involving animals were accepted by the Danish National Committee for Ethics in Animal Experimentation (2002/561-575). The animals were initially trained for 5 weeks to consume a palatable sucrose solution (1.5%). Animals were food and water deprived 14h before the sucrose consumption test, which consisted of a 1h exposure to a bottle of sucrose solution. During the training period the sucrose test was made twice weekly during the first 3 weeks, and once weekly during the last 2 weeks. During the stress period the sucrose consumption test was performed one time per week. On the basis of sucrose intake in three final baseline tests, animals were divided into two matched groups and placed in separate rooms. One group (n = 16) was exposed to 4 weeks of chronic mild stressors, while the other group (n = 8) was left undisturbed. The stress protocol was optimized in our lab (Jayatissa et al. 2006), and consisted of one period (10-14 hours) of intermittent illumination, stroboscopic light, grouping (alternating resident or intruder), or food or water deprivation; two periods of soiled cage and no stress; and three periods of 45^{o} box tilting. Total sucrose intake during CMS was determined for all animals as the area under the curve (AUC).

Drug administration

Male Sprague-Dawley rats (250-300g; Charles River, Germany) were housed in pairs on a 12 hr light/dark cycle with food and water freely available. For paroxetine administration the animals were divided into a paroxetine (n = 24) and a vehicle (dH_2O, n = 24) group, and subdivided into 3 groups that were treated for 1, 14, and 21 days, respectively. Animals received orally administered paroxetine (10 mg/kg, free base) or vehicle once daily. Animals were decapitated 24 hours after the last drug administration, and the brains rapidly removed, frozen on dry ice, and stored at -80 °C. The 5-HT depletion protocol closely followed Kornum et al (Kornum et al. 2006): Animals were injected with para-chlorophenylalanine (pCPA, 200 mg/kg i.p.) once daily for 3 consecutive days followed by a single fenfluramine (20 mg/kg s.c.) injection on day 4. Animals in the saline group (n = 10) were housed in pairs, while 5-HT depleted animals (n = 10) were housed individually to avoid aggressive behavior. Approximately 24 hours after the last injection, the animals were decapitated and the brains removed, frozen on dry ice, and stored at -80 °C. All animal procedures were in accordance with the European Communities Council Resolves of 24th November 1986 (86/609/ECC) and approved by the Danish State Research Inspectorate (J. No 2002/561-527).

Tissue preparation

The brains were sectioned on a cryostat in 10 μm coronal sections at -25 °C, thaw-mounted onto gelatinized glass slides, allowed to dry, and stored at -80 °C. Sections were taken between Bregma 5.64 and 4.20 mm; 1.56 and 1.32 mm; -0.24 and -0.72 mm; -2.52 and -2.92 mm; -5.28 and -5.52 mm; -5.88 and -6.12 mm (Paxinos and Watson 2005). Tissue sections were collected in 4 parallel series with three sections on each glass slide.

5-HT_{2A} and 5-HT_4 receptor autoradiography

The protocol for [^{3}H]SB207145 autoradiography was adapted from Parker *et al*., 2003 (Parker et al. 2003). Frozen tissue sections were brought to room temperature (RT) and allowed to dry prior to experimentation. For [^{3}H]SB207145 and [^{3}H]MDL100907 autoradiography sections were preincubated at RT for 15 or 30 min, respectively, in 50 mM Tris-HCl, pH 7.4, containing 0.01% ascorbic acid and 10 μM pargyline. Sections were incubated in the same buffer containing either 1 nM [^{3}H]SB207145 (1-5 times K_d) or 0.4 nM [^{3}H]MDL100907 (1-2 times K_d, (Kristiansen et al. 2005)) for 60 min. Sections used for non-specific binding were preincubated and incubated in the presence of 10 μM RS39604 (5-HT_4 antagonist) or 10 μM ketanserin (5-HT_{2A} antagonist). Sections were washed for 2 x 20 seconds in ice-cold 50 mM Tris-HCl followed by 20 seconds in ice-cold dH_2O. Slides were dried at RT in a gentle air stream for 1 hour. They were then fixed in paraformaldehyde vapor overnight at 4 °C. The sections were dried for 3 hours in a dessicator at RT, followed by exposure to a tritium-sensitive BAS TR2040 phosphor imaging plate (Fuji, Science Imaging Scandinavia AB) along with four autoradiographic [^{3}H]microscales (8 and 80 nCi, Amersham Biosciences) for 2 weeks at 4 °C. The imaging plate was scanned on a BAS-2500 bioimaging analyzer (Fuji Film Photo Co. LTD., Japan). Sections from each model and its respective control group were for each section level processed and exposed to imaging plates together.

5-HT transporter autoradiography

The protocol for (S)-[N-methyl-^{3}H]citalopram autoradiography was adapted from Thomsen *et al*., 2003 (Thomsen and Helboe 2003). The general procedure was as described for 5-HT_4 receptor autoradiography with the following changes: preincubation was 20 min in 50 mM Tris-HCl with 120 mM NaCl and 5 mM KCl, pH 7.4; and incubation was in the same buffer

with 2 nM (S)-[N-methyl-^{3}H]citalopram for 60 min. Non-specific binding was determined in the presence of 10 μM paroxetine. Sections were washed for 3 x 2 min in buffer, followed by a quick dip in ice-cold distilled water. Sections were exposed for 7 days to tritium-sensitive BAS TR2040 phosphor imaging plates.

Materials

$_{D,L}$-fenfluramine HCl (Sigma, Denmark), ketanserin tartrate (Tocris Cookson Ltd, UK), [^{3}H]MDL100907 (64 Ci/mmol, donated by C. Halldin, Karolinska Institutet, Sweden), paroxetine HCl (donated by GlaxoSmithKline, UK), *p*-chlorophenylalanine methyl ester HCl (pCPA, Sigma), RS39604 HCl (Tocris Cookson Ltd, UK), [^{3}H]SB207145 (66.7 Ci/mmol, donated by GlaxoSmithKline, UK), (S)-[N-methyl-^{3}H]citalopram (79.0 Ci/mmol, donated by H. Lundbeck A/S, Copenhagen, Denmark).

Image analysis of autoradiograms

Autoradiograms were analysed with ImageJ V.1.32j (htttp://rsb.info.nih.gov/ij/). Image optical density was converted to activity density in nCi/mg tissue equivalent (referred to as tissue) using the linear range of [^{3}H]microscale standards. Regions of interest (ROI) were defined by aligning representative brain section autoradiograms with anatomical line drawings from the digital versions of The Rat Brain in Stereotaxic Coordinates, Fifth Edition by Paxinos and Watson (2005). The alignment was performed with Adobe Illustrator CS2 (v12.0.0). The ROIs were drawn on the atlas-overlayed autoradiograms in ImageJ, and were then used to measure radioactive density on all sections from the same section level. For the [^{3}H]MDL100907 autoradiograms ROIs were drawn free-hand onto each section with

reference to the Rat Brain Atlas (Paxinos and Watson 2005). The activity density in nCi/mg tissue was converted to radioligand binding in fmol/mg tissue using the specific activity. The specific receptor binding was determined by subtracting the non-specific binding from the total radioactivity. The dorsal hippocampus was defined as 50% of the hippocampal volume starting at the septal pole, and the ventral hippocampus as the 50% starting at the temporal pole (Bannerman et al. 2004). The dorsal hippocampus was evaluated on sections taken between -2.52 and -2.92 mm from Bregma, and the ventral hippocampus between -5.28 and -6.12 mm from Bregma. Since there is a fronto-caudal gradient in 5-HT_4 receptor binding in the caudate putamen in rats (Vilaro et al. 2005), the 5-HT_4 receptor binding was determined separately for the frontal, medial, and caudal caudate putamen on sections taken between 1.56 and 1.32 mm, -0.24 and -0.72 mm, and -2.52 and -2.92 mm from Bregma, respectively.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software Inc.). The FST was analyzed by one-way ANOVA followed by a Tukey post-test. Correlations between AUC of sucrose intake and regional binding were analyzed by Pearson's correlation analysis, except in cases of non-normality where Spearman's correlation was used. The brain regional changes were analyzed by Student's *t*-test. For each depression model a post-hoc analysis of brain regions other than the caudate putamen and hippocampus was performed. The regional binding data was not corrected for multiple comparisons. Outliers were detected by Grubb's test. The level of significance was set at $p < 0.05$.

RESULTS

Flinders Line

FSL rats from our breeding colony displayed increased immobility in the FST compared to FRL rats (76%, $p < 0.001$) as well as to outbred Sprague-Dawley rats (37%, $p < 0.001$) (Fig. 1). Also, FRL rats were less immobile than the outbred Sprague-Dawley rats (22%, $p < 0.05$) (Fig. 1). Among the four hypothesis regions analyzed, the [^{3}H]SB207145 binding in the FSL was decreased in the dorsal (23%, $p = 0.014$) and ventral hippocampus (11%, $p = 0.045$) but unchanged in the medial and caudal caudate putamen compared to the FRL (Table 1). Post-hoc analysis of the hypothalamus and lateral globus pallidus (LGP) showed a significant downregulation of the [^{3}H]SB207145 binding in the LGP (7%, $p = 0.027$) of FSL compared to FRL (Table 1). In the dorsal and ventral hippocampus, the (S)-[N-methyl-^{3}H]citalopram binding was 12% ($p = 0.0001$) and 10% ($p = 0.020$) lower in the FSL than in the FRL (Table 2). By contrast, post-hoc analysis showed a 12% ($p < 0.0001$) larger (S)-[N-methyl-^{3}H]citalopram binding in the basolateral amygdala (BLA) of FSL rats (Table 2). We found no difference in (S)-[N-methyl-^{3}H]citalopram binding neither in the medial and caudal caudate putamen, nor in the post-hoc analyzed frontal cortex, hypothalamus, and LGP (Table 2). The [^{3}H]SB207145 and (S)-[N-methyl-^{3}H]citalopram binding showed a statistically significant correlation in the dorsal ($r = 0.48$, $p = 0.034$) but not in the ventral ($r = 0.24$, $p = 0.307$) hippocampus (Fig. 2).

Chronic Mild Stress

During the 4-week CMS period, stressed animals displayed larger increases or decreases in sucrose intake (relative to baseline) than the control animals (Fig. 3A). At baseline, the CMS and control group displayed similar variation in sucrose intake ($p = 0.952$, F test of variances)

but after 2 and 4 weeks ($p = 0.049$ and $p = 0.023$, F test of variances) of stress exposure there was a significant difference between the groups in the variation in sucrose intake (Fig. 3B). Given that there were too few anhedonic animals to subdivide the stressed group into anhedonic and CMS resilient (no decrease in sucrose intake) animals, we determined the total sucrose intake (area under the curve, AUC) during CMS for all animals and correlated this to regional changes in 5-HT_4 receptor and 5-HTT binding. There were no significant correlations between AUC sucrose intake during CMS and [^{3}H]SB207145 binding in the caudate putamen (frontal, medial, and caudal) or the hippocampus (dorsal and ventral) (Table 3). We did not detect any correlations within the post-hoc analyzed regions: hypothalamus, LGP, nucleus accumbens shell, or olfactory tubercles (Table 3). The (S)-[N-methyl-^{3}H]citalopram binding was inversely correlated with AUC sucrose intake during CMS in the frontal caudate putamen ($r = -0.41$, $p = 0.044$) but not in the caudal caudate putamen, hippocampus (dorsal and ventral), or in any of the post-hoc analyzed brain regions (Table 4).

Acute and chronic paroxetine administration

One day of paroxetine administration had no effect on 5-HT_4 receptor [^{3}H]SB207145 binding (Fig. 4, Table 5). However, as shown in Table 5, 14 days of paroxetine administration led to a significant 11-42% decrease in [^{3}H]SB207145 binding in the olfactory tubercles (32%, $p < 0.0001$), nucleus accumbens shell (36%, $p < 0.0001$), ventral pallidum (31%, $p < 0.0001$), LGP (frontal, 39%, $p < 0.0001$; and caudal, 42%, $p < 0.0001$), caudate putamen (medial, 19%, $p = 0.0031$; and caudal, 33%, $p < 0.0001$), hippocampus (dorsal, 24%, $p = 0.0050$; and ventral, 11%, $p = 0.0443$), and hypothalamus (38%, $p < 0.0001$). Among the 11 brain regions measured, only the frontal part of the caudate putamen (17%, $p = 0.0760$) did not show a statistically significant decrease in [^{3}H]SB207145 binding. As shown in Table 5 and Fig. 4,

after 21 days of paroxetine administration, the [^{3}H]SB207145 binding was 16-47% decreased in all measured brain regions, except dorsal hippocampus ($p = 0.068$): olfactory tubercles (32%, $p < 0.0001$), nucleus accumbens shell (34%, $p < 0.0001$), ventral pallidum (35%, $p < 0.0001$), LGP (frontal, 40%, $p < 0.0001$; and caudal, 47%, $p < 0.0001$), caudate putamen (frontal, 16%, $p = 0.0048$; medial, 23%, $p = 0.0031$; and caudal, 37%, $p < 0.0001$), ventral hippocampus (23%, $p = 0.0443$), and hypothalamus (42%, $p < 0.0001$).

One and 14 days of paroxetine treatment had no effect on [^{3}H]MDL100907 binding in frontal cortex, cingulate cortex, frontal caudate putamen, and dentate gyrus (Table 6). However, paroxetine administration for 21 days was associated with a significant decrease in [^{3}H]MDL100907 binding in the frontal cortex (10%, $p = 0.004$) and cingulate cortex (10%, $p = 0.049$) but not in the frontal caudate putamen or dentate gyrus (Table 6).

Sub-chronic 5-HT depletion

Sub-chronic 5-HT depletion induced a significant increase in [^{3}H]SB207145 binding in the dorsal hippocampus (41%, $p = 0.0007$), hypothalamus (22%, $p = 0.0002$), and LGP (13%, $p = 0.014$) (Fig. 5, Table 7), whereas no effect was seen in the ventral hippocampus, ventral pallidum, and caudate putamen (medial and caudal) (Table 7). The [^{3}H]MDL100907 binding was markedly decreased in the frontal cortex (24%, $p < 0.0001$), cingulate cortex (33%, $p < 0.0001$), medial caudate putamen (27%, $p = 0.0015$), and dentate gyrus (63%, $p = 0.0001$) after sub-chronic 5-HT depletion (Fig. 6, Table 8).

DISCUSSION

To the best of our knowledge, this is the first study to investigate 5-HT_4 receptor and 5-HTT binding in the Flinders Line and CMS models of depression, and to analyze the effect of acute and chronic antidepressant treatment on 5-HT_4 receptor binding in the investigated regions. The 5-HT_4 receptor [^{3}H]SB207145 binding density and pattern were comparable to those previously reported for this (Parker et al. 2003) and other 5-HT_4 receptor radioligands in rats (Jakeman et al. 1994).

The immobility times in the FST of FSL, FRL, and SD rats of our colony are within the previously reported range (Porsolt et al. 1977), and the FSL rats show increased immobility, a depression-like behavior (Cryan et al. 2005), compared to both FRL and SD rats. Within the hypothesis regions, the 5-HT_4 receptor binding was decreased in the dorsal and ventral hippocampus of FSL, while no change was detected in the caudate putamen. The FSL rats have higher hippocampal but similar striatal 5-HT levels compared to FRL rats (Zangen 1997). Given our present finding that chronic paroxetine administration downregulates hippocampal 5-HT_4 receptor binding, and is known to increase extracellular 5-HT levels (Owen and Whitton 2005;Hajos-Korcsok et al. 2000), we find it likely that the 5-HT_4 receptor binding is decreased in the FSL hippocampus in response to regionally elevated 5-HT levels. While we did not measure 5-HT levels in the hippocampus of our FSL and FRL animals, we determined 5-HTT binding as a marker for presynaptic serotonergic changes. Similar to the 5-HT_4 receptor binding changes, the 5-HTT binding was decreased in both dorsal and ventral hippocampus with no change in the caudate putamen of FSL rats. Furthermore, the changes in 5-HT_4 receptor and 5-HTT binding were directly correlated in the dorsal hippocampus but not in the ventral hippocampus. This may be due to a larger influence of variability in section level and angle on ventral hippocampal binding measurements. It is unlikely that the decreased 5-HTT binding is a result of degeneration of 5-HT fibers, as there are no

differences in 5-HT cell number in the DRN or in 5-HT fiber length in the dorsal hippocampus between the FSL and FRL rats (Husum et al. 2006). Rather, the decreased 5-HTT binding may be due to decreased expression, increased degradation, or increased internalization of the 5-HTT within serotonergic hippocampal projections. Though it has not been found in all studies, both chronic SSRI administration (Benmansour et al. 1999;Johnson et al. 2008) and 5-HT depletion (Rattray et al. 1996) have been shown to downregulate hippocampal 5-HTT binding, indicating changes in 5-HTT binding in response to changes in serotonergic tonus. Our own findings of decreased hippocampal 5-HT_4 receptor and 5-HTT binding in the FSL together with preliminary reports of increased hippocampal 5-HT_{1A} and 5-HT_2 receptor binding in this strain (Schiller 1991) indicates pronounced serotonergic changes in the hippocampus of the FSL model. The opposite directionality of hippocampal 5-HT_4 and 5-HT_{1A} receptor binding changes observed in these animals corroborates previous observations showing opposite regulation of hippocampal 5-HT_4 and 5-HT_{1A} receptor sensitivity by chronic antidepressant and corticosterone administration (Bijak et al. 2001).

Post-hoc analyses of additional brain regions in the FSL and FRL animals revealed downregulation of 5-HT_4 receptor binding in the LGP and no change in the hypothalamus. The unchanged 5-HT_4 receptor binding in the hypothalamus was unexpected given the previously reported elevated 5-HT levels in this region in FSL rats (Zangen et al. 1997). However, we did not detect differences in hypothalamic 5-HTT binding, suggesting no presynaptic 5-HT system differences in this region either. Whether our observation of a downregulation in 5-HT_4 receptor binding in the LGP in FSL rats can be explained by differences in serotonergic tonus in this area is difficult to say. While we in the present study have found that both 5-HT depletion and chronic paroxetine administration is capable of regulating 5-HT_4 receptor binding in the LGP in opposite directions, no study has analyzed 5-

HT levels in the LGP of FSL rats. We did not detect changes in 5-HTT binding in the LGP in the FSL animals, suggesting that the observed 5-HT_4 receptor changes in this region could be due to other factors than 5-HT system changes. Chronic dopamine depletion upregulates 5-HT_4 receptor binding in the LGP (Compan et al. 1996), and conversely, one might expect an increase in dopamine to downregulate pallidal 5-HT_4 receptor binding. The FSL have increased dopamine tissue levels in the caudate putamen (Zangen et al. 1999), which may affect the 5-HT_4 receptors in the LGP, as these receptors are expressed on GABA neurons projecting from the caudate putamen to the globus pallidus (Compan et al. 1996). Post-hoc analysis also revealed an increase in 5-HTT binding in the BLA of FSL rats. This is interesting as in humans 5-HTT binding in the amygdala is inversely correlated with amygdala activation in response to negative emotional faces, thereby linking increased 5-HTT binding with decreased amygdala excitability (Rhodes et al. 2007). As a parallel to these clinical observations, our finding of increased 5-HTT binding in the amygdala of FSL rats is in accordance with the FSL central amygdala being less responsive to acute stress (Zambello et al. 2008). The pattern of decreased hippocampal and increased amygdaloid 5-HTT binding in the FSL rats is similar to 5-HT_{2A} receptor expression changes in a reciprocal manner, with FSL rats having increased hippocampal and decreased (medial anterodorsal) amygdaloid 5-HT_{2A} receptor mRNA levels compared to FRL rats (Osterlund et al. 1999).

Apart from analyzing 5-HT_4 receptor binding in a congenital depression model, the FSL strain, we also examined a model of environmentally induced depression, the CMS depression model. In some laboratories, CMS induces both a decrease (anhedonia) as well as no change or an increase (CMS resilience) in sucrose intake (Strekalova et al. 2004;Bergstrom et al. 2007;Bergstrom et al. 2008;Bisgaard et al. 2007). The CMS induced difference in reward sensitivity is also evident in the conditioned place preference test (Bergstrom et al. 2008), and

is reflected in differential regulation of 156 genes in the hippocampus (Bergstrom et al. 2007). In the present study, CMS as expected induced both an increase and a decrease in sucrose intake, leading to increased variation in sucrose intake in the CMS group. Given that there were relatively few anhedonic animals in our cohort, we decided to relate the 5-HT_4 receptor and 5-HTT binding to accumulated sucrose intake during the entire CMS period. However, the present results should be treated with caution due to the induction of anhedonia in a limited number of animals. The 5-HT_4 receptor binding in the caudate putamen and hippocampus did not correlate with the total sucrose intake during CMS. However, the 5-HTT binding in the frontal caudate putamen was inversely correlated with total sucrose intake, associating increased 5-HTT binding with the anhedonic response to CMS. This is in agreement with the 5-HTT binding being increased in the striatum of depressed subjects in some studies (Cannon et al. 2007). The absence of a change in hippocampal 5-HT_4 receptor and 5-HTT binding after CMS is in accordance with anhedonic and CMS resilient animals not differing in hippocampal expression of genes related to the 5-HT system (Bergstrom et al. 2007).

In order to understand the mechanisms behind the 5-HT_4 receptor changes observed in the FSL depression model, we investigated regulation of 5-HT_4 receptor binding in response to alterations in cerebral 5-HT levels. For this normal rats were subjected to different treatment paradigms: acute and chronic paroxetine administration, and sub-chronic 5-HT depletion. While there was no effect of acute (1 day) paroxetine administration, a global and substantial downregulation of 5-HT_4 receptor binding was present after 14 and 21 days of treatment. Only one study has previously investigated the effect of chronic antidepressant treatment on 5-HT_4 receptor binding and reported no effect of citalopram on the 5-HT_4 receptor binding in the substantia nigra, which was the only region investigated (Gobbi et al. 1997). We did not

quantify 5-HT_4 receptor binding in the substantia nigra pars lateralis, as the small area of this region made it difficult to evaluate with the resolution of our method. We can exclude that the effect of chronic paroxetine administration on 5-HT_4 receptor binding is due to a direct effect of paroxetine or its metabolites on the receptor, as paroxetine does not bind to central 5-HT_4 receptors (Lucchelli et al. 1995), and only has polar metabolites which do not modify its pharmacological profile (Haddock et al. 1989). Further, we used a 24 hour drug washout period in our study, after which the inhibitory effect of paroxetine on 5-HT uptake is no longer present (Thomas et al. 1987). The absence of change in 5-HT_4 receptor binding after 1 day of paroxetine administration shows that the changes in 5-HT_4 receptor binding after 14 and 21 days are not due to the presence of paroxetine in the tissue. Our findings are in accordance with hippocampal slice electrophysiological studies, showing a decrease in 5-HT_4 receptor sensitivity after chronic (14 days) but not acute treatment with paroxetine (Bijak et al. 1997). The same group found a similar decrease in sensitivity after chronic administration of other antidepressant drugs (imipramine, citalopram, fluvoxamine) and after repeated electroconvulsive shock, indicating that this is a general effect of antidepressant treatment (Bijak 1997;Bijak et al. 2001;Bijak et al. 1997). Among available antidepressant drugs, paroxetine is the most potent inhibitor of 5-HT reuptake (Owens et al. 2001). Acute administration of paroxetine increases extracellular 5-HT levels in the hippocampus (Hajos-Korcsok et al. 2000) but not in the frontal cortex (Owen and Whitton 2005;Beyer et al. 2002). However, after 14 days of paroxetine administration the extracellular 5-HT baseline is increased in both the hippocampus (Hajos-Korcsok et al. 2000) and the frontal cortex (Owen and Whitton 2005). While paroxetine by acute blockade of the 5-HTT increases synaptic 5-HT in certain regions, chronic administration strongly downregulates 5-HTT binding globally, leading to a larger decline in 5-HT clearance from the synapse (Benmansour et al. 1999). Also, when administered for 3-21 days, paroxetine progressively desensitizes somatodendritic

5-HT_{1A} receptors without affecting 5-HT_{1A} receptor binding, leading to diminished negative feedback control of 5-HT neuronal activity in the raphe nuclei (Le Poul et al. 1995). We suggest that the present finding of decreased 5-HT_4 receptor binding after 14 and 21 days of paroxetine administration is due to an increase in serotonergic activity and/or extracellular 5-HT levels. The decreased 5-HT_4 receptor binding could reflect decreased expression of the 5-HT_4 receptor, or theoretically, a substantial decrease in receptor affinity. However, we will argue that the decrease in binding is due to increased agonist-induced internalization and degradation of the 5-HT_4 receptor, as prolonged 5-HT exposure decreases 5-HT_4 receptor binding in colliculi neurons (Ansanay et al. 1996) through endocytosis of plasma membrane receptors (Barthet et al. 2005). Given that 5-HT_4 receptor agonism increases the 5-HT neuron firing rate in the DRN, downregulation of the 5-HT_4 receptor level in response to increased serotonergic tonus may be interpreted as a compensatory downregulation of a positive regulator of 5-HT system function. While acute administration of 5-10 mg/kg paroxetine does not affect extracellular noradrenalin levels in the frontal cortex or hippocampus (Beyer et al. 2002;Hajos-Korcsok et al. 2000), hippocampal noradrenalin levels are increased after chronic (14 days) paroxetine administration (Hajos-Korcsok et al. 2000). From the present data it is not possible to determine if the increased noradrenalin levels contribute to the observed decrease in 5-HT_4 receptor binding after chronic paroxetine administration.

Given that paroxetine strongly affects 5-HT_4 receptor binding with a time-course similar to the clinical effect of paroxetine, the present data implicate the 5-HT_4 receptor in the effects of antidepressant treatment. While acute 5-HT_4 receptor antagonism does not inhibit the antidepressant-like effect of fluoxetine in the forced swim test (Cryan and Lucki 2000), a recent study has provided convincing experimental data of a fast-acting antidepressant effect of partial 5-HT_4 receptor agonists (Lucas et al. 2007). The antidepressant effect of 5-HT_4

receptor partial agonists (RS67333 and prucalopride) may relate to their ability to stimulate 5-HT neuron firing rate both after acute and chronic administration (Lucas et al. 2005). The absence of desensitization of this effect in response to chronic partial agonist administration is surprising given the present finding of chronic paroxetine induced downregulation of 5-HT_4 receptor binding. However, 3 days of RS67333 administration (1.5 mg/kg i.p.) has no effect on 5-HT_4 receptor binding in several brain regions (unpublished observations). The lack of desensitization after chronic RS67333 or prucalopride administration may be due to the partial agonist profile of these compounds (Duman 2007). To investigate whether the 5-HT_4 receptor changes are characteristic for this receptor, we compared the 5-HT system regulation of 5-HT_4 receptor binding to that of an extensively described 5-HT receptor, the 5-HT_{2A} receptor. In agreement with previous studies using [^{3}H]ketanserin (Maj et al. 1996;Nelson et al. 1989), we found a decrease in cortical 5-HT_{2A} receptor binding after chronic (21 days) but not acute paroxetine administration. This is also in accordance with the clinical observation that chronic paroxetine treatment downregulates cortical 5-HT_{2A} receptor binding, as measured with [^{18}F]setoperone, in depressed patients (Meyer et al. 2001). The decrease in 5-HT_{2A} receptor binding is not due to the presence of paroxetine, as paroxetine does not interact with the 5-HT_{2A} receptor (Thomas et al. 1987), or to paroxetine metabolites (see above). Whereas chronic paroxetine administration was associated with a profound and global reduction in the 5-HT_4 receptor binding, the effect on the 5-HT_{2A} receptor was smaller and confined to frontal and cingulate cortices.

The chronic 5-HT depletion paradigm in our study induced a moderate increase in 5-HT_4 receptor binding in the dorsal hippocampus, hypothalamus, and LGP. As previously published, our 4 day 5-HT depletion paradigm results in a 95% reduction in brain 5-HT on day 5 (Kornum et al. 2006). Our results support the previous report by Compan et al. of

increased 5-HT_4 receptor binding in the rostral caudate putamen, nucleus accumbens, substantia nigra, globus pallidus, and hippocampus 21 days after 5,7-DHT lesions of the raphe nuclei (Compan et al. 1996), and shows that this effect is also present after sub-chronic pharmacological 5-HT depletion. The hippocampal 5-HT_4 receptor binding was selectively increased in the dorsal part after 5-HT depletion, which is similar to the pattern of change in 5-HT_1 receptor binding after 5,7-DHT induced depletion, where binding is decreased in the anterior (dorsal) but not the posterior (ventral) hippocampus (Fischette et al. 1987). By contrast, the 5-HT_{2A} receptor binding was globally and markedly decreased after sub-chronic 5-HT depletion. While this is the first report to use [^{3}H]MDL100907 binding to evaluate 5-HT_{2A} receptor binding after 5-HT depletion, it has been reported that 5,7-DHT induced 5-HT depletion causes a 16-26% decrease in 5-$HT_{2A/2C}$ receptor [^{125}I]DOI binding in the cingulate, frontal, and parietal cortex (Compan et al. 1998b) but an increase in striatal [^{125}I]DOI binding, ascribed to 5-HT_{2C} receptors (Compan et al. 1998a). Also, chronic 5-HT depletion induced by chronic, high dose MDMA exposure causes dramatic decreases in cortical, striatal, thalamic, and hypothalamic [^{125}I]DOI binding (McGregor et al. 2003). However, 5-HT depletion by 5 days of pCPA administration has no effect on [^{125}I]DOI binding in the cingulate, frontal, and parietal cortex (Compan et al. 1998b), and other studies report an increase (Heal et al. 1985) or no change (Fischette et al. 1987) in [^{3}H]ketanserin binding after 5,7-DHT induced 5-HT depletion. The cause of these discrepancies may be the different 5-HT depletion protocols and radioligands used or the time point of receptor binding analysis.

Overall, the 5-HT_4 receptor binding is downregulated by chronic but not acute paroxetine administration, and upregulated after sub-chronic 5-HT depletion. In comparison, the 5-HT_{2A} receptor binding is downregulated by both chronic paroxetine administration and 5-HT depletion. While chronic paroxetine administration has a more widespread and pronounced

suppressing effect on the 5-HT_4 receptor, the effect of sub-chronic 5-HT depletion is most evident on 5-HT_{2A} receptor binding. The decrease in 5-HT_4 receptor binding in the hippocampus of the FSL depression model correlates with decreased 5-HTT binding, and may be a compensatory response to regionally increased 5-HT levels.

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TABLES

Table 1. 5-HT_4 receptor binding in Flinders Line

	FRL	FSL	Percent change[a]	p value
Caudate putamen, medial	19.1 ± 0.6	18.6 ± 0.9	-2.6	0.654
Caudate putamen, caudal	32.2 ± 0.9	31.8 ± 0.8	-1.4	0.733
Hippocampus, dorsal	10.7 ± 0.7	8.3 ± 0.5	-22.6	0.014*
Hippocampus, ventral	20.1 ± 0.6	17.9 ± 0.8	-11.0	0.045*
Hypothalamus	16.8 ± 0.6	17.1 ± 0.6	1.8	0.732
Lateral globus pallidus	33.2 ± 0.6	30.9 ± 0.8	-7.0	0.027*

Values are mean ± SEM in fmol/mg tissue. [a]FSL compared to FRL.

Table 2. 5-HT transporter binding in Flinders Line

	FRL	FSL	Percent change[a]	p value
Caudate putamen, medial	40.5 ± 1.0	39.6 ± 1.2	-2.2	0.587
Caudate putamen, caudal	42.2 ± 1.0	43.9 ± 0.8	4.0	0.207
Hippocampus, dorsal	33.0 ± 0.7	29.0 ± 0.5	-12.1	<0.0001***
Hippocampus, ventral	41.7 ± 1.0	37.5 ± 1.3	-10.2	0.020*
Basolateral amygdala	53.7 ± 0.8	60.1 ± 0.3	12.0	<0.0001***
Frontal cortex	50.6 ± 1.0	50.2 ± 1.0	-0.7	0.823
Hypothalamus	48.7 ± 0.7	48.2 ± 0.7	-1.0	0.614
Lateral globus pallidus	46.9 ± 1.4	48.0 ± 0.8	2.5	0.477

Values are mean ± SEM in fmol/mg tissue. [a]FSL compared to FRL.

Table 3. 5-HT_4 receptor binding after Chronic Mild Stress

	Control	CMS	r value[a]	p value
Caudate putamen, frontal	13.6 ± 0.5	13.3 ± 0.4	-0.21	0.329
Caudate putamen, medial	18.5 ± 1.1	19.1 ± 0.6	-0.36[b]	0.083
Caudate putamen, caudal	25.5 ± 0.6	25.0 ± 0.6	-0.12	0.586
Hippocampus, dorsal	10.6 ± 0.5	10.5 ± 0.5	-0.22	0.310
Hippocampus, ventral	14.6 ± 0.3	13.7 ± 0.5	-0.10[b]	0.651
Hypothalamus	16.6 ± 0.5	16.4 ± 0.5	-0.30	0.154
Lateral globus pallidus	26.1 ± 0.7	25.3 ± 0.8	-0.05	0.824
Nucleus accumbens shell	22.5 ± 0.4	23.1 ± 0.5	-0.27	0.207
Olfactory tubercles	31.1 ± 0.6[c]	30.9 ± 0.5	-0.13	0.563

Values are mean ± SEM in fmol/mg tissue. [a]Pearson's correlation coefficient between 5-HT_4 receptor binding and AUC sucrose intake during stress. [b]Spearman's correlation coefficient. [c]n = 7.

Table 4. 5-HT transporter binding after Chronic Mild Stress

	Control	CMS	r value[a]	p value
Caudate putamen, frontal	40.1 ± 1.2	38.6 ± 0.7	-0.41	0.044*
Caudate putamen, caudal	50.6 ± 1.0	51.0 ± 0.7	-0.16	0.449
Hippocampus, dorsal	37.7 ± 0.9	36.8 ± 0.8	-0.37[b]	0.076
Hippocampus, ventral	53.5 ± 1.7	55.9 ± 1.0	0.24	0.258
Basolateral amygdala	65.6 ± 1.1	64.9 ± 0.6	0.26	0.213
Frontal cortex	61.7 ± 1.9	60.9 ± 1.6	-0.30	0.150
Hypothalamus	54.1 ± 1.3	56.0 ± 0.5	-0.16	0.466
Nucleus accumbens shell	65.4 ± 1.0	67.2 ± 0.8	-0.02	0.915

Values are mean ± SEM in fmol/mg tissue. [a]Pearson's correlation coefficient between 5-HTT binding and AUC sucrose intake during stress. [b]Spearman's correlation coefficient.

Table 5. 5-HT_4 receptor binding after acute and chronic paroxetine administration

	1 day		14 days		21 days	
	Vehicle	Paroxetine	Vehicle	Paroxetine	Vehicle	Paroxetine
Caudate putamen, f	16.6 ± 0.8	17.4 ± 0.3	16.5 ± 1.0	13.8 ± 1.0	17.0 ± 0.6	14.3 ± 0.6**
Caudate putamen, m	20.6 ± 0.6[a]	21.8 ± 0.6	21.2 ± 0.5	17.1 ± 1.0**	22.8 ± 0.5	17.5 ± 0.7***
Caudate putamen, c	31.9 ± 0.8	30.7 ± 1.1	31.5 ± 1.1	21.0 ± 0.9***	34.1 ± 1.0	21.6 ± 1.1***
Hippocampus, dorsal	9.6 ± 0.5	8.1 ± 0.9[a]	9.7 ± 0.6	7.4 ± 0.3**	11.1 ± 0.8	8.4 ± 1.1
Hippocampus, ventral	16.2 ± 0.9[a]	15.4 ± 1.5	11.8 ± 0.6	10.5 ± 0.2*	13.4 ± 0.5	10.4 ± 0.9**
Hypothalamus	18.0 ± 0.6	16.9 ± 0.7	17.7 ± 0.9	11.0 ± 0.6***	19.4 ± 0.7	11.2 ± 0.9***
LGP, frontal	22.1 ± 0.9[a]	22.3 ± 0.7	22.4 ± 0.7	13.8 ± 1.2***	23.2 ± 0.5	13.9 ± 0.9***
LGP, caudal	31.6 ± 1.0	31.2 ± 1.6	29.9 ± 1.4	17.3 ± 1.1***	32.2 ± 1.1	17.1 ± 1.0***
N. accumbens shell	24.7 ± 0.7	25.6 ± 0.8	27.6 ± 1.1	17.6 ± 1.0***	28.1 ± 0.7	18.6 ± 1.0***
Olfactory tubercles	32.9 ± 0.8	33.6 ± 1.1	36.6 ± 0.9	24.8 ± 1.0***	36.6 ± 0.9	24.9 ± 1.1***
Ventral pallidum	25.8 ± 0.9[a]	26.1 ± 0.6	28.2 ± 0.8	19.6 ± 1.1***	28.9 ± 0.8	18.8 ± 1.0***

Values are mean ± SEM in fmol/mg tissue. f: frontal, m: medial, n: nucleus, c: caudal. [a]n = 7. *p < 0.05, **p < 0.01, ***p < 0.0001 for paroxetine versus vehicle.

Table 6. 5-HT_{2A} receptor binding after acute and chronic paroxetine administration

	1 day		14 days		21 days	
	Vehicle	Paroxetine	Vehicle	Paroxetine	Vehicle	Paroxetine
Caudate putamen, frontal	14.4 ± 0.8	13.7 ± 0.7	12.5 ± 0.7	13.4 ± 0.5	11.1 ± 0.7[a]	11.5 ± 0.8
Cingulate cortex	31.7 ± 0.4	30.2 ± 1.4	26.7 ± 0.7	26.8 ± 0.6	26.7 ± 0.7[a]	24.1 ± 1.0*
Dentate gyrus, dorsal	16.9 ± 0.6	15.5 ± 0.4	12.4 ± 1.1	11.3 ± 0.8[a]	17.9 ± 0.7	16.9 ± 0.9
Frontal cortex	40.4 ± 1.0	40.3 ± 1.7	43.3 ± 1.1	42.0 ± 0.8	39.8 ± 0.8	35.5 ± 0.9**

Values are mean ± SEM in fmol/mg tissue. [a]n = 7. *p < 0.05, **p < 0.01 for paroxetine versus vehicle.

Table 7. 5-HT_4 receptor binding after sub-chronic 5-HT depletion

	Saline	pCPA + FEN	Percent change[a]	p value
Caudate putamen, medial	17.6 ± 0.7	17.8 ± 0.7	1.0	0.871
Caudate putamen, caudal	27.4 ± 0.7	28.9 ± 0.6	5.7	0.102
Hippocampus, dorsal	7.8 ± 0.4	11.0 ± 0.7	40.7	0.0007***
Hippocampus, ventral	15.1 ± 0.9[b]	16.4 ± 0.4	8.6	0.163
Hypothalamus	14.8 ± 0.2	18.1 ± 0.6	22.2	0.0002***
Lateral globus pallidus	26.6 ± 0.7	30.0 ± 1.0	12.8	0.014*
Ventral pallidum	26.9 ± 0.6	29.3 ± 1.1	8.8	0.087

Values are mean ± SEM in fmol/mg tissue. [a]pCPA + FEN compared to saline. [b]n = 7.

Table 8. 5-HT_{2A} receptor binding after sub-chronic 5-HT depletion

	Saline	pCPA + FEN	Percent change[a]	p value
Caudate putamen, medial	28.5 ± 1.7	20.9 ± 1.2	-26.7	0.0015**
Cingulate cortex	33.8 ± 1.5[b]	22.5 ± 1.2	-33.3	<0.0001***
Dentate gyrus, dorsal	17.4 ± 1.8	6.5 ± 0.7	-62.5	0.0001**
Frontal cortex	50.3 ± 0.8	38.2 ± 1.4	-24.1	<0.0001***

Values are mean ± SEM in fmol/mg tissue. [a]pCPA + FEN compared to saline. [b]n = 9.

FIGURE LEGENDS

Figure 1. Forced Swim Test of Flinders Line rats from our colony. Immobility time (seconds) of FSL (n = 10), FRL (n = 10), and Sprague-Dawley (SD) (n = 12) rats, representing the phenotype of our Flinders Line colony. Horizontal lines are mean ± SEM. *p < 0.05, ***p < 0.0001

Figure 2. Correlation between 5-HT_4 receptor and 5-HT transporter binding in Flinders Line rats. [^{3}H]SB207145 versus (S)-[N-methyl-^{3}H]citalopram binding in A) dorsal hippocampus (r = 0.48, p = 0.034), and B) ventral hippocampus (r = 0.24, p = 0.307) of FSL (triangles, n = 10) and FRL (squares, n = 10). Values are fmol/mg tissue.

Figure 3. A. Individual sucrose intake (g) of rats during 4 weeks of Chronic Mild Stress (CMS, n =16) and control (n = 8). B. Variation in sucrose intake (g) within control and CMS group during CMS procedure. *p < 0.05

Figure 4. 5-HT_4 receptor binding after acute and chronic paroxetine administration. Autoradiograms of specific [^{3}H]SB207145 binding (1 nM) at four section levels after 1 (left panel) and 21 (right panel) days of vehicle (n = 7-8; A, D, G, J) or paroxetine (10 mg/kg, n = 7-8; B, E, H, K) administration. C, F, I, L) Non-specific binding in the presence of 10 μM RS39604.

Figure 5. 5-HT_4 receptor binding after sub-chronic 5-HT depletion. Autoradiograms of specific [^{3}H]SB207145 binding (1 nM) at 3 section levels after 4 days of A, D, G) saline (n = 9) or B, E, H) 3 x pCPA + 1 x FEN (n = 10) administration. C, F, I) Non-specific binding in the presence of 10 μM RS39604.

Figure 6. 5-HT_{2A} receptor binding after sub-chronic 5-HT depletion. Autoradiograms of specific [^{3}H]MDL100907 binding (0.4 nM) at 3 section levels after 4 days of A, D, G) saline (n = 10) or B, E, H) 3 x pCPA + 1 x FEN (n = 10) administration. C, F, I) Non-specific binding in the presence of 10 μM ketanserin.

FIGURES

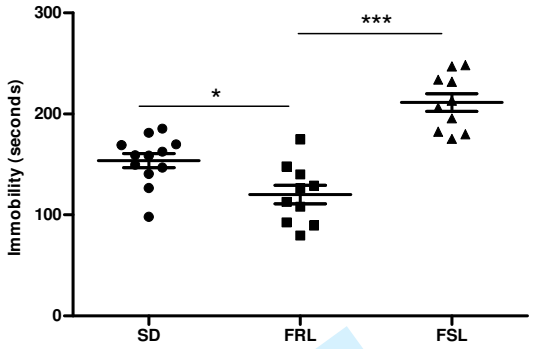


Figure 1

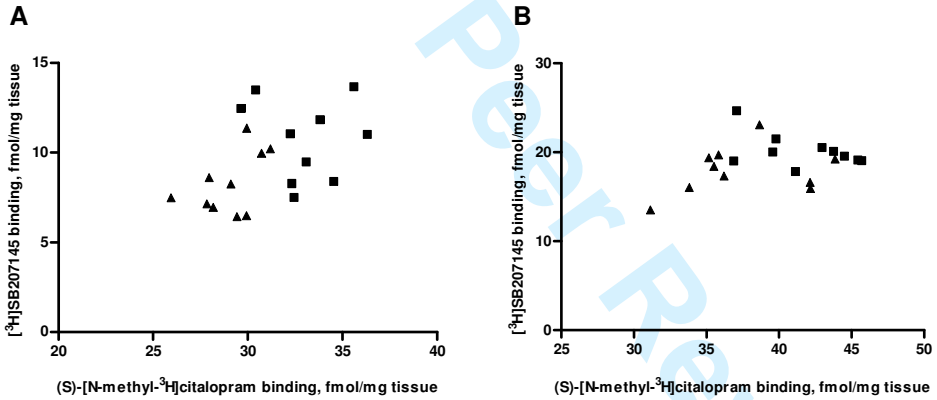
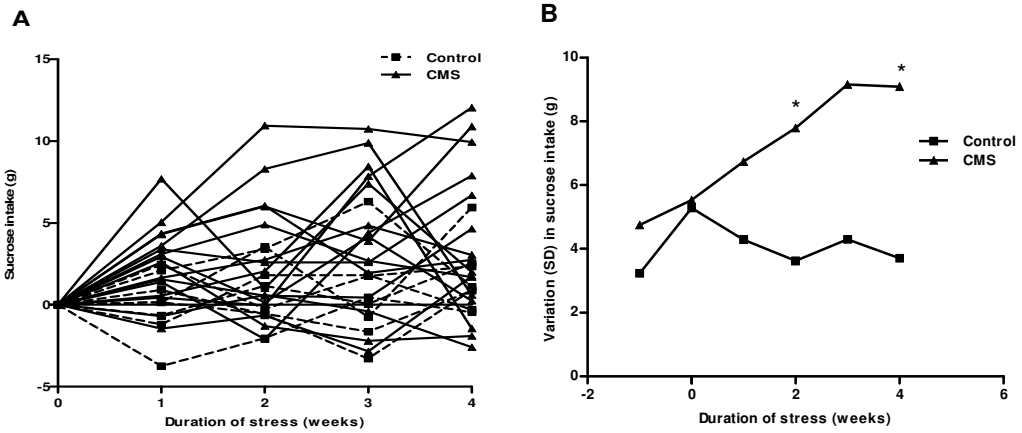


Figure 2



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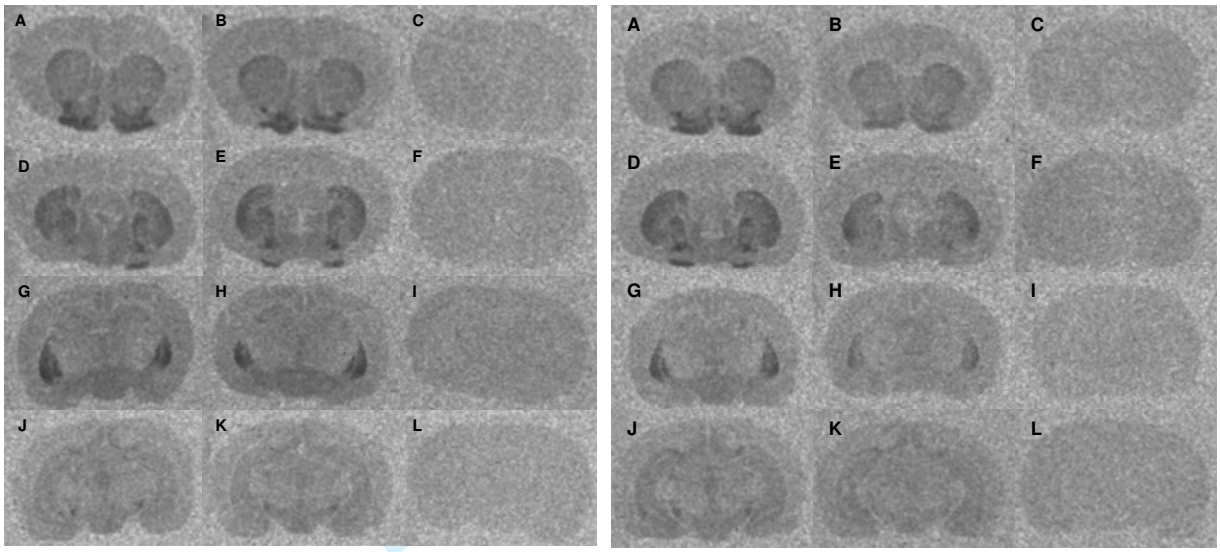

Figure 4

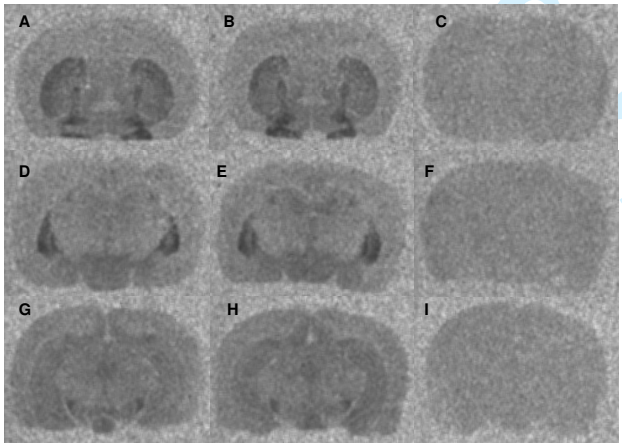

Figure 5

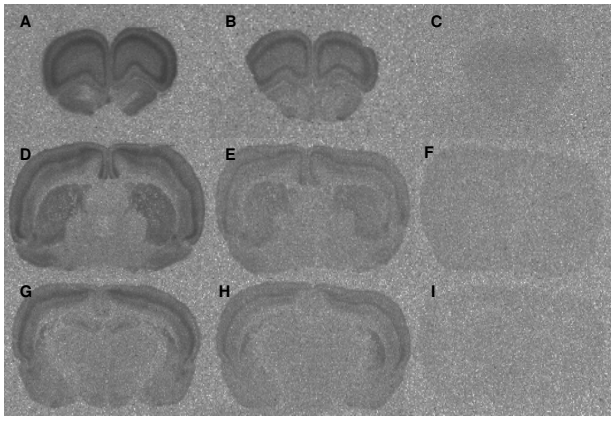

Figure 6

Manuscript III

Effects of 5-HT$_4$ receptor agonism and paroxetine administration on hippocampal extracellular 5-HT levels

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Running title: Effects of 5-HT$_4$ receptor partial agonism on 5-HT levels

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Abstract

The 5-HT_4 receptor stimulates 5-HT neuron activity and is a new target for antidepressant treatment. We here evaluated the effect of the 5-HT_4 receptor partial agonist, RS67333, on extracellular 5-HT levels in the ventral hippocampus of the rat by in vivo microdialysis, and explored the ability of 5-HT_4 receptor agonism to augment the acute effects of the selective serotonin reuptake inhibitor (SSRI) paroxetine on extracellular 5-HT levels. As 3 days of RS67333 administration shows antidepressant-like effects in animal models of depression, we also examined the effect of this treatment on extracellular 5-HT levels. Acute administration of RS67333 (1.5 mg/kg i.v.) alone had no effect on extracellular 5-HT or 5-HIAA levels, while acute paroxetine administration (0.5 mg/kg i.v.) caused the expected increase in 5-HT (276% of baseline) and decrease in 5-HIAA levels. When administering RS67333 after the effect of paroxetine had stabilized, an additional increase in 5-HT levels (to 398% of baseline) was observed. After 3 days of RS67333 administration basal extracellular 5-HT levels were increased by 73%, 5-HIAA levels were decreased, and acute paroxetine administration induced higher absolute 5-HT levels. There was no additional effect of acute RS67333 administration on 5-HT levels after paroxetine in the RS67333 pretreated animals. The present data suggest that the 5-HT_4 receptor agonist RS67333 is able to augment the acute effect of paroxetine on 5-HT levels in the ventral hippocampus. Also, the ability of sub-chronic RS67333 administration to increase extracellular 5-HT levels suggests antidepressant potential.

Key words: selective serotonin reuptake inhibitor, 5-HT_4 receptor, in vivo microdialysis, ventral hippocampus

INTRODUCTION

Antidepressant drugs of the selective serotonin reuptake inhibitor (SSRI) type require a minimum of three weeks to have therapeutic effect (Bourin et al. 2001) and are only effective in 50% of cases, although partial responses can be achieved for 80% of patients (Nestler et al. 2002). To increase the effectiveness and reduce the time required for antidepressant effect, pharmacological strategies have explored co-administration of 5-HT receptor ligands (Sharp et al. 1997;Boothman et al. 2006;Hjorth 1993). The SSRIs inhibit 5-HT reuptake by the 5-HT transporter (5-HTT), leading to acute increases in extracellular 5-HT levels (Hajos-Korcsok et al. 2000). However, the increased 5-HT levels inhibit 5-HT neuron activity in the raphe nuclei (Hajos et al. 1995) through activation of somatodendritic 5-HT_{1A} autoreceptors (Gartside et al. 1995;Sprouse and Aghajanian 1987), constraining the increase in 5-HT levels. Addition of 5-HT_{1A} receptor antagonists to acute SSRI administration prevents the inhibition of 5-HT neuronal activity and increases the release of 5-HT in the frontal cortex (Gartside et al. 1995) and ventral hippocampus (Hjorth 1993). This is similar to the effect of chronic SSRI administration, where the inhibitory effect of 5-HT_{1A} receptor activation is desensitized (Invernizzi et al. 1994;Kreiss and Lucki 1995) and extracellular 5-HT levels in the hippocampus and frontal cortex increased (Hajos-Korcsok et al. 2000;Owen and Whitton 2005).

Apart from 5-HT autoreceptors, the postsynaptic 5-HT_4 receptors also regulate 5-HT neuron activity and 5-HT release. In contrast to the 5-HT autoreceptor inhibition, however, the 5-HT_4 receptor stimulates 5-HT neuron firing rate in the dorsal raphe nucleus (DRN) (Lucas and Debonnel 2002;Lucas et al. 2005), and this effect does not desensitize in response to chronic partial agonist administration (Lucas et al. 2005). Also, both local and systemic acute

administration of non-selective 5-HT_4 receptor agonists increases extracellular 5-HT levels in the hippocampus by 200% (Ge and Barnes 1996). It is therefore likely that stimulation of the 5-HT_4 receptor will increase general serotonergic activity, be able to potentate the effect of SSRI treatment, and possibly have antidepressant properties in itself. In support of the latter, a recent study has demonstrated antidepressant-like effects after 3 days of 5-HT_4 receptor partial agonist administration in rodents (Lucas et al. 2007). The aim of the present study was to examine by in vivo microdialysis, the ability of acute 5-HT_4 receptor partial agonism to augment the effect of paroxetine on hippocampal extracellular 5-HT levels, and to determine the effect of sub-chronic 5-HT_4 receptor partial agonism on 5-HT levels.

We have evaluated the effects of acute administration of a 5-HT_4 receptor partial agonist, RS67333, on extracellular 5-HT levels in the ventral hippocampus by in vivo microdialysis. We also examined the ability of RS67333 to increase the effect of acute SSRI (paroxetine) administration on hippocampal 5-HT levels, and evaluated the effects of 3 days of RS67333 administration on basal 5-HT levels.

EXPERIMENTAL METHODS

Animals

Male Sprague-Dawley rats (270-410 g, Harlan, UK) were housed in groups of up to 6 under controlled conditions of temperature (21°C) and humidity (50%) on a 12 h light/dark cycle with food and water freely available. In the acute experiments, the following groups were analyzed: paroxetine (0.5 mg/kg, n = 6), RS67333 (1.5 mg/kg, n = 5), paroxetine + RS67333 (n = 7), and vehicle (distilled water, n = 3). The sub-chronic experiments consisted of 5 animals pretreated

for 3 days with RS67333 (1.5 mg/kg i.p.) followed by acute paroxetine + RS67333, and 2 animals pretreated with vehicle and used for baseline determination only. All animal procedures were covered by a project license (PPL 302445) issued by the Home Office, under the UK Animals (Scientific Procedures) Act 1986 and associated guidelines.

Microdialysis procedure

Rats were anaesthetized with chloral hydrate (400-500 mg/kg i.p., followed by supplementary doses as necessary), and local analgetic (lignocaine, s.c.) was applied to the neck and ears before placing the animals in a stereotaxic frame (Kopf) with the incisor bar set at -3.3 mm. Animal body temperature was maintained at 36°C by means of a homoeothermic heating pad with rectal probe. The skull was exposed and burr holes were drilled for the stereotaxic placement of a self-made (Sharp and Zetterström 1992) single cannula microdialysis probe (4 mm membrane) into the right ventral hippocampus. Stereotaxic co-ordinates were: rostro-caudal -5.0 mm, medio-lateral -4.5 mm, dorso-ventral -8.0 mm from Bregma and dura surface according to (Paxinos and Watson 2005). The microdialysis probe was secured in place using dental cement and two skull screws. The probe was perfused (2 µl/min) for 1 hour before and during implantation, and throughout the experimental period with artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 140, KCl 3.0, $CaCl_2$ 2.4, $MgCl_2$ 1.0, Na_2HPO_4 1.2, NaH_2PO_4 0.27, and glucose 7.2, pH 7.4, via polyethylene tubing (0.38 mm i.d.) connected to a 1 ml syringe mounted on a microinjection pump (CMA/100, Carnegie Medicine, Stockholm, Sweden). Perfusates were collected every 20 min (app. 40 µl) in small tubes, containing 1 µl 0.06 M perchloric acid, placed over the outlet cannula of the microdialysis probe throughout the experiment (300 min). A 25 gauge hypodermic needle was placed in the lateral tail vein to

allow intravenous administration of drugs. After surgery, the animal was removed from the stereotaxic frame.

Experimental protocol

Animals were anaesthetized throughout the experimental period. In all experiments, a period of at least 1.5 hours was allowed after probe insertion before sample collection for 5-HT levels to stabilize. Baseline samples were then collected for 2 hours (6 samples) before administration of paroxetine (0.5 mg/kg i.v.), RS67333 (1.5 mg/kg i.v.), or vehicle (distilled water, i.v.) over 3-4 min. in the middle of the following sample. Samples were then collected for another 2 hours. When combining paroxetine and RS67333, RS67333 was administered 80 min. after paroxetine injection. For sub-chronic RS67333 administration prior to microdialysis, animals were injected with RS67333 (1.5 mg/kg i.p.) once daily for 3 consecutive days, and microdialysis was performed on day 4. At the end of the experiment animals were given an overdose of anesthetic, and the brain was removed to allow histological verification of probe location. Animals with a probe location outside of the ventral hippocampus were excluded.

High performance liquid chromatography (HPLC) analysis

Dialysate samples were analyzed for 5-HT, 5-HIAA, and DOPAC using HPLC with electrochemical detection. Samples were separated using a Rainin Dynamax HPLC Reverse Phase Analytical (100 x 4.6 mm, Microsorb C_{18} 3 μm particles, Varian) and 5-HT was detected at RT using a 3 mm glassy carbon electrode maintained at +0.75 V versus an Ag/AgCl reference electrode (BAS LC-4 potentiometer, BASi). The mobile phase was 0.13 M NaH_2PO_4 buffer containing 12.5% (v/v) methanol, 0.85 mM EDTA, and 0.05 mM $_L$-octane sulphonic

acid (brought to pH 3.55 with orthophosphoric acid) at a flow rate of 1 ml min^{-1} (LKB 2150 HPLC pump). Dialysates were injected directly onto the chromatography system immediately following collection. The sample run time was app. 8 min, and samples from two animals could therefore be analyzed during the experiment. Chromatograms were displayed, integrated, and stored (Milton Roy CI 4000 integrator) prior to analysis. Chromatographic peaks for 5-HT, 5-HIAA, and DOPAC were converted to absolute amounts in pmol by external calibration of the integrator before each experiment. The limit of detection for 5-HT was approximately 0.005 pmol per sample.

Drugs

Laboratory chemicals were Analar or HPLC grade. The drugs were: chloral hydrate (Centaur, Somerset, UK), lignocaine HCl (Centaur, Somerset, UK), paroxetine HCl (donated by GlaxoSmithKline, UK), RS67333 HCl (Tocris Cookson Ltd, UK), WAY100635 HCl (donated by Wyeth Research, UK). All drugs were prepared fresh in distilled water, calculated on the basis of the respective salt.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software Inc.). Microdialysis data after acute drug challenge are expressed as percentage change compared to baseline, calculated as the average of the last three samples collected before drug or vehicle injection. Baseline 5-HT levels after 3 days of RS67333 administration are presented as absolute levels (pmol/sample of 40 µl), uncorrected for in vitro recovery. Between group analyses were performed using two-way ANOVA with repeated measures, and Drug and Time

as independent factors. Group differences after significant ANOVAs were analyzed by Bonferroni posttests. The acute microdialysis data was analyzed in two steps: first, we compared the effects of paroxetine, RS67333, paroxetine + RS67333, and vehicle on 5-HT levels for the time span 0-260 min. We then compared the effects of paroxetine + RS67333 on 5-HT levels with that of paroxetine alone for the time span 140-300 min. Basal 5-HT and metabolite levels after 3 days of RS67333 were calculated as the average of the last four samples collected before drug or vehicle administration, and compared with a control group of combined untreated (n = 16) and 3 day vehicle treated (n = 2) animals by Student's *t*-test. The level of significance was set at $p < 0.05$.

RESULTS

Acute effects of RS67333 and paroxetine

When comparing the effects of paroxetine, RS67333, paroxetine + RS67333, and vehicle on 5-HT levels, there was a significant effect of Drug ($F(3,204) = 13.30$, $p = 0.0001$) and Time ($F(12,204) = 18.97$, $p < 0.0001$), and an interaction ($F(36,204) = 8.541$, $p < 0.0001$) between the two. Bonferroni posttests showed a significant effect of paroxetine on 5-HT levels but no effect of RS67333, when administered alone (Fig. 1). Paroxetine (0.5 mg/kg) injection caused a rapid increase in 5-HT levels in the ventral hippocampus to 276% of baseline levels (Fig. 1). The increase in 5-HT levels was significant 50 min after paroxetine injection, reaching a plateau after approximately 90 min. When comparing the effects of paroxetine + RS67333 on 5-HT levels to that of paroxetine alone, there was a significant effect of Time ($F(8,88) = 29.11$, $p < 0.0001$) and an interaction ($F(8,88) = 2.479$, $p = 0.0179$) between Drug and Time. Bonferroni posttests showed a significant effect on 5-HT levels, when RS67333 was administered 80 min after paroxetine ($p < 0.01$ at 240 min time point), giving a further increase

in 5-HT levels to 398% of baseline 30 min after RS67333 i.v. administration (Fig. 1). The additional effect of RS67333 was no longer significant at the 260 min time point.

When comparing all treatment groups there was an effect of Time ($F(12,204) = 16.79$, $p < 0.0001$) and an interaction between Drug and Time ($F(36,204) = 1.520$, $p = 0.0381$) on 5-HIAA levels. Bonferroni posttests showed a decrease in 5-HIAA levels after paroxetine at the 260 min ($p < 0.05$) time point and after paroxetine + RS67333 at the 240 ($p < 0.05$) and 260 min ($p < 0.001$) time point (Fig. 2A). Comparison of the combination of paroxetine and RS67333 with paroxetine alone (140-300 min), showed a significant effect of Time ($F(8,88) = 95.42$, $p < 0.0001$) and an interaction between Drug and Time ($F(8,88) = 2.243$, $p = 0.0314$) on 5-HIAA levels but no time points were significant by Bonferroni posttests. Two-way ANOVA of the effects of all treatments on DOPAC levels showed no effect of Drug or Time but a significant interaction ($F(36,204) = 1.885$, $p = 0.0033$) between the two. Bonferroni posttests showed lower DOPAC levels at the 240 and 260 min time point of all drug treatments (except paroxetine at 240 min) compared to vehicle (Fig. 2B). Inspection of the data in figure 2B suggests that this is due to an increase in DOPAC levels in the vehicle group at these time points. Comparison of paroxetine + RS67333 and paroxetine alone (140-300 min) showed a significant effect of Time on DOPAC levels ($F(8,88) = 4.543$, $p = 0.0001$) and an interaction ($F(8,88) = 2.562$, $p = 0.0147$) between Drug and Time but Bonferroni posttests showed no significant differences.

Three days of RS67333 administration increases 5-HT baseline levels

Since the 5-HT levels of 3-day vehicle administered animals were within the range of the untreated controls, a control group consisting of both untreated and vehicle administered animals was used (Fig. 3A and 3B). The analysis of 3 days of RS67333 administration on

extracellular 5-HT baseline levels showed a significant effect of Pretreatment with RS67333 ($F(1,105) = 18.91$, $p = 0.0003$) and Time ($F(5,105) = 5.671$, $p = 0.0001$) and no interaction between Pretreatment and Time. Bonferroni posttests showed a significant increase in 5-HT levels after 3 days of RS67333 administration in baseline sample 1-3 and 5-6, i.e. over a period of 120 min from sample collection start until administration of drug (Fig. 3A). When expressed as average 5-HT level (pmol/sample) of the four last samples before drug administration (sample 3-6), there was a significant 73% increase in 5-HT levels after 3 days of RS67333 compared to controls ($p = 0.0001$, Fig. 3B). Three days of RS67333 administration also induced a 27% decrease in baseline 5-HIAA levels in the ventral hippocampus ($p = 0.047$) but had no effect on DOPAC levels (Table 1).

We compared the influence of 3 days of RS67333 administration on the effect of paroxetine + RS67333 on 5-HT, 5-HIAA and DOPAC levels. The two-way ANOVA analysis showed a significant effect of Time ($F(14,140) = 39.59$, $p < 0.0001$) and Pretreatment with RS67333 ($F(1,140) = 5.083$, $p = 0.048$) on 5-HT levels but no interaction between Time and Pretreatment. Bonferroni posttests showed that the two groups differed at the 280 min time point ($p < 0.05$), where the 3-day RS67333 pretreated group had higher 5-HT levels (Fig. 4). There was no change in the effect of paroxetine + RS67333 on 5-HIAA and DOPAC levels after 3 days of RS67333 pretreatment (data not shown).

DISCUSSION

To our knowledge this is the first study to examine potential SSRI augmenting effects of systemic 5-HT_4 receptor agonism, and to evaluate the effects of sub-chronic 5-HT_4 receptor agonism on extracellular 5-HT levels. We found that while acute pharmacological stimulation

of the 5-HT_4 receptor alone had no effect on extracellular 5-HT or 5-HIAA levels in the ventral hippocampus, 5-HT_4 receptor partial agonism after acute paroxetine administration increased extracellular 5-HT levels further than paroxetine administration alone. In contrast to the acute experiments, three days of 5-HT_4 receptor partial agonist administration increased basal extracellular 5-HT levels, and when followed by a paroxetine challenge induced higher 5-HT levels than in animals, which were not pretreated with RS67333.

Acute effect of 5-HT_4 receptor partial agonism on hippocampal 5-HT levels

Acute administration of RS67333 had no effect on extracellular 5-HT levels in the ventral hippocampus of the anaesthetized rat. This was unexpected as a previous microdialysis study has shown markedly increased 5-HT levels in the ventral hippocampus after systemic administration of the 5-HT_4 receptor agonist renzapride (Ge and Barnes 1996). The reason for this discrepancy could be that RS67333 is a partial agonist (Eglen et al. 1995), while renzapride is a full agonist. We chose RS67333 because of its higher selectivity for the 5-HT_4 receptor, although RS6733 also has affinity for σ_1 and σ_2 receptors in vitro (Eglen et al. 1995). Another difference between the two studies is that Ge et al. performed their experiments in wake rats, while we used chloral hydrate anaesthetized animals. However, administration of RS67333 at the dose used in our study increases 5-HT neuron activity in the DRN of the chloral hydrate anaesthetized rat (Lucas et al. 2005), and stimulation of the DRN is normally reflected in increased 5-HT release in the ventral hippocampus (Sharp and Hjorth 1990). We speculate that acute increases in 5-HT release in response to acute RS67333 administration could not be detected in our study because of rapid reuptake of 5-HT by the 5-HT transporter.

5-HT_4 receptor stimulation augments 5-HT response to acute paroxetine

When administered 80 min after the SSRI paroxetine, RS67333 increased hippocampal 5-HT levels above the levels induced by paroxetine alone. The increase in 5-HT levels after acute systemic paroxetine administration is influenced by serotonergic tonus, which is affected by both inhibitory effects of 5-HT_{1A} (Sprouse and Aghajanian 1987), 5-HT_{2A} and 5-$HT_{2B/C}$ receptors (Boothman et al. 2003), and by stimulatory effects of 5-HT_4 receptors on 5-HT neuron firing. Addition of a 5-HT neuron activating compound, RS67333, may shift the balance between inhibitory and stimulatory 5-HT receptor effects on 5-HT neuron activity, increasing the 5-HT release in the ventral hippocampus. The transient nature of the additional increase in 5-HT levels after RS67333 administration is unlikely to be due to acute desensitization of the 5-HT_4 receptors, as the 5-HT neuron response to 5-HT_4 receptor partial agonism does not desensitize even with chronic partial agonist administration (Lucas et al. 2005). Rather the response may be inhibited by negative feedback, as the increase in 5-HT release in response to RS67333 will increase the activation of inhibitory 5-HT receptors. In comparison, the SSRI augmenting effect of acute 5-HT_{1A} receptor antagonism is long lasting and of a larger scale (Hjorth 1993), possibly due to a larger influence of 5-HT_{1A} receptors on 5-HT neuron activity. Our results are in line with the ability of renzapride to increase 5-HT levels in the ventral hippocampus in the presence of paroxetine after local administration of both compounds via the microdialysis probe (Ge and Barnes 1996). However, the effect of renzapride on paroxetine elevated 5-HT levels was larger and longer lasting, possibly because the inhibitory effects of 5-HT_{1A} autoreceptors were avoided due to the local administration route.

Three days of 5-HT_4 receptor stimulation increases hippocampal 5-HT levels

Three days of RS67333 administration increased the 5-HT baseline levels in the ventral hippocampus. Given that the acute RS67333 administration did not have a similar effect, this is

most likely due to the 5-HT_4 receptor stimulatory effect on 5-HT neuron activity (Lucas et al. 2005), in combination with a desensitization of 5-HT_{1A} autoreceptors in the raphe nuclei (Lucas et al. 2007). Our finding is in agreement with the observed increased tonus on hippocampal postsynaptic 5-HT_{1A} receptors after sub-chronic RS67333 administration (Lucas et al. 2007).

RS67333 shows antidepressant potential in the Forced Swim Test, and sub-chronic administration has antidepressant-like effects in the Chronic Mild Stress and Olfactory Bulbectomy animal models of depression (Lucas et al. 2007). However, the increase in 5-HT levels after three days of RS67333 administration is smaller than the 2-3-fold elevation, which has been observed after 14 days of SSRI treatment (Hajos-Korcsok et al. 2000;Kreiss and Lucki 1995). Together with the paroxetine augmenting effect of acute RS67333 administration this suggests that a combination of SSRI administration and 5-HT_4 receptor partial agonism enhances 5-HT system activity. This is supported by the observation that a paroxetine challenge can still elevate 5-HT levels after three days of RS67333 administration and does so to a higher absolute level than reached with paroxetine alone.

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FIGURE LEGENDS

Figure 1. 5-HT levels in the ventral hippocampus in response to paroxetine and RS67333 administration. Extracellular 5-HT levels in the ventral hippocampus, expressed as percentage of baseline, after paroxetine (0.5 mg/kg, n = 6), RS67333 (1.5 mg/kg, n = 5), or vehicle (n = 3) administration, and in response to combined paroxetine and RS67333 (n = 7) administration. Arrows indicate injection times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: paroxetine relative to vehicle (0-260 min); $^{\#\#}$ $p < 0.01$: paroxetine + RS67333 compared to paroxetine (140-300 min).

Figure 2. 5-HIAA and DOPAC levels in the ventral hippocampus in response to paroxetine and RS67333 administration. Extracellular 5-HIAA (A) and DOPAC (B) levels, expressed as percentage change, after administration of paroxetine, RS67333, vehicle, or paroxetine and RS67333 combined. Arrows indicate injection times. * $p < 0.05$: paroxetine relative to vehicle, $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$: paroxetine + RS67333 relative to vehicle. § $p < 0.05$: vehicle relative to RS67333, paroxetine, and paroxetine + RS67333.

Figure 3. Effect of sub-chronic RS67333 administration on 5-HT levels in the ventral hippocampus. Baseline extracellular 5-HT levels (pmol) after 3 days of RS67333 (1.5 mg/kg i.p., n = 5) administration compared to controls (untreated and vehicle treated combined, n = 18). A) 5-HT levels in the 6 baseline samples are compared by two-way ANOVA with RM and Bonferroni post-tests. B) Baseline 5-HT levels, expressed as the average of the four last baseline samples before drug administration (sample 3-6). Vehicle treated animals (n = 2) are indicated by triangles, and untreated controls (n = 16) by circles. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4. Effect of paroxetine and RS67333 administration on 5-HT levels after sub-chronic RS67333 pretreatment. Changes in extracellular 5-HT levels (pmol/40 μl sample) in the ventral hippocampus after paroxetine (0.5 mg/kg) and RS67333 (1.5 mg/kg) administration in animals receiving 3 days of RS67333 (1.5 mg/kg, n = 5) pretreatment or no pretreatment (n = 7). Arrows indicate injection times. * $p < 0.05$,

TABLES AND FIGURES

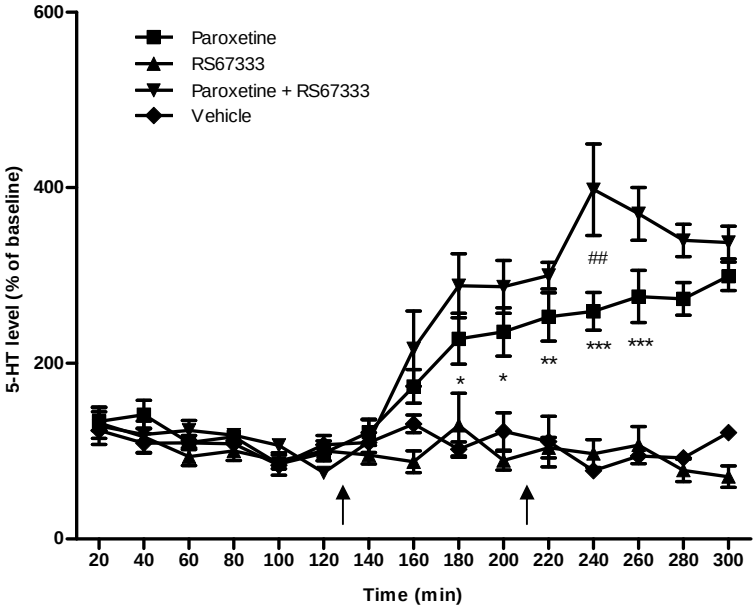
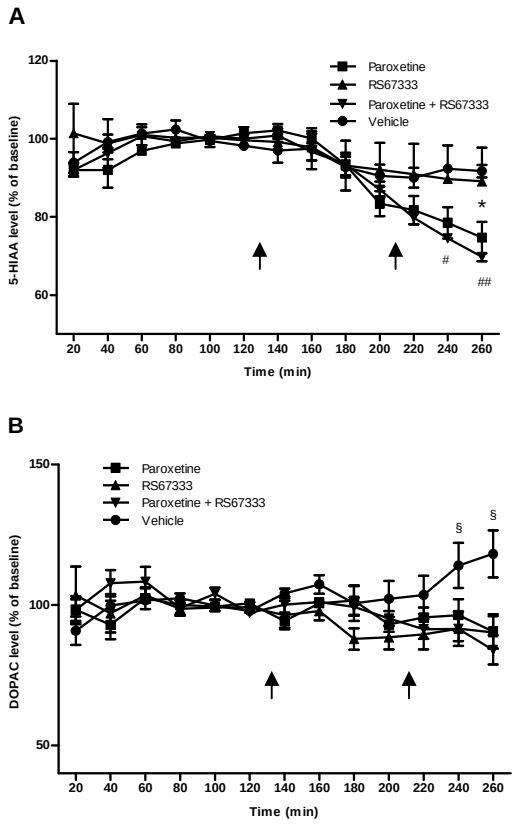


Figure 1



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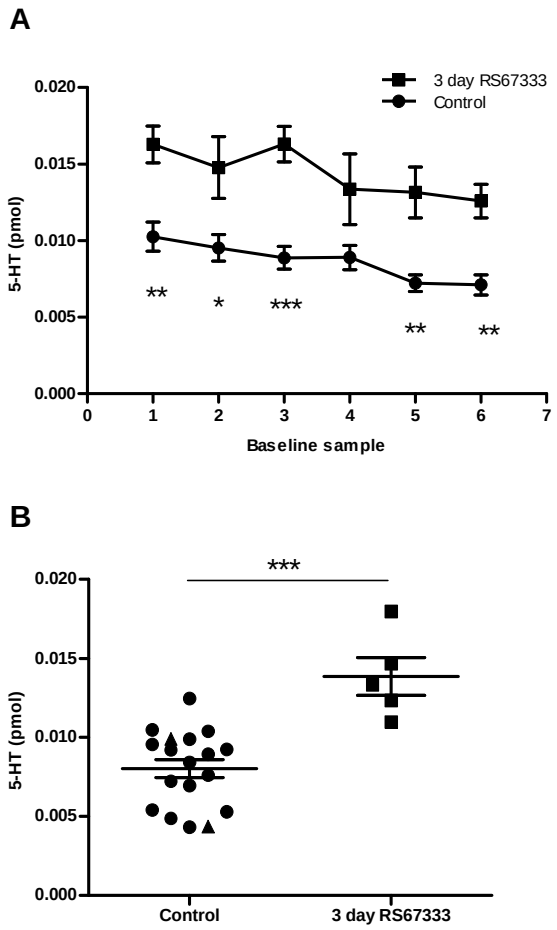

Figure 3

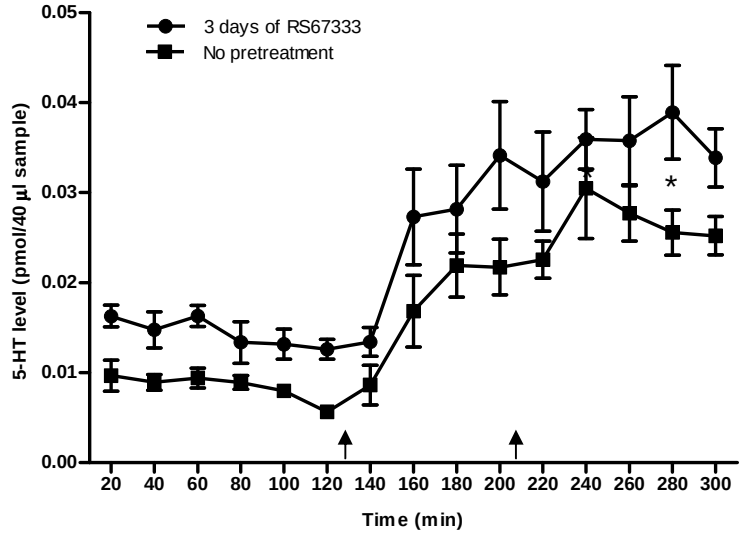

Figure 4

Table 1. Extracellular 5-HT, 5-HIAA, and DOPAC levels after sub-chronic RS67333 administration

	Control	3 days RS67333	p value
5-HT	0.008 ± 0.001	0.014 ± 0.001	0.0001***
5-HIAA	7.536 ± 0.459	5.478 ± 0.834	0.0474*
DOPAC	0.424 ± 0.034	0.352 ± 0.055	0.3209

Values are mean ± SEM in pmol.