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GALANIN RECEPTOR SUBTYPES IN RODENT MODELS OF MOOD DISORDERS

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MSc

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Cover: The painting is by Nabeel H. Wardi (1952-2002), whom was an architect, a poet and a painter. By using his painting as the cover image of my thesis, I would like to express my sincere gratitude to my late uncle Nabeel H. Wardi. I am honored to have one of his paintings in my possession.

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To my family
with love

ABSTRACT

Major depressive disorder (MDD) is a serious mental illness, with a complex etiology. It is a multifactorial disorder caused by a combination of genetic, environmental and epigenetic factors. Exposure to stressful life events seems to be a common, contributing cause of morbidity. Discoveries during the last 60 years have provided increasing evidence for a dysfunctional serotonergic and/or noradrenergic neurotransmission in MDD. However, other transmitter systems also appear to be involved, e.g. GABAergic, glutamatergic and cholinergic systems, as well as several neuropeptide systems. Since its discovery in the 1980s at Karolinska Institutet, the neuropeptide galanin has been implicated in a diversity of physiological processes including not only feeding and nociception, but also anxiety and stress.

The main aim of this thesis was to investigate the role of galanin and galanin receptors in rodent models of mood disorders, and to evaluate the possible interaction between exposure to stress and galanin receptor subtype stimulation by measuring the expression of neuronal chemical markers of relevance for depression in serotonin/5-hydroxytryptamine (5-HT) and noradrenaline (NA) neurons. Several behavioral tests were employed including the forced swim test. The validity of this test was examined by the use of the 5-HT selective reuptake inhibitor fluoxetine, a widely used antidepressant.

Galanin, given intracerebroventricularly (i.c.v.) to rats, increased immobility time in the forced swim test indicative of depression-like behavior. Co-infusion of the unspecific galanin receptor antagonist M35 blocked the effect of galanin. Importantly, M35 alone decreased immobility time. The galanin receptor 1 (GalR1) agonist M617 and the galanin receptor 2 (GalR2) antagonist M871 both increased immobility time in the FST, whereas, the GalR2 agonist M1896 caused a decrease.

In situ hybridization studies performed after infusion of galanin (i.c.v.) and exposure to swim stress showed elevated levels of galanin mRNA in the locus coeruleus versus control animals. However, a significant increase of galanin mRNA levels was also observed in the locus coeruleus in saline- and fluoxetine-treated animals indicating that the stress of injection is adding to the stress of swimming. In contrast, in the dorsal raphe nucleus, the swim/injection procedure did not change the mRNA levels of galanin or tryptophan hydroxylase, indicating that 5-HT neurons are not as sensitive as the NA neurons in locus coeruleus to stress. The levels of 5-HT_{1A} receptor mRNA were reduced in the dorsal raphe nucleus following galanin or the GalR2 agonist M1896 infusion. These results support an involvement of brain galanin systems in depression-like behavior in rodents, and galanin receptor subtypes appear to play differential roles in modulation of depression-like behavior. The action of galanin and galanin receptor ligands seems to modulate depression-like behavior in rodents partly via changes in expression of certain monoaminergic molecules relevant for depression.

To further analyze the functional role of GalR2 in rodent models of depression, mice overexpressing this receptor (GalR2OE mice) under the platelet-derived growth factor B promoter were generated. These mice displayed decreased immobility time in the FST compared to the wild-type controls indicative of antidepressant-like behavior. However, anxiety-like behavior, emotional memory and locomotor activity measures did not differ between the genotypes. Overexpression of the GalR2 transcripts and receptor protein was detected in limbic areas such as subregions of the medial prefrontal cortex and subiculum. Thus, GalR2 in these areas may contribute to the depression-like behavior observed in these mice.

The present thesis gives evidence for a role of the neuropeptide galanin and its receptors in rodent models of depression-like behavior and suggests that GalR1 antagonists, and GalR2 agonists may represent new candidates for the development of drugs for treatment of mood disorders.

Keywords: 5-HT_{1A} receptor, galanin, galanin receptor 2 overexpressing mice, locus coeruleus, dorsal raphe, depression-like behavior, forced swim test

LIST OF PUBLICATIONS

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[1] These authors contributed equally to the study

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LIST OF ABBREVIATIONS

5-HIAA	5-hydroxyindolacetic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HTT	5-hydroxytryptamine transporter
8-OH-DPAT	8-hydroxy-2-dipropylaminotetralin hydrobromide
aCSF	Artificial cerebrospinal fluid
AC	Adenyl cyclase
ACTH	Adrenocorticotropin
ANOVA	Analysis of variance
BDNF	Brain-derived nerve growth factor
CA	Cornu Ammonis; area of the hippocampus
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CRF	Corticotropin-releasing factor
CSF	Cerebrospinal fluid
DA	Dopamine
DBH	Dopamine β-hydroxylase
DNA	Deoxyribonucleic acid
DRN	Dorsal raphe nucleus
dS	Dorsal subiculum
FITC	Fluorescein isothyocianate
FST	Forced swim test
GABA	Gamma-amino-butyric acid
Gal-KO	Galanin-knockout
GalOE	Galanin-overexpressing
GalOE/D	Galanin overexpression under the DBH promoter
GalR	Galanin receptor
GalR-KO	Galanin receptor-knockout
GMAP	Galanin message-associated peptide
GPCR	G protein-coupled receptor
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
i.v.	Intravenous
ir	Immunoreactive
LC	Locus coeruleus
LDCV	Large dense-core vesicle
LI	Like immunoreactivity
MAOI	Monoamine oxidase inhibitor
MAP	Mitogen activated protein
MR	Median raphe
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
NK1	Neurokinin (substance P) receptor 1
PaS	Parasubiculum
PBS	Phosphate-buffered saline
PDGF-B	Platelet-derived growth factor B
PFC	Prefrontal cortex
PLSD	Protected least significant difference
PNS	Peripheral nerve system
PrS	Presubiculum
RT-PCR	Reverse transcription polymerase chain reaction
S	Subiculum
s.c.	Subcutaneous
SNRI	Selective noradrenaline reuptake inhibitor
SSC	Standard saline sodium citrate
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TH	Tyrosine hydroxylase
TNB	Tris-sodium blocking buffer
TNT	Tris-sodium washing buffer
TPH	Tryptophan hydroxylase
TSA	Tyramide signal amplification
TST	Tail suspension test
VS	Ventral subiculum
WT	Wild type

1. INTRODUCTION

1.1 Major Depressive Disorder (MDD)

MDD is a severe, life-threatening, and highly prevalent psychiatric disorder, predicted to soon become one of the major causes of death worldwide (Manji *et al*, 2001; Nestler *et al*, 2002). The lifetime prevalence of MDD is as high as 21.4% worldwide (Gabilondo *et al*, 2009), and is associated with high disability rates. Currently, it accounts for almost 12% of total years lived with disability according to the World Health Organization (2008). Furthermore, MDD causes a significant financial burden to the individual/society, and the estimated cost of this illness corresponds to 1% of the total economy of Europe (Sobocki *et al*, 2006).

1.2 Symptoms and Diagnosis

MDD is a state of intense sadness, despair or melancholia that is disruptive to an individual's wellbeing and social functioning in everyday life. The diagnostic criteria for MDD, as defined by DMS-IV 2000, include depressed mood, diminished ability to experience pleasure (anhedonia), and vegetative symptoms such as abnormalities in appetite and sleep. At least five of the symptoms listed in Box 1 have to be present during the same two-week period to signify a diagnosis. Suicidal ideation or attempts are recurrent in MDD, making suicide one of the major causes of death among depressed patients. MDD is the most common psychiatric illness among individuals with suicide-related behaviors (Kessler *et al*, 2005). In addition to mortality associated with suicide, depressed patients are more prone to develop coronary artery disease and type 2 diabetes (Knol *et al*, 2006).

Box 1: DMS-IV diagnostic criteria for MDD (DMS IV, 2000)

1. Depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad or empty) or observation made by others (e.g., appears tearful).
2. Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation made by others).
3. Significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day.
4. Insomnia or hypersomnia nearly every day.
5. Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down).
6. Feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick).
7. Fatigue or loss of energy nearly every day.
8. Diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others).
9. Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide

Five (or more) of the above symptoms have to be present during the same two-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure.

1.3 Pathophysiology of MDD

Knowledge of the pathophysiology of depression has evolved from Hippocrates' speculation about an excess of black bile causing melancholia to theories focused on chemical imbalances in the brain, and to current hypotheses that incorporate gene-environmental interaction, neurotrophins and neurogenesis.

Despite centuries of research, our understanding of the etiology of depression is still rudimentary. There are several reasons for this: first, depression, like many other mood disorders, is a heterogeneous illness, and there is no consensus regarding the site of pathology. Several brain regions and circuits regulate emotion, reward and executive function, and dysfunctional changes within these highly interconnected regions (limbic areas, e.g. hippocampus, amygdala and prefrontal cortex) have been implicated in depression and antidepressant action (Berton and Nestler, 2006; aan het Rot *et al*, 2009). Second, observing pathological changes in the brain remains markedly more difficult than other organs. Available techniques to detect subtle deviant functions of brain areas or interconnected circuits depend on either post-mortem tissue, which has numerous limitations, or neuroimaging techniques which rely on measuring neuronal activity utilizing indirect markers for neuronal activation (Phelps and LeDoux, 2005). Simple decreases or increases of regional brain activity are probably insufficient to explain the complex array of symptoms caused by depression. In short, there is still no well documented biomarker for MDD.

1.3.1 Genetic and Environmental Causes of MDD

The heritability of MDD is estimated to be at a range of 30-40% (Malhi *et al*, 2000; Sullivan *et al*, 2000; Kendler *et al*, 2006). Yet, the search for specific depression vulnerability genes that confer this risk has so far not been successful, most likely because of the heterogeneity of MDD (Burmeister, 1999). Furthermore, genetic predisposition is thought to interact with environmental factors, such as stressful life events, initiating a depressive episode in some patients (Kessler, 1997; Kendler *et al*, 1999). Among the genes that have been studied in relation to MDD is the one encoding the serotonin transporter (SERT). Functional polymorphism in the promoter region of this gene was predicted to have a role in susceptibility to affective disorders and to interact with a number of stressful life events in depression (Collier *et al*, 1996; Caspi *et al*, 2003).

Recent research have demonstrated that many psychiatric disorders are not due to mutations in a single gene but rather involve molecular disturbances entailing multiple genes and signals that control their expression, i.e. epigenetic modifications (Tsankova *et al*, 2007). The epigenetic modifications include DNA methylations and post-translational modifications (acetylation and methylation) of histone N-terminal tails (Tsankova *et al*, 2007). Therefore, epigenetic changes might help explaining high discordance rates between monozygotic twins, individual differences among rodents and the greater prevalence of depression in women (Krishnan and Nestler, 2008). Epigenetic modification might also be a mechanism by which stressful life events can produce long-lasting changes in gene expression (Krishnan and Nestler, 2008). For example, chronic social defeat stress induced long-lasting downregulation of the brain-derived neurotrophic factor (BDNF) transcripts and increased histone methylation at their corresponding promoters (Tsankova *et al*, 2006). Chronic treatment with the antidepressant imipramine reversed this downregulation and increased histone acetylation at these promoters (Tsankova *et al*, 2006).

1.3.2 The Hypothalamic-Pituitary-Adrenal Axis and MDD

Neuroendocrine studies strongly suggest that hyperactivity of central corticotropin-releasing hormone (CRH) circuits, resulting in a characteristic dysregulation of the hypothalamic-pituitary-adrenal axis (HPA), plays a critical role in the development and course of affective disorders such as MDD (Holsboer, 2001). Clinical studies have reported increases in serum cortisol concentrations in a majority of depressed patients (Parker *et al*, 2003; Raison and Miller, 2003). Exposure to trauma and stress in susceptible individuals are predisposing factors for the development of MDD (Nestler *et al*, 2002; Caspi *et al*, 2003; Lesch, 2004; Holsboer and Ising, 2009). It is well documented that stress increases serum corticosterone levels, and some depression-like symptoms can be produced in rodents by chronic administration of glucocorticoids (Gourley *et al*, 2008). Further, excess glucocorticoids, through the activation of glucocorticoid receptors, can reduce cell proliferation rates and produce atrophic changes in hippocampal subregions (McEwen and Milner, 2007). This could contribute to the hippocampal volume reductions seen in depression. The hippocampus is known to provide negative feedback to the HPA axis (Jacobson and Sapolsky, 1991; Brown *et al*, 2004). Thus damage to the hippocampus might contribute to glucocorticoid elevations through impaired negative feedback to the HPA axis, and subsequently cognitive impairment in depressed persons. Hyper-responsiveness to circulating adrenocorticotropic hormone (ACTH) (Parker *et al*, 2003) and hypersecretion of CRH (Nemeroff, 1996; Nemeroff and Owens, 2002) might also represent mechanisms underlying the dysregulation of the HPA-axis in mood disorders. In line with these findings, glucocorticoid and CRF receptor antagonists have been tested in clinical trials (Zobel *et al*, 2000).

1.3.3 Hippocampal plasticity and MDD

Volumetric changes observed in the hippocampus in a subgroup of depressed patients (Sheline *et al*, 1996; Bremner *et al*, 2000) have supported the "neurotrophic hypothesis" of depression, involving decrements in neurotrophic factors which regulate plasticity in the adult brain (Monteggia *et al*, 2004; Duman and Monteggia, 2006). These studies have focused on BDNF, which is abundantly expressed in adult limbic structures. Preclinical studies show that several forms of stress reduce BDNF-mediated signaling, whereas chronic antidepressant treatment increases BDNF signaling (Nestler *et al*, 2002; Duman *et al*, 2006). Decreased levels of neurotrophins in the hippocampus of suicide victims and in serum of depressed patients (Karege *et al*, 2005a,b) further support the neurotrophic hypothesis. Moreover, it has been proposed that hippocampal 5-HT might play a role in synaptic plasticity and that the 5-HT_{1A} receptor has a significant role in neurogenesis and for the actions of SSRIs (Jacobs *et al*, 2000; Santarelli *et al*, 2003). However, some preclinical studies have failed to confirm the above findings, or even have reported opposite results (Groves, 2007; Martinowich *et al*, 2007). It may, therefore, be necessary to carefully reexamine the neurotrophic hypothesis.

1.4 Neurotransmitter Systems Studied in Depression

1.4.1 The Brain Serotonin System: Raphe Nuclei

Serotonin (5-HT)-containing neurons form clusters of cells located in the pontine raphe region and lower brainstem. Dahlström and Fuxe (1964) using the Falck-Hillarp fluorescence method were the first to describe nine 5-HT nuclei in the raphe region of the rat. The caudal groups of 5-HT nuclei project largely to the medulla and spinal cord, whereas the rostral groups (raphe dorsalis, raphe medianus, and centralis superior, or B7-B9) innervate the telencephalon and diencephalon. The raphe medianus (B8) provides extensive 5-HT innervations to the limbic system. The dorsal raphe or B7 projects to the neostriatum, cerebral and cerebellar cortices, and thalamus (Azmitia and Segal, 1978; Pineyro and Blier, 1999).

1.4.1.1 Serotonin Receptors

There are multiple 5-HT receptors, at least 15 molecularly distinct subtypes (Hoyer *et al*, 1994), which are grouped into seven families based on various criteria including their coupling to signaling mechanisms (Kroeze *et al*, 2002; Iversen *et al*, 2009). The majority of 5-HT receptors (5-HT_1 and 5-HT_2 and 5-HT_4-5HT_7) belong to the guanine protein-coupled receptor family (GPCR), whereas the 5-HT_3 receptor is a ligand-gated ion channel receptor. Finally, 5-HT transporter is a Na^+ and Cl^--dependent transporter protein responsible for 5-HT reuptake (Amara and Kuhar, 1993).

There are at least five isoforms of the 5-HT_1 receptor (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}). These receptors are coupled to $G_{i/o}$ proteins, inhibiting adenyl cyclase (AC) activity, opening K^+ or closing Ca^{2+} channels (Beer *et al*, 1993). The 5-HT_{1A} receptor serves as the predominant autoreceptor of the raphe nuclei, reducing the firing rate of these neurons, the amount of 5-HT released per action potential, and neurotransmitter synthesis (Aghajanian and Lakoski, 1984; Verge *et al*, 1985). The 5-HT_{1A} receptors are abundantly expressed in the dorsal raphe nucleus (DRN), frontal cortex hippocampus and amygdala (Chalmers and Watson, 1991), and have been implicated in affective disorders such as anxiety and MDD. 5-HT_2 family is mainly post-synaptic, existing in regions such as the hippocampus and cortex. Stimulation of these receptors leads to activation of phospholipase C (PLC), presumably via coupling to G_q protein. 5-HT_4, 5-HT_6, and 5-HT_7 receptors are also stimulatory, mediating their action via activation of G_s protein and an increase in AC activation. These receptors are mainly post-synaptic and are expressed in hippocampus, amygdala, thalamus and hypothalamus (Iversen *et al*, 2009).

1.4.2 The Brain Noradrenaline System: Locus Coeruleus

Histochemical mapping studies have demonstrated the existence of groups of noradrenergic neurons in the locus coeruleus (LC) of the pons and in the lateral tegmental portion of the midbrain (Dahlström and Fuxe, 1964; Moore and Bloom, 1979). The LC innervates the spinal cord, cerebellar cortex, hypothalamus, thalamus, septum, hippocampus, and the cerebral cortices, that is essentially the entire central nervous system (Jones *et al*, 1977; Iversen *et al*, 2009).

1.4.2.1 Noradrenergic Receptors

Adrenergic receptors are divided into three major classes defined by their differential coupling to G proteins, α_1, α_2, and β. The three subtypes of the β-adrenergic receptors (β_1, β_2, and β_3) are all coupled to stimulation of AC via G_s protein. The α_1 receptors are generally excitatory in nature and are usually coupled to Gq protein. Stimulation of these receptors leads to activation of PLC and phospholipase A (PLA) (Iversen *et al*, 2009). Activation of α_1 receptors on central noradrenergic target neurons, such as the 5-HT neurons, produce depolarization response, e.g. an excitatory response due to decrease in K^+ conductance (Baraban and Aghajanian, 1980). On the other hand, the α_2 receptor is inhibitory via coupling to G_i protein decreasing AC activity. It is thought that this receptor also mediates its inhibitory effect via another, yet undetected signal transduction mechanism (Iversen *et al*, 2009). The main function of the α_2 receptor is the presynaptic regulation of neurotransmitter release, exerting an inhibitory control of noradrenergic neurons innervating the frontal cortex (Cedarbaum and Aghajanian, 1976; Limberger *et al*, 1986).

1.5 Noradrenaline and Serotonin in Behavior

Serotonergic transmission has been suggested to play a role in a large number of physiological processes including sleep, appetite, memory/cognitive functions, impulsivity, sexual behavior, and motor function, as well as modulation of limbic/affective responsiveness, such as fear and anxiety.

Additionally, evidence links 5-HT to neuronal homeostasis and trophic mechanisms in neuronal growth and differentiation (Lucki, 1998; Ressler and Nemeroff, 2000; Berger *et al*, 2009). However, 5-HT transmission does not appear to be essential for behavioral and physiological functions based on studies in rodents (Jacobs and Azmitia, 1992). This conclusion is consistent with serotonin's presumed modulatory influence, its widespread and divergent anatomical organization (5-HT projections and receptors), and the tonic steady state nature of 5-HT neurotransmission.
Several lines of evidence suggest that NA has a prominent role in organizing the behavioral state of mammalian organism such as vigilance, arousal, stress responses and modulation of memory systems. At the cellular level NA seems to change the "signal-to-noise" ratio of both excitatory and inhibitory activity, making synaptic transmission in target neurons (circuits) more effective (Woodward *et al*, 1991).

1.6 Role of Monoamines in MDD

1.6.1 The Revised Monoamine Hypothesis of MDD

The serendipitous discovery of the first antidepressant drug imipramine more than half a century ago (Kuhn, 1958) and the subsequent observation that this compound inhibit the neuronal reuptake of the monoamines NA and 5-HT (Glowinski and Axelrod, 1964; Ross and Renyi, 1967) laid the foundation for the monoamine hypothesis of depression, which posits that MDD is due to a relative deficiency of brain monoaminergic activity (Bunney and Davis, 1965; Schildkraut, 1965; Coppen, 1968).
However, there are several problems with the monoamine hypothesis. First, and most importantly and intriguingly, changes in synaptic monoamine levels after treatment with antidepressant take place within hours, while the therapeutic response requires administration of these drugs for weeks (Baldessarini, 1989). Second, not all patients respond to antidepressant treatment.
The revised monoamine theory suggests that the acute increase in the levels of the monoamines at the synapse is only the first step in a complex cascade of events that ultimately results in antidepressant activity. Altered receptor sensitivity (desensitization or hypersensitivity of receptors), or changes on the transcriptional/translational levels of receptors in the monoaminergic system are examples of adaptive changes in the brain that temporally better correlate with the onset of therapeutic effects of antidepressants. More recent studies indicate that NA and 5-HT activities in limbic circuits probably are dysregulated in affective disorders such as MDD. These abnormalities most likely interact with other neurobiological systems causing deficits in concentration, attention, memory, arousal states, and sleep regulation, leading to symptoms such as lack of concentration and attention and sleep disturbances.

1.6.2 Evidence for the Involvement of Monoamines in MDD

1.6.2.1 The Serotonin System

Many studies on the mechanisms underlying depression have focused on the brain 5-HT system. The 5-HT_{1A} receptor has received particular attention. A subgroup of patients suffering from MDD show decreased levels of the metabolite 5-hydroxyindoleacetic acid (5-HIAA) in plasma and cerebrospinalfluid (CSF) (Åsberg *et al*, 1976; Roy *et al*, 1989; Mann, 1999). However, this observation seems to correlate better with suicidal behavior rather than depressive behavior (Faustman *et al*, 1991). Early post mortem studies reported reduced 5-HT_{1A} number in the hippocampus and lowered receptor affinity in the amygdala of depressed suicide victims (Cheetham *et al*, 1990). Further, 5-HT_{1A} receptor gene expression and binding were reduced in the prefrontal cortex (Bowen *et al*, 1989) as well as in the caudal aspects of the DRN (Arango *et al*, 2001) of post mortem brains. Positron emission tomography (PET) imaging studies found that 5-HT_{1A} receptor binding was reduced

both pre-synaptically in the DRN, and post synaptically in cortical and limbic areas as compared to healthy subjects (Drevets *et al*, 1999; 2000). In contrast, higher 5-HT_{1A} binding was detected both pre- and post-synaptically (Parsey *et al*, 2006).
Early preclinical studies have shown that sustained treatment (14 days) with the selective serotonin reuptake inhibitor (SSRI) fluoxetine (FLX) increased extracellular levels of 5-HT in the striatum and hippocampus measured using in vivo microdialysis (Kreiss and Lucki, 1995). In the same study, repeated FLX treatment attenuated the ability of the 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) to decrease 5-HT release in both striatum and hippocampus (Kreiss *et al*, 1995). Later studies showed that chronic treatment with FLX also desensitized DRN 5-HT_{1A} autoreceptors as measured by receptor autoradiography (Hervas *et al*, 2001). These results suggested that repeated treatment with antidepressant drugs increases extracellular levels of 5-HT and possibly desensitizes the 5-HT_{1A} receptors.

1.6.2.2 The Noradrenaline System

Findings regarding NA, its metabolites, and pre- and postsynaptic adrenergic receptor dynamics in relation to mood disorders vary greatly. Hypersecretion of NA in plasma and CSF has been reported in unipolar depression and anxiety states (Wyatt *et al*, 1971; Roy *et al*, 1988; Sevy *et al*, 1989), in contrast to the early monoamine hypothesis (Schildkraut, 1965). NA receptor kinetics have been shown to be altered in depression and anxiety states. For example, altered α_2 binding in depression led to the "supersensitivity hypothesis", proposing that supersensitive presynaptic α_2 receptor activity resulted in decreased overall NA activity (Charney *et al*, 1981). Increases in α_2 receptors and β-adrenergic receptors binding in post-mortem tissue have also been reported (Mann *et al*, 1986; Meana *et al*, 1992), indicating possible receptor supersensitivity. Increases in tyrosine hydroxylase (TH) expression (Ordway *et al*, 1994a), α_2 receptor density (Ordway *et al*, 1994b), and decreased NA transporter density (Klimek *et al*, 1997) have also been demonstrated.
Studies in rodents have shown decreased β-adrenergic receptor numbers and function in rat cortex after chronic treatment with desipramine (DES) or electroconvulsive shock (Heal *et al*, 1987; 1989). Moreover, the α_2 receptor is reported to be desensitized by repeated treatment with DES without changing its density or binding affinity (Sacchetti *et al*, 2001), in agreement with the original idea of α_2 receptor supersensitivity in MDD.
In conclusion, abnormal NA turnover coupled with increased receptor sensitivity might be part of the mechanisms underlying depression. The NA levels may indeed be lower than normal under basal firing conditions, but that rapid increase in LC firing in response to stress may lead to a greater than normal NA transmission due to supersensitive receptors (Ressler and Nemeroff, 2000; Harro and Oreland, 2001).

1.7 Brain Areas Implicated in MDD

1.7.1 The Hippocampal Formation

The hippocampal formation comprises the dentate gyrus (DG) and the hippocampus proper with three subdivitions: CA1, CA2, and CA3 (CA=cornu ammonis), subiculum (S), presubiculum (PrS), parasubiculum (PaS) and entorhinal cortex (EC). The DG receives input from the EC via the perforant pathway. The EC terminals innervate the dendrites of the granular cells of the DG (in the molecular layer) (Cajal, 1893; Andersen *et al*, 2007). The granular cells of the DG project to the CA3 pyramidal cells via the mossy fibers (Blackstad *et al*, 1970; Swanson *et al*, 1978; Andersen *et al*, 2007). The pyramidal cells of CA3 then project to the pyramidal cells of the CA1 via Schaffer collaterals,

completing the trisynaptic circuitry. CA1 projects to the subiculum, the main output station of the hippocampus (see below). The major excitatory neurotransmitter in the trisynaptic circuit is glutamate. For information about the involvement of hippocampus in MDD, see section 1.3.3.

1.7.2 Subiculum

The subiculum is the major source of efferent projections from the hippocampal formation (Swanson and Cowan, 1975). The cytoarchitectonic characteristics of subiculum are similar to other hippocampal subfields (CA1, CA3, etc.), that is three layers: pyramidal layer, molecular layer and polymorph layer (Andersen *et al*, 2007). The CA1 pyramidal cell axons project to the pyramidal and molecular layers of the subiculum (Amaral *et al*, 1991). The pyramidal cell layer of the subiculum then projects to the EC. Thus, the EC is not only the main entry point to the hippocampal formation, but it also relays processed information back to cortex (Andersen *et al*, 2007). Further, the subiculum gives rise to prominent projections to the medial and ventral orbitofrontal cortices, the prelimbic and infralimbic cortices and the anterior olfactory nucleus (Verwer *et al*, 1997). The anterior cingulate, retrosplenial and the perirhinal cortices also receive inputs from the subiculum. Subiculum also projects to the PrS and PaS. There is some controversy regarding whether or not the PrS and subiculum (and PaS) should be grouped together (Andersen *et al*, 2007). The PrS and PaS have a similar cytoarchitectonic organization as the EC, that is multilaminated (typically six layers). Further, PrS and PaS connect with the anterior thalamic nucleus. Given the anatomical and connectivity differences, the subiculum, the PrS and PaS should not be considered as one functional group.

Subiculum is implicated in stress integration through its connections to the HPA axis. Depending on the modality and/or intensity of the stressor in question, the role of the ventral subiculum (VS) in the HPA-axis regulation can vary (Mueller *et al*, 2006). Lesions restricted to the VS can increase corticotrophin releasing hormone (CRH) mRNA and peptide expression in the paraventricular nucleus (PVN), and increase corticosterone responses following novelty and restraint stress (Herman *et al*, 1998). Anatomical studies have shown an indirect pathway from the subiculum to the principal stress-effector, the CRH producing neurons of the PVN.Tracing data indicate that glutamatergic neurons in the VS innervate GABAergic neurons in the anterodorsal, dorsal and intrafasicular subnuclei of bed nucleus of the stria terminalis (BNST) (Canteras and Swanson, 1992; Cullinan *et al*, 1993), which in turn innervate the PVN. There are reports implicating GABAergic mechanisms in the negative control of the HPA-axis. For example, intracerebroventricular (i.c.v) infusion of GABA has been shown to inhibit CRH release (Plotsky *et al*, 1987), as well as ACTH secretion (Makara *et al*, 1978). In addition, in vitro studies have demonstrated that GABA inhibits basal CRH secretion (Jones *et al*, 1976), and that GABA also antagonizes serotonin- and acetylcholine-induced CRH release (Calogero *et al*, 1988; Hillhouse and Milton, 1989).

1.8 Neuropeptides

More than 100 small peptides, ranging from 3-100 amino acid residues, have been discovered during the past 30 years (Hökfelt *et al*, 2003). The first neuropeptide, substance P, was identified by von Euler and Gaddum in 1931, but its exact chemical structure was not described until 1971 (Chang *et al*, 1971). Neuropeptides are always formed from a larger precursor polypeptide (prepro-peptide) by specific cleavage enzymes that recognize pairs of amino acid residues. Neuropeptides are synthesized and released from neurons in the CNS and peripheral nervous system (PNS) and they almost always coexist with classic neurotransmitters (Hökfelt *et al*, 1980). They can function as neurotransmitters, hormones, and growth factors, and their actions are mediated through GPCRs. Another common feature shared by some neuropeptides is the presence of an amide group at the C-terminal (instead of

the usual carboxylic acid), which provides protection against enzymatic degradation and is required for biological activity. This property has been used to detect novel neuropeptide structures as in the case of galanin. Much evidence indicates that neuropeptides are of particular importance when the nervous system is challenged, e.g. by stress, traumatic events, injury, or drug abuse, modulating the activity of co-expressed neurotransmitters (Hökfelt *et al*, 2003). Binding affinities of neuropeptides are commonly in the nanomolar range, that is a thousand–fold or more higher than the classical neurotransmitters. Consequently the selectivity of neuropeptide receptors is high, possibly making pharmacological interventions with antagonists less prone to side-effects. These features and the large number of neuropeptides and neuropeptide receptors provide many opportunities for the discovery of new drug targets for the treatment of disorders affecting the nervous system.

A wide range of physiological processes and possibly associated pathologies have been attributed to neuropeptides (Hökfelt *et al*, 2003), including nociception (Saria, 1999; Xu *et al*, 2000), feeding (Leibowitz, 1995; Kuhar and Dall Vechia, 1999), mood (Fuxe *et al*, 1998; Ögren *et al*, 2006; Refojo and Holsboer, 2009), alcohol intake and addiction (Van Ree *et al*, 2000; Heilig and Thorsell, 2002), sleep (Kilduff and Peyron, 2000; Willie *et al*, 2001) and learning and memory (Ögren *et al*, 1999).

A number of neuropeptides have been identified as potential targets for the development of antidepressant drugs (see Table 1). For instance, it has been reported that neurokinin 1 antagonist is as efficient as an SSRI in the treatment of depression (Kramer *et al*, 1998). Other neuropeptides implicated in mood regulation and anxiety are neuropeptide Y (NPY), CRF, nociceptin and galanin (Table 1). The focus of this thesis is galanin's role in mood and mood disorders.

Table 1. Neuropeptide receptors as potential targets for the treatment of mood disorders.

Peptide	**Receptor(s)**	**Mouse model and behavioral phenotype**	**Potenial drug candidate and effect in rodent models of MDD**	**References**
Substance P	NK_1	Null mutant mice-antidepressant-like	NK_1antagonists-antidepressant-like	(Rupniak *et al*, 2001) (Kramer *et al*, 1998)
Corticotropin-releasing hormone (CRH)	CRF_1	Null mutant mice-antidepressant-like	CRF_1antagonists-antidepressant-like	(Timpl *et al*, 1998) (Nielsen *et al*, 2004)
Neuropeptide Y (NPY)	Y_1, Y_2	Y_2 null mutant mice anxiolytic-like	Y_2agonists-antidepressant-like	(Redrobe *et al*, 2002)
Galanin	GalR3	Galanin overexpressing mice-depression-like	Nonselective galanin antagonists M15 and M35-antidepressant-like GAL3 antagonists-antidepressant-like	(Swanson *et al*, 2005) (Kuteeva *et al*, 2005) (Weiss *et al*, 2005)
Nociception	NOP	Null mutant mice-antidepressant-like	NOP antagonists antidepressant-like	(Gavioli *et al*, 2003) (Trapella *et al*, 2006)

1.8.1 What is Different About Neuropeptides?

Neurotransmitters, like monoamines, e.g. NA and 5-HT, are synthesized in cell bodies and nerve terminals from diet-derived amino acids by several intracellular enzymatic steps. Neuropeptides, on the contrary, are formed in the cell body or dendrites of a neuron. The synthesis takes place on ribosomes and the created prepro-peptide is packaged into vesicles in the Golgi apparatus and then cleaved by specific enzymes to the bioactive peptide. Further, classic neurotransmitters are mainly stored in small synaptic vesicles (40-60 nm in diameter), accumulated close to the active zone of the synapse, whereas neuropeptides are packed in large dense core vesicles (LDCVs, 90-250 nm in diameter) are located away from the active zone. The LDCVs are transported from the cell body of a neuron to the axonal terminal through fast axonal transport. The release of neuropeptides usually requires a high frequency or burst activity for exocytosis as compared to classic neurotransmitters, but like the monoamine neurotransmitters, their release is Ca^{2+} dependent (Lundberg and Hökfelt, 1986).

1.8.2 Galanin and its Localization in Neuronal Circuits Implicated in Mood

Galanin was initially isolated from extracts of porcine gut by Tatemoto, Mutt and collaborators in 1983 at Karolinska Institutet (Tatemoto *et al*, 1983). Galanin is a 29 amino acid (aa) residues peptide with an amidated C-terminal. It consists of 30 aa residues in humans, lacking the C-terminal amide (Evans and Shine, 1991). The actions of galanin are mediated via three receptor subtypes: galanin receptor 1 (GalR1), galanin receptor 2 (GalR2), and galanin receptor 3 (GalR3), widely expressed in the brain (O'Donnell *et al*, 1999; Waters and Krause, 2000). As mentioned earlier, all these receptors are GPCRs, mediating both inhibitory and stimulatory effects, thus contributing to the complex changes in the activity of neurons expressing these receptors.

Galanin is widely distributed in the CNS and the PNS in different species (Rökaeus *et al*, 1984; Skofitsch and Jacobowitz, 1985; Melander *et al*, 1986a; Kordower *et al*, 1992; Perez *et al*, 2001). In the rat, high levels of galanin immunoreactivity were found in a number of brain regions, e.g. amygdala, hypothalamus, septum and LC, while e.g. the hippocampal formation contain lower levels of galanin (Skofitsch and Jacobowitz, 1986) (See Figure 1).

Importantly, galanin is synthesized in 5-HT DRN (Melander *et al*, 1986b; Xu *et al*, 1998c; Larm *et al*, 2003) and NA LC (Melander *et al*, 1986b; Holets *et al*, 1988; Xu *et al*, 1998b) neurons, thus coexisting with these monoamines. Galanin also coexists with other neurotransmitters and peptides such as acetylcholine, e.g. in the basal forebrain (Melander *et al*, 1985; Dutar *et al*, 1989; Senut *et al*, 1989) and substance P and enkephalin in the tuberomammilary nucleus (Köhler *et al*, 1986).

The extensive anatomical distribution of galanin led to the suggestion that this neuropeptide is involved in a variety of physiological processes and possibly associated pathologies, including feeding behavior (see Leibowitz, 1995), nociception (Xu *et al*, 2008), learning and memory processes (see Ögren *et al*, 1998; Crawley, 2008) and anxiety- and depression-like behaviors (Fuxe *et al*, 1998; Weiss *et al*, 1998; Karlsson and Holmes, 2006; Ögren *et al*, 2006; Lu *et al*, 2007).

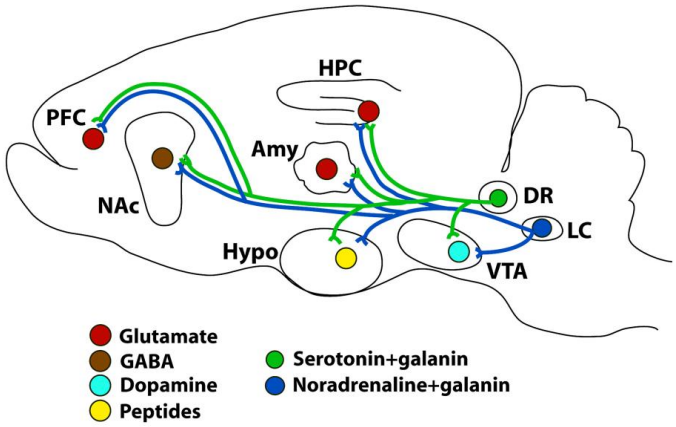

Figure 1. Galanin in neuronal circuity of mood (Courtesy of Eugenia Kuteeva).

1.8.3 Galanin Receptors

As many other neuropeptide receptors, galanin receptors belong to the group of 7-transmembrane (7-TM) GCPRs, of which three have been cloned, GalR1-3 (Branchek *et al*, 2000) (Figure 2). As mentioned previously, galanin and its receptors may be of considerable interest in relation to major depression, as galanin coexists with 5-HT in the DRN as well as with NA in LC and has an inhibitory action on both NA and 5-HT neurons (Pieribone *et al*, 1998).

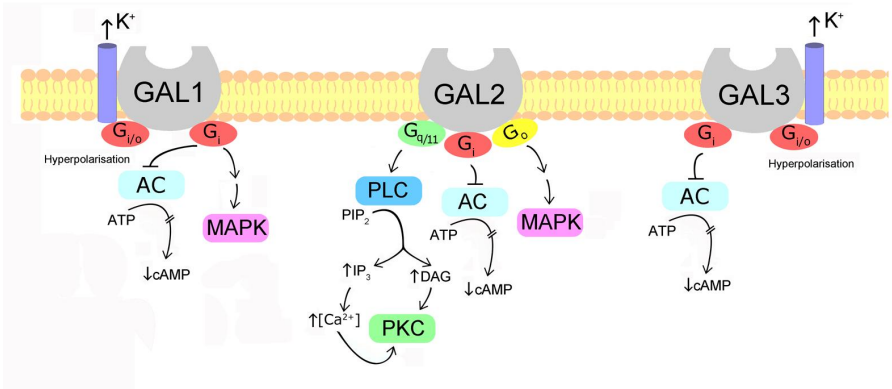

Figure 2. Schematic illustration of the three galanin receptor subtypes (GalR1, GalR2 and GalR3). **AC** = adenyl cyclase; **ATP** = adenosine triphosphate; **cAMP** = cyclic adenosine monophosphate; **DAG** = diacylglycerol; **G** = G protein; **IP_3** = inositol triphosphate; **MAPK** = mitogen activated protein kinase; **PIP_2** = phosphatidyl 4,5-biphosphate; **PKC** = protein kinase C; **PLC** = phospholipase C Modified from *T.P. Iismaa and J.Shine* (Results Probl Cell Diff 1999; 26:257-291), with kind permission from the authors and Springer Science and Business Media. (Illustration by Sofia Wadenberg).

1.8.3.1 The Galanin Receptor 1

The GalR1 was isolated from the human Bowes melanoma cell line (Habert-Ortoli *et al*, 1994), and then the rat receptor was cloned (Burgevin *et al*, 1995; Parker *et al*, 1995). The GalR1 receptor is coupled to G_i/G_o types of G-proteins, which means that the activation of the receptor: i) inhibits AC activity, resulting in reduction of the concentration of cAMP, ii) opens G-proteins coupled K^+ channels, inducing hyperpolarisation, and iii) stimulates MAP-kinase activity (Figure 2). Thus GalR1 primarily mediates inhibitory action, in the brain (Iisma and Shine, 1999; Branchek et al., 2000)

Using *in situ* hybridization, GalR1 was detected in the brain in hypothalamus, amygdala, thalamus, ventral hippocampus, medulla oblongata nuclei and in the dorsal horn of the rat spinal cord (Burgevin *et al*, 1995; O'Donnell *et al*, 1999). Importantly, a moderate expression of GalR1 was detected in the rat LC using *in situ* hybridization and a low level of mRNA expression in the DRN region (O'Donnell *et al*, 1999). An immunohistochemical study reported high levels of GalR1-like immunoreactivity in catecholaminergic nuclei of the mouse brain (Hawes and Picciotto, 2004). In DRN, GalR1-like immunoreactivity was detected in the rat but not the mouse (Larm *et al*, 2003).

1.8.3.2 The Galanin Receptor 2

The GalR2 was isolated from rat hypothalamus extract (Howard *et al*, 1997; Smith *et al*, 1997; Wang *et al*, 1997a). This receptor is either coupled to G_i/G_o types or G_q/G_{11} types of G-proteins (see Figure 2), which means that this subtype of galanin receptors can mediate inhibitory as well as stimulatory effects. When coupled to G_i/G_o, the stimulation of GalR2 reduces the concentration of cAMP and stimulates MAP-kinase pathway. On the other hand, when coupled to G_q/G_{11}, the receptor activates the PLC pathway, resulting in an increase of intracellular Ca^{2+}, stimulating neurotransmitter release (Iismaa and Shine, 1999; Branchek *et al*, 2000).

The distribution of GalR2 is also widespread within the rat CNS but is different from that of GalR1. The dorsal root ganglia expresses the highest level of GalR2 in the rat (O'Donnell *et al*, 1999). In the brain, GalR2 is expressed at high levels in the DG, hypothalamus and amygdala (O'Donnell *et al*, 1999; Waters and Krause, 2000), while low levels of GalR2 mRNA were detected in the rat LC and in the DRN region (O'Donnell *et al*, 1999). The presence of a GalR2 protein has been reported in the mouse brain but not in the DRN (Hawes *et al*, 2004).

1.8.3.3 The Galanin Receptor 3

Similar to GalR1, GalR3 mediates inhibitory effects via activation of G_i/G_o types of G-proteins, and inhibition of the AC activity (see Figure 2) (Iismaa *et al*, 1999; Branchek *et al*, 2000).

GalR3 is widely distributed in peripheral tissue such as heart, spleen, liver, stomach as well as the the kidney (Wang *et al*, 1997b; Smith *et al*, 1998; Waters and Krause, 2000). GalR3 transcripts were detected in the hypothalamus, pituitary, cerebral cortex and olfactory bulb (Smith *et al*, 1998; Waters and Krause, 2000).

1.8.4 Modulatory Effects of Galanin on 5-HT and NA Neurotransmission

1.8.4.1 The Serotonin System

Numerous studies suggest that exogenous galanin can modulate both 5-HT and NA systems. In the late 1980s, evidence was obtained that the neuropeptide galanin can interact with central 5-HT neurons at both the pre- and at the post-synaptical level (Fuxe *et al*, 1988a,b). I.c.v. injection of porcine galanin was found to reduce 5-HT metabolism in the limbic forebrain of the rat, an effect that was suggested to be due to an inhibitory action of galanin at the level of the raphe nuclei (Fuxe *et al*, 1988a). Recent studies have shown that i.c.v. galanin causes a long-lasting and dose-dependent

reduction of basal extracellular 5-HT levels in the ventral hippocampus of awake rats as measured by microdialysis (Kehr *et al*, 2002). Local administration of galanin in the vicinity of the DRN indicates that this reduction is mediated by galanin receptors acting at the level of the DRN, an effect blocked by the unselective galanin receptor antagonist M35 (Kehr *et al*, 2002). In the same study, galanin reduced the levels of tryptophan hydroxylase (TPH) mRNA in the DRN. Other studies showed that infusion of a GalR1/R2 agonist into the DRN in both rats and mice reduced 5-HT concentrations in the DRN and hippocampus as measured by immunofluorescence or High-Performance Liquid Chromatography (HPLC) (Mazarati and Lu, 2005). In contrast, injection of a GalR2(R3) agonist increased serotonin concentration in both areas. Swanson et al. (2005) showed the GalR3 antagonist SNAP 37889 partially antagonized the galanin-evoked reduction in hippocampal 5-HT, as did the 5-HT_{1A} receptor antagonist WAY100635 (Swanson et al, 2005). Their combination produced a complete reversal of the effect of galanin. Another GalR3 antagonist, SNAP 398299, also partially reversed the galanin-evoked inhibition of DRN cell firing and galanin-evoked hyperpolarizing currents. These results indicate that galanin receptors might have differential role in regulating the 5-HT neurotransmission.

Studies suggest a possible interaction between galanin and 5-HT_{1A} receptors. The affinity of presynaptic 5-HT_{1A} receptors was significantly and time-dependently reduced by i.c.v galanin infusion (Razani *et al*, 2000). The 5-HT_{1A} receptor mRNA levels in the DRN were also reduced in rats treated with i.c.v galanin (Razani *et al*, 2000). These data provide evidence for a modulatory action of in vivo galanin on presynaptic 5-HT_{1A} receptors. Behavioral studies have shown a possible regulatory effect of galanin on postsynaptic 5-HT_{1A} receptors. In the passive avoidance (PA) paradigm, which is a reliable test for assessing the role of 5-HT in forebrain systems (Ögren *et al*, 1985), i.c.v. galanin attenuated the impairment in PA retention caused by prior treatment with the 5-HT_{1A} agonist 8-OH-DPAT (Misane *et al*, 1998; Razani *et al*, 2001). These findings give support for the hypothesis that 5-HT_{1A} and galanin can interact at pre- and postsynaptic sites, and that this interaction is mediated by different galanin receptors.

1.8.4.2 The Noradrenaline System

Galanin applied directly onto an LC neurons caused hyperpolarization, presumably by increasing the K^+ conductance (Seutin *et al*, 1989; Sevcik *et al*, 1993; Pieribone *et al*, 1995). M15, a galanin antagonist, blocked galanin-induced hyperpolarization of LC, indicating that these inhibitory effects are mediated by galanin receptors (Bartfai *et al*, 1991). It is likely that these effects are mediated by GalR1 receptors, since a mixed GALR1/R2 agonist but not a GalR2(R3) agonist evoked the same effects as galanin on LC neurons (Ma *et al*, 2001). In agreement, there is evidence for presence of both GalR1 and GalR2 mRNA in the LC (O'Donnell *et al*, 1999), where GalR1 may act as an autoreceptor.

Electrophysiological studies have also shown that galanin can inhibit NA release in the cortex, a projection area of the LC neurons. This suggests a presynaptic action on NA terminals, probably mediated via autoreceptor GalR2 on cortical NA/galanin terminals (Xu et al, 2001). In vivo microdialysis studies have provided evidence for a NA/galanin interaction (Yoshitake *et al*, 2003). Thus, i.c.v galanin infusion reduced basal NA levels in rat hippocampus. In addition, galanin transiently attenuated the effect of DES-induced increase in extracellular NA levels. These results suggest that i.c.v galanin attenuates both basal and antidepressant-induced accumulation of extracellular NA levels, most likely via a predominant inhibitory action on noradrenergic neurons in the LC.

1.8.5 Galanin Modulation of Rodent Depression-Like Behaviors

In the context of the above mentioned galaninergic modulation of monoamine neurons, it is relevant to study how galanin might affect depression-like behavior. Early work by Weiss and colleagues found that infusion of galanin into the dopaminergic ventral tegmental area (VTA) produced an increase in immobility time in the rat FST (Weiss *et al*, 1998; 2005), consistent with increased depression-like behavior. The nonselective galanin receptor antagonist M15 decreased immobility time in the FST, indicative of antidepressant-like effect when given intra-VTA (Weiss *et al*, 1998). In contrast, systemic injection with the non-peptide GalR3 antagonist SNAP 37889, produced antidepressant-like effects in different models of depression/anxiety (Swanson *et al*, 2005; Barr *et al*, 2006). Further, the Flinders Sensitive Line of rats (rats selected for high FST immobility) showed elevated levels of galanin binding in the DRN as well as reduced galanin-like immunoreactivity in the DRN and the hippocampus (Bellido *et al*, 2002).

Genetically engineered mice overexpressing galanin under the PDGF-B promoter (Holmberg *et al*, 2005) exhibit increased depression-like behavior and altered monoaminergic response in the FST (Yoshitake *et al*, 2004). However, GalR1 and GalR2 knockout mice display a normal phenotype in the tail suspension test (TST) (Gottsch *et al*, 2005; Holmes *et al*, 2005) (see Table 2).

In fact, the effect of galanin on depression-like behavior has not been consistent. Thus, there is evidence that increased galanin function may have antidepressant-like effects. For example, the non-peptide galanin agonists galmic and galnon decreased immobility time in the FST in rats after systemic (intraperitoneally, i.p.) treatment, suggesting an antidepressant-like effect of galanin receptor stimulation (Bartfai *et al*, 2004; Lu *et al*, 2005). In addition, levels of galanin mRNA in the LC as well as in the DRN were increased after chronic treatment with FLX (Lu *et al*, 2005).

A potential explanation for the complexity of galanin's effect on monoamine function and depression-related behaviors could be that galanin exerts its effects through the three functionally different receptors, GalR1-GalR3 (Branchek *et al*, 2000). These receptors have different but partially overlapping distribution in midbrain and limbic areas mediating emotional behaviors (Iismaa *et al*, 1999; O'Donnell *et al*, 1999; Branchek *et al*, 2000; Waters and Krause, 2000).

Table 2. Studies on pharmacological and genetic manipulations of galanin in depression behavior models.

Treatment/mutation	Species	Behavioral paradigm	Behavioral effect	References
Galanin i.c.v.	Rat	FST	None	(Weiss *et al*, 1998)
Galanin intra-VTA	Rat	FST	Prodepressive	(Weiss *et al*, 1998)
Galanin antagonist (M15) intra-VTA	Rat	FST	Antidepressant-like	(Weiss *et al*, 1998)
Galanin intra-hypothalamus	Rat	FST	None	(Weiss *et al*, 1998)
Galanin i.c.v.	Mouse	TST	None	(Holmes *et al*, 2005)
Galanin agonist (galmic)	Rat	FST	Antidepressant-like	(Bartfai *et al*, 2004)
Galanin agonist (galnon)	Rat	FST	Antidepressant-like	(Lu *et al*, 2005)
GalR3 antagonist (SNAP 37889)	Rat	FST	Antidepressant-like	(Swanson *et al*, 2005)
Galanin overexpression (DβH promoter)	Mouse	FST	None	(Holmes *et al*, 2005)
Galanin overexpression (PDGF-B promoter)	Mouse	FST	Prodepressive	(Kuteeva *et al*, 2005)
GalR1 knockout	Mouse	TST	None	(Holmes *et al*, 2005)
GalR2 knockout	Mouse	TST	None	(Gottsch *et al*, 2005)

2. AIMS OF THE THESIS

- To study the effect of galanin and galanin receptor ligands on depression-like behavior in rats (**papers I and II**).

- To investigate the possible interaction between exposure to stress and galanin receptor subtype stimulation by measuring the expression of neurochemical markers of relevance for depression (**paper II**).

- To develop a transgenic mouse overexpressing the GalR2 and to characterize the behavioral phenotype of this mouse with focus on depression-like behavior (**paper III**).

- To study the distribution of GalR2 transcripts and receptor protein in the GalR2OE mouse brain (**paper III**).

- To investigate whether the antidepressant-like effect of the SSRI FLX depends on the number of drug injections and their temporal relation to the swim stress (**paper IV**).

- To examine the effects of 5-HT reuptake inhibition on explorative behavior in the rat locomotor activity test, as well as the effect of exposure to acute swim stress on anxiety-like and social behavior (**paper IV**).

3. MATERIALS AND METHODS

3.1 Animals

Both adult mice and rats were used in the experiments. GalR2OE mice were generated at Karolinska Institutet as described below. At the time of experiment the mice were between 3-18 months old. Male Sprague-Dawley rats (10-12 weeks old), weighing 290-320 g at the time of surgery, were obtained from Scanbur (Sollentuna, Sweden).

GalR2OE and WT mice were housed in groups of two to six in standard plastic cages (Macrolon® Type A3). Rats were housed in groups of four in standard plastic cages (Macrolon® Type A4) until surgery, after which they were housed separately (two animals per cage separated by a plastic wall). All animals were kept in colony under standardized conditions (12 h light/dark cycle, lights on at 7 a.m.), temperature of 22±0.5°C and 40-50% relative humidity). Food and water were provided *ad libitum* up to the time of the experiment. Animals were allowed to habituate to the animal facility for at least five days before starting the experiments. In all behavioral experiments, animals were handled for at least three days prior to the experiments to reduce the influence of stress. Animal housing and experimental procedures followed the provisions and general recommendations of the Swedish animal protection legislation. All experimental procedures were approved by the local Animal Ethics committee (Stockholm Norra djurförsöksetiska Nämnd).

3.2 Generation of Transgenic Mice (Paper III)

The 1.3 kb PDGF-B promoter (Collins *et al*, 1985; Sasahara *et al*, 1991) was excised from the psisCAT6a plasmid with *Xba*I and *Hind*III (New England Biolabs Inc., Beverly, MA). The GalR2 gene construct, containing the restriction sites *Xba*l and and *Sac*ll, was ligated into the EGFP-N1 plasmid that was cut using *Nhe*I and *Sac*II. The EGFP-N1 plasmid contained a CMV promoter that was excised and replaced by the PDGF-B promoter. The GalR2 was created by polymerase chain reaction (PCR) from C57BL/6N mouse genomic DNA using PfuUltra II DNA polymerase (Stratagene) and the following primers: 5'-ATCCTCTAGAATACCATGAATGGCTCG GACAGC-3' and 5'-TATTCCGCGGACAGGCTGGATCGAGGGTTCTAC-3'. The linearized recombinant DNA was injected at a concentration of 2-3 ng/µl into pronuclei from fertilized mouse oocytes (Hogan *et al*, 1986). The transgenic mice were back-crossed to C57BL/6N strain for 7 generations. The experimental procedures were approved by the local ethics committee (Stockholm Norra Djurförsöksetiska Nämnd).

3.3 DNA Preparation and Genotyping

The transgenic status of animals was confirmed by PCR as described. Briefly, mouse-tail biopsies were used for genotyping and DNA was amplified by using PCR with specific primers for EGFP-GALR2 5´ GTCAACCCCATCGTTTATGCTCTGGTCTCC-3´ and 5´-TGGGTGCTCAGGTAGTGG TTGTCGG-3´ and Taq DNA polymerase (2.5 units; Sigma). Incubations were performed at 94°C for 20 s, at 62°C for 30s, and at 72°C for 1 min for 30 cycles and, finally, at 72°C for 10 min. The PCR product showed an 880-bp EGFP-GALR2OE band.

3.4 Surgical and Microinfusion Procedures

3.4.1 Stereotaxic Surgery in Rats (Papers I and II)

For **paper I** rats were anaesthetized with Hypnorm/Midasolam anaesthesia (fentanyl citrate 0.2 mg/kg, fluanisone 6.7 mg/kg, midasolam 2 mg/kg) and placed in a stereotaxic frame. For **paper II** rats were

anaesthetized with isoflurane (Apoteket, Stockholm, Sweden; induction 4.7%, maintenance 2.1–3.4% and airflow of 364-380 ml/min) using an anesthesia pump (AgnTho's, Lidingö, Sweden). The anesthetized rat was placed in a stereotaxic frame (David Kopff Instruments, Tujunga, CA), and body temperature was maintained at 37°C using a temperature-controlled heating pad (CMA/105, CMA/Microdialysis, Stockholm, Sweden). A permanent steel guide cannula (26 GA; diameter 0.45 mm, Plastics One, Roanoke, VA) was implanted into the right lateral ventricle at the following coordinates: 1.3 mm posterior to the bregma, 1.8 mm lateral to the mid-sagittal line and 3.7 mm ventral to the surface of the skull (infusion site 0.5 mm below the ventral coordinate) (Paxinos and Watson, 1998). The guide cannula was secured to the skull using three microscrews (AgnTho's) and dental cement (Dentalon®, AgnTho's). Finally, a dummy cannula was inserted into the guide cannula. Animals received post surgical pain relief as a single intramuscular injection of buprenorphin (Temgesic ® Shering-Plough AB, Stockholm, Sweden) at a dose of 0.05 mg/kg, as well as the local anaesthetic lidocaine (10 mg/dose spray; Xylocain ®; AstraZeneca, Södertälje, Sweden), which was sprayed onto the wound. Rats were given a single injection of saline to compensate for possible fluid loss during the surgery. The animals were allowed to recover for 6-7 days in the colony room before the start of the experiments, with a regular check of body weight.

3.4.2 Intracerebral Microinfusions (Papers I and II)

Microinfusions of peptidergic ligands were performed using a microinfusion pump (CMA/100, CMA/Microdialysis) and a Hamilton syringe (25 µl), which was connected through a plastic tubing to an injection cannula (33 GA; 0.2 mm in diameter; Plastic One) that was 0.5 mm longer than the guide cannula. The infusion rate was 0.5 µl/min with a duration of 2 min, after which cannula was left inside for additional 60 s to avoid back-flow. The animals were lightly held during the infusion procedure.

3.5 Compounds

The following compounds were used in the experiments: the antidepressant drugs DES and FLX (Sigma Aldrich, St Louis, MO), rat galanin (Bachem, Budendorf, Switzerland) and the chimeric galanin antagonist M35 (galanin-(1-13)-bradykinin-(2-9)-amide) (Bartfai et al., 1992) (Bachem, Budendorf, Switzerland), the GalR1 agonist M617 (galanin-(1–13)-Gln^{14}-bradykinin-(2–9)-amide) (Lundström et al., 2005), the GalR2(R3) agonist AR-M1896 (galanin-(2–11)-amide)(Liu et al., 2001), and the GalR2 antagonist M871 (galanin-(2–13)-Glu-His-$(Pro)_3$-$(Ala\text{-}Leu)_2$-Ala-amide) (Sollenberg et al., 2006).

The peptides were freshly dissolved in artificial cerebral fluid (aCSF), which was used as control, and the solutions were coded before i.c.v. infusion. All the compounds were stored in a refrigerator (4°C) or on ice until used. The concentration of galanin and the galanin antagonist M35 was calculated on basis of the purity of the peptide.

Table 2. Summary of galaninergic ligands/compounds used in the different experiments, route of administration, time interval and supplier. i.c.v.- intracerebroventricular, i.p.-intraperitoneal.

Paper	Compound/ peptide	Route of administration	Dose	Time interval	Supplier/ Reference
I,II	Galanin (rat)	i.c.v.	3 nmol	20 min	Bachem
I	M35	i.c.v.	1 nmol	90;20 min	Bachem
II	M617	i.c.v.	3 nmol	20 min	(Lundström *et al*, 2005)
II	M1896	i.c.v.	1 nmol	20 min	(Liu *et al*, 2001)
II	M871	i.c.v.	3 nmol	20 min	(Sollenberg *et al*, 2006)
I	DES-HCl	i.p.	10 mg/kg	23, 5, 1 h	Sigma Aldrich
I,II	FLX-HCl	i.p.	10 mg/kg	23, 5, 1 h	Sigma Aldrich

3.6 Histological Techniques

3.6.1 Tissue Preparation (Papers I, II and III)

For both oligo- and riboprobe *in situ* hybridization animals were killed by decapitation. The brains were rapidly dissected out, immersed in ice-cold 0.9% NaCl and immediately frozen on dry ice. For the histological control of cannula position in the right ventricle, sections were cut in a cryostat (Microm, Heidelberg, Germany) at 20 µm thickness, thaw-mounted on aluminum gelatin-coated slides, stained with cresyl violet and viewed in a microscope. For immunohistochemistry, mice were deeply anesthetized with sodium pentobarbital (60 mg/kg i.p.) and perfused through the ascending aorta via the heart with Tyrode's buffer (37°C), followed by fixative containing 4% paraformaldehyde and 0.2% picric acid diluted in 0.16 M phosphate buffer (pH 6.9; Pease, 1962; Zamboni and De Martino, 1967). The brains were dissected out and postfixed in the same fixative for 90 min at 4°C and finally immersed for 48 h in 10% sucrose dissolved in phosphate-buffered saline (PBS; pH 7.4) containing 0.01% sodium azide (Sigma) and 0.02% Bacitracin (Sigma) at 4°C, before rapid freezing by CO_2. Sections were cut in a cryostat (Microm, Heidelberg, Germany) at a thickness of 14 µm, and thaw-mounted on aluminum gelatin-coated slides.

Sections were cut in a cryostat (Microm) at 14 µm thickness and thaw-mounted on SuperFrost®Plus slides (VWR International AB, Stockholm, Sweden).

3.6.2 Immunohistochemistry (Paper III)

To investigate the cellular and regional distribution of receptor proteins and peptides in the mouse brain, the tyramide signal amplification (TSA) (Adams, 1992) method was applied using a commercial kit (TSA+, NENLife Scientific Products, Boston, MA). The sections were rinsed in phosphate-buffered saline (PBS) and incubated overnight at 4°C, in a humid chamber, with either rabbit anti-galanin (Theodorsson and Rugarn, 2000) or rabbit anti-GFP antiserum (Abcam, Cambridge, UK) diluted, respectively, 1:8,000 and 1:2,000 in PBS containing 0.3% Triton X-100 (Sigma), 0.02% Bacitracin (Sigma) and 0.01% sodium azid (Sigma). The sections were rinsed in Tris-NaCl-Tween (TNT) buffer for 15 min followed by blocking with Tris-NaCl-blocking reagent (TNB) buffer for 30 min at room temperature (RT), incubated with a secondary antibody, swine-anti-rabbit immunoglobulin (DAKO A/S, Copenhagen, Denmark) conjugated with horseradish peroxidase 1:200 in TNB, for 30 min at RT, and rinsed in TNT buffer for 15 min at RT. Finally, sections were incubated with Fluorophore Tyramide (FT) diluted 1:100 in amplification reagent (kit) for 10 min, rinsed in TNT

at RT for 15 min and mounted for fluorescence microscopy in 2.5% DABCO (1,4-diazabicyclo [2.2.2] octane) (Sigma).
For double-labeling, the immunohistochemical procedure for visualizing GFP using rabbit anti-GFP antiserum and the TSA procedure with FT-FITC was completed, and then the sections were incubated with rabbit anti-galanin with TSA visualization followed by FT-tetramethylrhodamine (TRM) conjugate.

3.6.3 Oligoprobe In Situ Hybridization (Papers II and III)

Oligonucleotide probes complementary to the mRNA of interest were labeled with ^{33}P (dATP) (NEN) at the 3'-end using terminal deoxynucleotidyltransferase (Amersham, Amersham, UK) and purified using ProbeQuant™ G-50 microcolumns (Amersham Pharmacia Biotech, Piscataway, NJ). Probes were added to the hybridization solution containing 50% of deionized formamide (Ambion Inc., Austin, TX), 4 ×standard saline citrate (SSC), 1 ×Denhardt's solution and 50 mg/l denaturated salmon testis DNA (Sigma). Sections were air-dried overnight and hybridized with the oligonucleotide probes (0.5 ng per slide) for 16–24 h at 42°C. After hybridization the sections were rinsed for 2 h (4 ×30 min) in 1 ×SSC at 55–60°C followed by 1 h at room temperature. Sections were then rinsed in distilled water, rapidly dehydrated in ethanol and air-dried for at least 2 h. For the control of specificity, an excess (100 ×) of the unlabeled probe(s) was added to the hybridization solution. This procedure completely abolished the hybridization signal. The sections were exposed together with ^{14}C standards (Amersham) to a Kodak Biomax MR film (Kodak, Rochester, NY). The films were developed in Kodak LX 24 and fixed in Kodak AL4, rinsed and air-dried.

3.6.4 Riboprobe In Situ Hybridization (Paper III)

For prehybridization the slides were preincubated for 4 to 6 h with a mix (1:1) of 2x prehybridization cocktail and deionized formamide. To prepare the ^{35}S-riboprobe, 7 µl of water, 4 µl of the DNA template, 2 µl of 10x transcription buffer, 3 µl of nucleotide mix, 2 µl of the ^{35}S-UTP and 2 µl of the RNA polymerase T7 or SP6 were mixed at RT. After incubation for 20 min at 37°C, 1 µl of TURBO DNAse was added, and the mix was incubated for another 15 min at 37°C, and finally 1 µl of 0.5 M EDTA was added to stop the reaction. The riboprobe solution was filtered in a NucAwayTM Spin Columns (Ambion), and counted for radioactivity. The hybridization solution contained: 50% formamide, 1X Grundmix, 0.33 M NaCl, 0.1 M DTT, 10% Dextran sulfate, the RNA probe at $5x10^6$ cpm/ml, and water to the right volume. Sections were covered with 200 µl of the hybridization cocktail and incubated for 16 h at 55°C. After hybridization, sections were washed with stirring as follows: 30 min in 1X SSC at 55°C (twice), 1 h in 50% formamide/0.5X SSC at 55°C, 15 min in 1X SSC at 55°C, 1 h in RNase A buffer at 37°C, 15 min in 1X SSC at 55°C (twice), then the slides were dehydrated for 2 min in 50% ethanol, 2 min in 70% ethanol and 2 min at 100% ethanol. Slides were finally air dried for at least 1 h and then put against x-ray film or dipped in liquid emulsion.

Table 3. Summary of oligoprobes and riboprobes used for *in situ* hybridization.

Paper	Neurotransmitter/ Enzyme	Nucleotides	Species	Reference
II	Galanin	152-199	Rat	(Vrontakis *et al*, 1987)
II	Tyrosine hydroxylase (TH)	1441-1488	Rat	(Grima *et al*, 1985)
II	Tryptophan hydroxylase 2 (TPH2)	489-536	Rat	(Walther *et al*, 2003)
II	5-HT_{1A}receptor	762-809, 1048-1095	Rat	(Albert *et al*, 1990)
II	5-HT transporter (5-HTT)	305-352	Rat	(Hoffman *et al*, 1991)
III	GalR2	1364-1675	Mouse	Paper III

3.6.5 Dipping Procedure (Paper III)

Slides were exposed to an autoradiographic emulsion for 10 sec, and dried for 3 h at RT, and stored in boxes with silica gel in a cold room. After varying exposure times, boxes were moved to RT in front of a fan for 1 h. The slides were dipped in D19 developer (Kodak) for 3 min, in water for a few seconds and for 7 min in AL4 fixative (Kodak), followed by rinsing under tap water for 30 min, dried, and mounted with glycerol (90%)/PBS (10%) medium.

3.6.6 Imaging and Density Measurements (Papers II and III)

Sections were analysed using a Nikon Eclipse E600 microscope equipped with a dark filter condenser and epi-polarization, and epi-fluorescence with appropriate filters combinations connected to a digital camera (Nikon DXM 1200). In some cases, a Kodak T-MAX 400 black-and-white film was used for photography. Sections were then scanned using a Nikon LS-2000 film scanner (Nikon corporation, Tokyo, Japan). Scanned and digital images were imported into Adobe PhotoShop 6.0 (Adobe System Incorporated, San Jose, CA) and optimized for brightness, contrast and sharpness.

In paper II, analysis of film autoradiograms was carried out by computerized microdensitometry using NIH Image 1.63 software after scanning films. ^{14}C standards (Amersham) coexposed with the slides were used to monitor of radioactivity (nCi/g). Measurements were performed on four sections for the LC (left and right independently), and on 4–6 sections for the DRN and MR, and the mean values were calculated for each animal. Since there exist significant anterior–posterior differences in the expression of various genes in the DRN the analysis was performed on sections taken at identical levels (mid-rostra), the area projecting to the forebrain. The DRN, was subdivided into lateral (lDRN), dorsal (dDRN), and ventral (vDRN) subnuclei, which were each assessed individually.

3.7 Behavioral Experiments

All behavioral tests were performed between 8 a.m. and 3 p.m. Animals were brought to the experimental room and were allowed to habituate to the room for at least 60 min prior to experiment.

The following behavioral methods were used to examine various domains of anxiety and depression-like behaviors in rats and mice and to assess the behavioral consequences of mutations in transgenic mice.

3.7.1 Locomotor Activity (Paper IV)

The examination of spontaneous locomotor activity and exploratory behavior (rearing) was conducted by means of a multicage red and infrared-sensitive motion detection system (Ögren *et al*, 1986). The

system is fully computerized and uses beams of red and infrared lights in combination with vertical (infrared light sensitive photocells in the walls of the apparatus) and horizontal (red light sensitive photocells in the floor of the apparatus) arrays. The distance between photocells is 4 cm. Locomotor activity was measured as all movements of a distance of 4 cm or more detected by 48 vertical photocells, and represents a measurement of general activity. Rearing was measured by counting the number of times an animal stands on its hind limbs. Rats were individually tested in an activity monitor (a standard transparent type III Macrolon® cage with 50 ml of wooden shavings on the floor) linked to a data collecting system, which was activated immediately after the animal was placed in the activity box.

3.7.2 Anxiety-Like Behaviors

3.7.2.1 Social Interaction (Paper IV)

Social interaction (SI) has been defined as active or passive (File and Hyde, 1978), but in this thesis only active social interaction was studied. The SI test was carried out according to methods described previously with minor modifications (Toth *et al*, 2008). Each experimental animal (resident rat) was tested with a partner rat (target rat), whose body weight differed no more than 45 g. The partner animal was a socially housed male rat that was not subjected to any stress and did not receive any treatment before. During the 3 min test, the following social behaviors were scored: sniffing the partner, contact interaction (physical contact with mutual responses and orientation toward the other), following the partner, climbing over or burrowing under it, and walking around it (social behaviors). Boxing, biting, or threatening the partner rat are classed as aggressive behaviors, self-grooming and remaining alone, away from the partner as non-social behaviors. The total duration of social and non-social behavior for the resident rat was recorded by the operator.

3.7.2.2 Open Field (Papers III and IV)

The open field (OF) test can be considered a non-conditioned anxiety test based on the creation of a conflict between the exploratory drive of the rat and its innate fear of exposure to an open area (Broadhurst, 1975; Angrini *et al*, 1998). This test was used to study general locomotor activity as indicated by total distance travelled, exploratory behavior as measured by total number of visits/crossings (transition between quadrants) and "anxiety" as measured by percentage of time spent in the center of the OF. The OF test can be considered as a non-conditioned anxiety test based on the creation of a conflict between the exploratory drive of the rat and its innate fear of exposure to an open area (Broadhurst, 1975; Angrini *et al*, 1998). Animals were placed individually in the center of the OF arena located in a dimly lit room and allowed to explore for 3 min (rats) or 5 min (mice). The measures of the OF arena differed between rats and mice (for more details, see **papers III and IV**). The behavior of animals were scored for rats by the experimenter or mice by a TES digital analyzing system based on a video camera connected to a PC-compatible software (TSE, Hamburg, Germany). The arena was cleaned with 70% ethanol between each animal.

3.7.2.3 Elevated Plus Maze (Paper III)

The elevated plus maze (EPM) is an etiologically-based animal model of anxiety-related behavior (Rodgers *et al*, 1997) that does not require any previous training. Rodents have an innate tendency to avoid open or brightly lit areas, and this behavior is related to a safety assessment strategy. The apparatus, which is a plus-shaped maze, is made from dark grey glacial polyvinyl chloride and consists of four arms (each arm 30×5 cm) and a central area (5×5 cm) elevated 1 m above the floor (Figure 3). Two arms were open and two closed, with 10-cm high walls made from the same material.

Mice were individually placed in the center facing an open arm and allowed to explore for 5 min. Total distance travelled during the experiment, number of crossings between closed and open arms, as well as time spent in the open and closed arms were detected using the TSE video-tracking system (TSE). The percentage of time spent in the open area (including the central arena) was used to describe anxiety-like behaviour. The apparatus was cleaned with 70% ethanol after each tested animal.

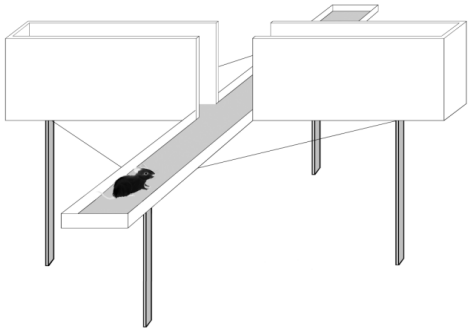

Figure 3. The Elevated Plus-Maze (Illustration by Sofia Wadenberg).

3.7.3 Learning and Memory

3.7.3.1 Passive Avoidance (Paper III)

Passive avoidance (PA) involves both the hippocampal and amygdala (Ögren et al., 2008). The task is used to evaluate emotional memory and is based on Pavlovian conditioning. The experimental procedure was performed as described earlier for rats (Misane and Ögren, 2000) and later modified for mice (Madjid *et al*, 2006). The step-through PA box (10x16x18 cm, Ugo Basile, Comerio-Varese, Italy) consisted of two equal-sized compartments, with stainless-steel grid floor and adjoined with a sliding door (4x4 cm) (Figure 4). The compartments were made of white or black plastic, with black plastic as ceiling of the dark box, and clear plastic ceiling in the light compartment, illuminated by a lamp bulb (24 V, 5 W). The light intensities in the two compartments were 3 lx (dark compartment) and 330 lx (light compartment). PA training was conducted as a single trial, during which the mouse firstly explored the light compartment for 120 s, followed by opening of the sliding door. Upon the animal's entering to the dark compartment, the door was closed and a weak electrical current (scrambled current, 0.3 mA, 2 s) was delivered through the grid floor, serving as an unconditioned stimulus (US).

After delivery of the US, the mice were kept in the dark compartment for an additional 30 s before being removed. This procedure allows the mice to strengthen the association between the dark context and the US, and to avoid US-experimenter association (Madjid *et al*, 2006). After the mouse was removed from the PA box, it was temporarily transferred to a clean holding cage with non-tested animals.

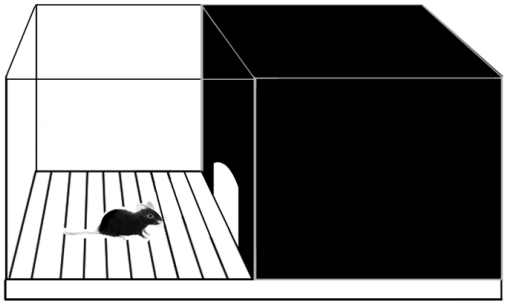

Figure 4. The passive avoidance apparatus (Illustration by Sofia Wadenberg)

3.7.4 Depression-Like Behavior

3.7.4.1 Forced Swim Test (Papers I, II, III and IV)

Most rodent models of depression-related behaviors are based on exposure to acute inescapable stressful events such as the TST, the FST or the chronic mild stress paradigms (CMS). The FST described originally by Porsolt (Porsolt *et al*, 1977; Porsolt *et al*, 1978b) is the most frequently used behavioral paradigm in rodents for assessing antidepressant activity. The FST is based upon the failure to maintain escape-directed behavior (swimming and climbing), in response to inescapable swim stress (Cryan *et al*, 2005b). In this thesis depression-like behavior was assessed in the FST (Porsolt *et al*, 1977; Detke and Lucki, 1996). Animals were individually placed in a vertical glass cylinder (25 cm high, 13 cm diameter for mice and 50 cm high, 18 cm in diameter) containing tap water (25±0.5 °C, 16 cm for mice and 30 cm for rats) (Figure 5). To increase the sensitivity of the test (Borsini *et al*, 1989), two swimming sessions were conducted: a 15 min (**papers I and III**) or 10 min (**papers II and IV**) pre-test followed 24 h later by a 5 min test (rats, **papers I, II, and IV**) or 6 min test (mice, **paper III**). The total duration of immobility and climbing behavior was recorded during the second test. Immobility was defined as floating passively in an upright position in water, with only small movements necessary to keep the head above the water surface. Climbing was defined as vigorous forepaw movements directed toward the walls of the cylinder.

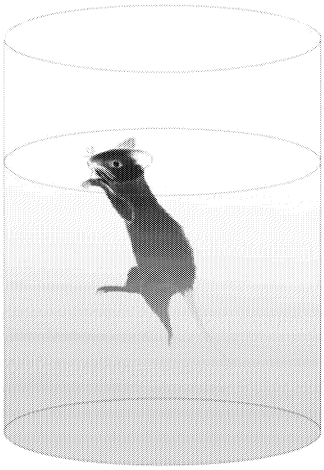

Figure 5. The forced swim test setup for rats and mice used in all experiments (Illustration by Sofia Wadenberg).

4. RESULTS AND DISCUSSION

4.1 Paper I

The pathophysiology of MDD involves dysfunction of the monoamine neurotransmitter circuits in the CNS, particularly 5-HT and NA. The current pharmacotherapy of MDD targets monoamine transporter proteins, thus blocking the reuptake of released transmitter from the extracellular space. The most frequently used antidepressants are either selective 5-HT reuptake inhibitors SSRIs such as FLX, or selective NA reuptake inhibitors SNRIs such as DES.

The exact mechanism(s) behind the dysfunction of monoamine systems (neurotransmission) is still largely unknown. However, recent studies have also implicated several neuropeptide systems in the pathophysiology of MDD. In this context the neuropeptide galanin (Tatemoto *et al*, 1983) is of particular interest, since it coexists both with 5-HT in the DRN and with NA in the LC. Moreover, several neurochemical, electrophysiological, and behavioral studies have shown that galanin can modulate the brain 5-HT and NA systems.

In view of these findings the purpose of paper I and II was to examine the effect of galanin and galanin receptor agonists/antagonists on depression-like behavior in rats.

4.1.1 Effects of Intracerebroventricular Galanin and the Unspecific Galanin Antagonist M35 on Depression-Like Behavior in the Rat Forced Swim Test

I.c.v galanin administration resulted in increased immobility time in the FST in the rat. This effect was probably mediated by galanin receptors, since co-infusion of the galanin receptor antagonist M35 blocked the effect of galanin on immobility time. M35 itself significantly reduced immobility time indicative of an antidepressant-like profile.

The increase in immobility time is interpreted either as the failure of persistence in escape-directed behavior or as the development of passive behavior (learned helplessness) (Cryan *et al*, 2002). Importantly, the failure of active coping with stressful stimuli represents a feature of the depressive syndrome, probably related to dysfunction of 5-HT and NA brain systems (Robbins, 2005).

Studies have shown that acute stress increases the release of endogenous galanin leading to facilitation or inhibition of anxiety-like behavior, depending on the area of limbic system involved (Khoshbouei *et al*, 2002). The increase of immobility during inescapable stress caused by galanin may represent an enhanced inhibition of on-going behavior, probably related to dysfunction of serotonergic and/or noradrenergic neurotransmission involved in coping mechanisms. It has been proposed that galanin released under stressful conditions from the LC terminals in the ventral tegmental area can inhibit activity of the mesolimbic dopaminergic system, resulting in manifestation of depression-like symptoms (Weiss *et al*, 1998; Weiss *et al*, 2005). This hypothesis is supported by the finding that mice overexpressing galanin are clearly more stress-sensitive (Kuteeva *et al*, 2005), indicative of a defect in the regulation of the response to stressful stimuli (Yoshitake *et al*, 2004).

Since galanin and galanin receptors are expressed in both the NA and 5-HT systems, it is possible that the role of galanin in depression-like behavior may, at least partially, be related to modulation of brain NA and/or 5-HT functions at the cell body level and/or their projection areas. In agreement, previous studies have shown that galanin is a potent inhibitory modulator of the 5-HT system in vivo under basal (non-stressful) conditions. Thus, i.c.v. galanin caused a significant long-lasting reduction of the basal (Kehr *et al*, 2002) and SSRI-induced (Yoshitake *et al*, 2003) 5-HT release in the hippocampus, as measured by in vivo microdialysis. Moreover, galanin decreased TPH2 mRNA expression in the DRN (Kehr *et al*, 2002), suggesting that galanin also inhibits 5-HT synthesis. In contrast to a profound

inhibition of 5-HT release, i.c.v. galanin only marginally and transiently decreased basal and DES-induced NA release in the hippocampus (Yoshitake *et al*, 2003).
In our study M35 given alone displayed antagonistic properties blocking the action of galanin. However, since M35 has equal affinities to all three receptors, it is not possible to attribute its action to a particular receptor subtype. A possible explanation for the action of M35 alone is that stress-related events increase galanin expression (Holmes *et al*, 1995; Sweerts *et al*, 1999) and galanin release in the DRN/LC and also in limbic areas of the brain. The blockade of galanin receptors by M35 possibly normalizes the DRN and LC cell activity and thereby the processing of stressful stimuli in the forebrain areas.

4.2 Paper II

It is critical to define which galanin receptor subtype(s) is involved in the action of exogenous galanin. Therefore, paper II focused on the effect of specific galanin receptor agonists/antagonists on depression-like behavior in rat. Additionally, changes in several chemical markers in the 5-HT and NA systems were studied in the DRN and the LC following the FST, antidepressant treatment, and galanin receptor activation/antagonism.

4.2.1 Galanin Receptor 1 Agonist, M617, in Depression-Like Behavior

Similarly to galanin, the GalR1 agonist M617 increased immobility time indicative of pro-depressive effects in the rat FST. *In situ* hybridization indicates the presence of GalR1-GalR3 in the DRN and LC (O'Donnell *et al*, 1999; Mennicken *et al*, 2002), but the exact cellular distribution (i.e. expression in the 5-HT and/or NA neurons) of these receptors in DRN is still uncertain. However, coexistence of 5-HT and GalR1-like immunoreactivity was reported in a subpopulation of cells in the rat DRN (but not mouse) (Larm *et al*, 2003) and in catecholaminergic neurons in mouse (Hawes *et al*, 2004). Thus, GalR1 activation might lead to inhibition of both the DRN and the LC and cause depression-like behavior in rat FST. It should, however, be noted that a clear association of GalR1 with DRN 5-HT neurons was not observed in the *in situ* hybridization studies by O'Donnell et al (1999). Also, the specificity of available galanin receptor antibodies has been questioned (Lu and Bartfai, 2009).
Electrophysiological data also suggest that GalR3 is expressed on DRN 5-HT neurons. It is notable that the GalR3 selective antagonist SNAP 37889 exerted an antidepressant-like effect in several behavioral models relevant for anxiety- and depression-like behavior, including the FST (Swanson *et al*, 2005; Barr *et al*, 2006). Thus, the inhibitory effects of galanin are probably mediated via activation of GalR1/GalR3 at the level of DRN.

4.2.2 Galanin Receptor 2 Agonist/Antagonist in Depression-Like Behavior

The GalR2 M1896 agonist significantly reduced immobility time in the rat FST, while the GalR2 antagonist M871 caused an increase to the same extent as galanin and a GalR1 agonist. These finding supports the hypothesis that stimulation of the GalR2 has an antidepressant-like effect (Lu *et al*, 2005). The results with the GalR2 antagonist indicate that there exists a tonic activation of the GalR2 receptor under in vivo conditions of forced swim, which is sufficiently strong to have physiological consequences. This finding gives functional evidence for the view that galanin is released under stressful conditions (Lundberg and Hökfelt, 1986), stimulating multiple receptor subtypes. Blockade of the GalR2 receptor attenuates the "antidepressant" effect related to the GalR2 stimulation. This leads to a shift towards the inhibitory actions of the GalR1/GalR3 receptors causing "prodepressive" effects in the rat FST (Lu *et al*, 2005).

Further, studies showed that GalR2 activation increased 5-HT release in the hippocampus, conceivably through the regulation of firing of 5-HT neurons (Mazaratti and Lu, 2005), an effect consistent with the intracellular signaling cascades coupled to GalR2 (Branchek *et al*, 2000). Interestingly, chronic administration of FLX, up-regulated GalR2 but not GalR1 in the DRN (Lu *et al*, 2005). If GalR2 does enhance serotonergic transmission, this action of FLX might be one of the mechanisms underlying its antidepressant effect (Lu *et al*, 2005; Mazarat and Lu, 2005). Thus, GalR2-mediated positive regulation of serotonergic transmission implies that GalR2 agonists might be beneficial for the treatment of MDD.

4.2.3 In Situ Hybridization Studies

The mRNA levels of TH, the rate limiting enzyme for NA synthesis, and galanin, were studied in the LC with in situ hybridization. The TPH2 transcript (the rate limiting enzyme for 5-HT synthesis), 5-HT_{1A} and 5-HTT receptors were studied in the same way.

4.2.3.1 Locus Coeruleus: Tyrosine Hydroxylase and Galanin

Exposure to FST and repeated i.p. injections of saline (SAL) increased the levels of TH and galanin. This indicates that the combination of these two events may result in an increased activity of the LC, since increased TH levels by exposure to stress may reflect activation of LC neurons (Biguet *et al*, 1986; Berod *et al*, 1987; Richard *et al*, 1988). Moreover, increased TH and galanin mRNA levels following swim/injection stress indicate enhanced release and compensatory synthesis of both NA and the co-released galanin (Schalling *et al*, 1989; Meister *et al*, 1990). Stress-induced hyperactivity of the LC neurons and increased release of NA have been proposed to contribute to development of human depression (Nestler *et al*, 1990; Mongeau *et al*, 1997; Grant and Weiss, 2001).

Galanin, under basal conditions, has been shown to reduce LC neuronal activity and NA release in rats (Pieribone *et al*, 1995; Yoshitake *et al*, 2003), probably via stimulation of the GalR1 receptor subtype (Ma *et al*, 2001). However, i.c.v galanin did not reduce the TH mRNA levels, when rats were exposed to the FST in our study, suggesting that the effects of galanin differ under basal (Counts *et al*, 2002) and stressful conditions. Taken together, LC activity is probably augmented under stressful conditions, and the released galanin probably increases the stress-reactivity of the LC NA system (Yoshitake *et al*, 2004) and exerts effects on LC projection areas, further aggravating behavioral depression (Weiss *et al*, 1998; 2005).

4.2.3.2 Dorsal Raphe Nucleus: Tryptophan Hydroxylase 2, galanin, 5-HT_{1A} and 5-HTT

In contrast to the LC, the levels of TPH2 and galanin mRNA levels in the DRN were not altered by the swim/injection stress. This finding agrees with the view that the LC NA system is more sensitive to the acute stress than the DRN 5-HT system (Abercrombie and Jacobs, 1987; Wilkinson and Jacobs, 1988). However, i.c.v galanin increased the galanin mRNA levels in the DRN, suggesting that the release of galanin might be increased. It is known that galanin exerts inhibitory effects on basal 5-HT neurotransmission, most likely mediated via GalR1 (Xu *et al*, 1998c; Kehr *et al*, 2002; Yoshitake *et al*, 2003; Mazarat and Lu, 2005). Taken together, these data indicate that the hypothesized increase of galanin release might inhibit the 5-HT release in DRN projection areas, thus causing depression-like behavior in the FST.

Galaninergic modulation of 5-HT neurotransmission is presumably partly mediated via its interaction with the 5-HT_{1A} receptors. Galanin has been shown to have antagonistic interactions with the 5-HT_{1A} autoreceptors in DRN and postsynaptic 5-HT_{1A} receptors, e.g. in the limbic areas (Razani *et al*, 2000; Kehr *et al*, 2002). The level of 5-HT_{1A} receptor mRNA was decreased by i.c.v galanin, confirming the

results of the above mentioned study (Razani et al., 2000). Additionally, the GalR1 agonist M617 and GalR2 agonist also decreased the levels of 5-HT_{1A} mRNA, suggesting that both receptors contribute to this effect.
Down-regulation of presynaptic 5-HT_{1A} receptor has been proposed to underlie the increase of 5-HT neurotransmission and the therapeutic action of SSRIs (Artigas et al., 1996; Le Poul et al., 2000). Therefore, the antagonistic interaction between galanin and 5-HT_{1A} might seem paradoxical, since galanin decreases 5-HT neurotransmission (Xu *et al*, 1998c; Kehr *et al*, 2002). However, the decrease of 5-HT_{1A} receptor mRNA may be an effect secondary to reduced 5-HT neurotransmission caused by i.c.v galanin infusion. Thus, decreased level of 5-HT_{1A} mRNA might be a compensatory effect to counteract the decreased 5-HT neurotransmission caused by galanin.
Swim stress and the treatments used in this study (galanin and FLX) did not alter the level of 5-HTT mRNA in the raphe nuclei.

4.3 Paper III

The lack of systematically active, blood-brain barrier (BBB)-penetrating and receptor specific ligands has limited our understanding of the functional role of galanin and how each receptor mediates the effect of galanin in the brain. However, non-peptidergic GalR3 selective antagonists (Swanson *et al*, 2005; Barr *et al*, 2006) as well as non-selective galanin receptor agonists (Saar *et al*, 2002; Bartfai *et al*, 2004; Lu *et al*, 2005) penetrating into brain tissue have been developed and studied in recent years.
Transgenic mice overexpressing galanin under different promoters (Cai *et al*, 1999; Holmberg *et al*, 2005; Holmes *et al*, 2005), knockout mice lacking galanin (Wynick *et al*, 1998) and knockout mice lacking either GalR1 (Jacoby *et al*, 2002) or GalR2 (Gottsch *et al*, 2005), have provided an alternative approach to help comprehend the functional role of endogenous galanin and to delineate receptor subtype functions.
In paper III we have generated a transgenic mouse that overexpresses GalR2 under the PDGF-B promoter to examine a possible role of the GalR2 receptor subtype in depression- and anxiety-like behaviors as well as locomotor activity and learning and memory. Additionally, the neuroanatomical distribution of GalR2 receptor protein and GalR2 mRNA was studied using immunohistochemistry and *in situ* hybridization. The GalR2OE mice were compared to their WT littermates in behavioral tests relevant for affective domains of behavior.

4.3.1 Behavioral Characterization of the Galanin Receptor 2 Overexpressing Mice

The behavioral phenotype of the GalR2OE mice was assessed in various tests, including FST, OF, EPM and PA.

4.3.1.1 Locomotor Activity

Locomotor activity of the GalR2OE mice was studied in the OF and the EPM. No differences between the GalR2OE mice and their WT littermates were observed in the OF (distance travelled) and in the EPM (distance travelled and number of total arm entries). Previous studies have indicated that deletion of GalR2 does not influence locomotion (Gottsch *et al*, 2005; Bailey *et al*, 2007). Also, mice overexpressing galanin did not display an overall significant difference in locomotor activity compared to control mice (Kuteeva *et al*, 2005). Studies with exogenous galanin reported either no effect on exploratory behavior or a slight reduction of locomotor activity in the rat OF when given i.c.v or into the VTA (Ericson and Ahlenius, 1999). Thus, galanin signaling in general and GalR2 signaling in particular might have a modest or no effect on spontaneous locomotor activity in rodents.

4.3.1.2 Anxiety-Like Behavior

Anxiety-like behavior was assessed in two different tests, the OF and the EPM. Based on previous data, galanin and its receptors have been implicated in anxiety-like behavior in rodents. However, no differences between genotypes in the two behavioral models were detected. A previous study using GalR2-deficient mice also failed to observe any alteration in stress-induced hyperthermia or in OF (Gottsch *et al*, 2005). In another study, GalR2 null mutant mice displayed significant decreases in open arm entries in the EPM test (Bailey *et al*, 2007). Since anxiety and depression are linked together, the present findings are intriguing (see below) since they suggest that GalR2 might not play a role in anxiety-like behavior.

4.3.1.3 Learning and Memory

Several reports suggest an inhibitory role of galanin in hippocampal-dependent learning and memory processes (Ögren *et al*, 1996; 1998; Rustay *et al*, 2005). In the present study, no differences between the genotypes, in retention time or in training latencies, were observed in the PA task. Notably, intracerebral galanin failed to alter PA-learning as well as spatial acquisition. Overexpression of GalR2 subtype mRNA and protein was not detected neither in the amygdala nor in hippocampus, brain areas implicated in emotional learning. Also mice lacking the GalR2 receptor displayed no impairment in the cued trace fear conditioning test (Gottsch *et al*, 2005; Bailey *et al*, 2007). Further, the role of galanin in emotional learning task is not well defined. A PA study detected no differences in latency during training or memory retention between galanin overexpressing mice (PDGF-B promoter) and their WT littermates (Kuteeva *et al*, 2005). However, mice expressing galanin under the DβH promoter showed impairments in the cued trace fear conditioning test (Kinney *et al*, 2002). Taken together, the results from transgenic mice suggest that galanin might be involved in certain aspects of emotional learning and memory, and that GalR2 probably has at most a minor role in emotional learning in mice.

4.3.1.4 Forced Swim Test

Depression-like behavior was assessed in the FST. As mentioned earlier, increased time of immobility may indicate a failure of persistence in escape-directed behavior (behavior despair), or development of passive behavior (Cryan *et al*, 2002). In these studies, a two-exposure design was used to be able to study the ability of coping with stress (Kuteeva et al., 2005). This contrasts with most studies in mice which only use a single exposure to water-stress. GalR2OE mice demonstrated decreased immobility time compared to the WT controls in all three age groups studied (Figure 6), indicative of antidepressant-like behavior or increased stress resistance. In fact, the effect can was most pronounced in the oldest group suggesting an age related sensitivity.

Previous studies have suggested that GalR2 might be involved in neuronal mechanisms underlying depression-like behavior. Repeated FLX treatment to rats increased the number of GalR2 binding sites in DRN (Lu *et al*, 2005) (see results and discussion of paper II). Further, GalR2 activation increased 5-HT release in the hippocampus, conceivably through an excitatory regulation 5-HT neuronal firing (Mazarati and Lu, 2005). Taken together, these studies suggest that the FLX-induced increase of the GalR2 mRNA might lead to increased 5-HT signaling, and thereby to the antidepressant-like effect.

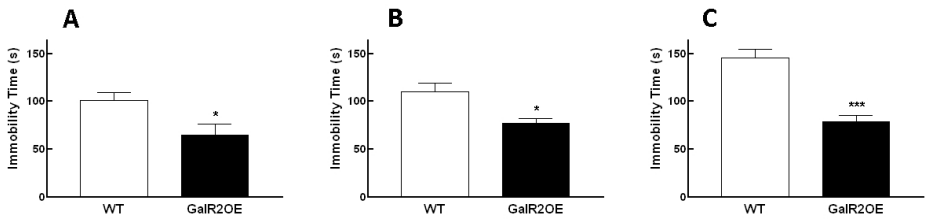

Figure 6. Immobility time in the Porsolt forced swim test (6 min) for mice 5-7 months old (WT, n=21 and GalR2OE, n=14) (A), 8-11 months old (WT, n=6 and GalR2OE, n=6) (B) and 18-20 months old (WT, n=7 and GalR2OE, n=12) (C). Data represent mean±S.E.M. Open bars, WT mice; closed bars, GalR2OE mice. (Mann-Whitney test).

4.3.2 Distribution of Galanin Receptor 2 in the Central Nervous System

The PDGF-B promoter construct was used to overexpress GalR2, since this promoter is widely distributed in the brain (Sasahara *et al*, 1991), and has been shown to produce strong overexpression of β-amyloid precursor protein (Games *et al*, 1995) and galanin (Kuteeva *et al*, 2004) in the mouse brain.

In the olfactory bulb, a high expression/overexpression of GalR2 was detected in the mitral cells, the principle output neurons of the olfactory bulb that convey information to higher olfactory structures, e.g. the anterior olfactory nucleus and piriform cortex (Shipley et al., 1995). Interestingly the receptor protein-EGFP construct was also seen in the olfactory tract running along the ventro-ventral cortex. This suggests that the receptor indeed is transported centrifugally and could have a presynaptic function. Additionally, GalR2 was found to be expressed, to a lesser extent, in the glomerular cell layer of the olfactory bulb (Figure 7).

A widespread expression of the GalR2 mRNA and receptor protein was found in the prefrontal cortex, e.g. prelimbic and infralimbic cortices. Moreover, a strong expression of GalR2 transcript and receptor protein was found in the cingulate cortex and the retrosplenial granular and agranular cortices. Additionally expression of GalR2 mRNA and receptor protein was detected in the other cortical areas, as well as perirhinal and motor cortex. Of particular interest, overexpression/expression of GalR2 was detected in pre- and para-subiculum, and subiculum (Figure 7 C-E).

Galanin levels were very low in the cortical/hippocampal nerve terminals of the GalR2OE mice, but could clearly be detected in the WT mice. This could indicate increased galanin release. However, we did not observe increased galanin mRNA levels in the cell bodies in the LC, which should be expected as a mechanism to compensate for release of the peptide. A similar situation was observed in a GalOE mouse (Kuteeva *et al*, 2004). Presently there is no obvious explanation for this unexpected finding.

Several mechanisms may underlie the antidepressant-like phenotype of the GalR2OE mice. Our neurochemical data indicate that GalR2 mice exhibit expression/overexpression of the GalR2 in several brain areas involved in regulation of mood. Overexpression/expression of GalR2 in these areas might also be involved in the decreased time of immobility in the GalR2OE mice.

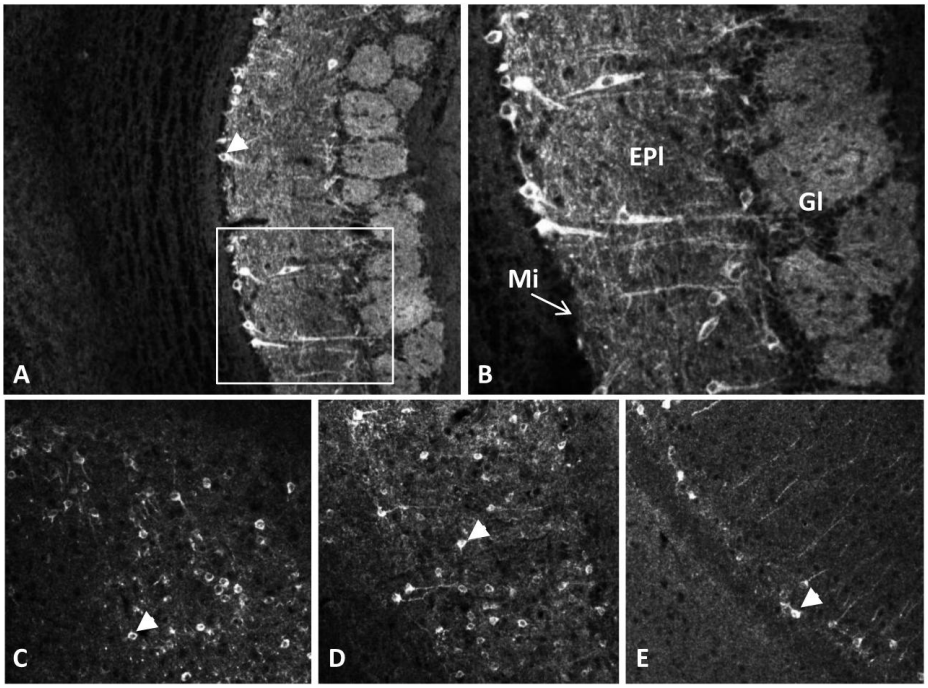

Figure 7. Immunofluorescence photomicrographs showing the distribution of GalR2 positive cells in mitral cells (arrowheads) of the olfactory bulb. B shows box in A. C, D and E show the dorsal part of subiculum, the presubiculum and the ventral part of subiculum respectively.

4.4 Paper IV

The FST is the most frequently used behavioral paradigm in rodents for assessing antidepressant activity. The test is based on the failure to maintain escape-directed behavior (swimming and climbing) in response to inescapable swim stress (Cryan *et al*, 2005b). When placed in a cylinder of deep water, rodents immediately struggle to escape by vigorous swimming, but subsequently develop a state of immobility as demonstrated by passively floating or making only the movements necessary to remain afloat. Increased immobility is indicative of depression-like behavior (Porsolt *et al*, 1977).

Despite its predictive validity, the use of FST as a model of depression is controversial. First, antidepressant drugs decrease immobility in the FST after a single-dose or subchronic administration, while their clinical efficacy require chronic administration (Nestler *et al*, 2002; Dulawa *et al*, 2004). Second, immobility is measured during the exposure to the stressful event itself, and with the current behavioral sampling technique it is therefore difficult to distinguish between the immobility as a "mental state" from the expression of locomotor behavior. Moreover, a number of contradictory results have also been reported in studies using SSRIs in the FST, probably reflecting inter-laboratory differences in the test procedures, such as the number of sessions, acute vs. chronic or subchronic treatment, time and route of administration as well as the chosen rodent strain (Lucki, 2001; Petit-Demouliere *et al*, 2005). In view of these considerations, this study investigated whether the

antidepressant-like effect of an SSRI depends on the number of drug injections and their temporal relation to the swim stress sessions. Additionally, the study included behavioral measures, e.g. locomotor activity and anxiety-like behaviors, to assess sensorimotor alterations following antidepressant treatment and the behavioral consequences of exposure to the stressful event.

4.4.1 Effects of Serotonin Reuptake Inhibition on Immobility and Climbing in the Pretest

A single injection of FLX, given 1 h prior to the pretest, failed to reduce immobility time. Thus, previous exposure to an inescapable swim stress seems to be important for revealing the antidepressant effects of the SSRI FLX in the FST. FLX significantly increased immobility during the second 5 min period of the first session; an effect that can be explained by an enhanced feedback inhibition of serotonin release by activation of autoinhibitory 5-HT_{1A} receptors. Both the inescapable stress and the 5-HT uptake inhibition increases serotonin release at the level of the DRN, resulting in increased feedback inhibition of serotonin release. However, in the same experiment FLX did not alter immobility during the first 5 min period of the first session. Climbing was not different between the FLX-treated and SAL-treated rats.

4.4.2 The Temporal Role of Serotonin Reuptake Inhibition in the Forced Swim Test

Rats treated with a single (i.p.) injection of FLX, independent of time of administration, did not differ significantly in the immobility and climbing times compared to the corresponding control rats. This indicates that a single injection is not sufficient to alter immobility and climbing behaviors even in rats pre-exposed to swim stress. In contrast, when FLX was injected twice, either 1 h before the pretest plus 1 h prior to the test or 1 h after the pretest plus 1 h prior to the test, immobility time was significantly reduced compared to control animals. The double injection schedule seems to be as efficient in reducing immobility time on the second day of experiment as the injection schedule used by Porsolt (23, 5, 1 h before the 5 min test session) (Porsolt *et al*, 1977, 1978a). Climbing was significantly reduced in rats treated with FLX 1 h prior to pretest plus 1 h prior to test session, while no significant changes were detected in climbing for any of the other treatment schedules used in this study. Variable effects of the 5-HT reuptake blockers on climbing behavior have been reported. Some studies show that SSRIs have no effects on climbing behavior but rather decrease immobility by increasing swimming (Lucki and O'Leary, 2004; Cryan *et al*, 2005a), while other studies showed that SSRIs like FLX decrease climbing behavior (Kuteeva *et al*, 2007). This variability might depend on the rat strain used or the experimental procedure.

4.4.3 Effects of Antidepressants on Locomotor Activity and Exploratory Behavior

Locomotor activity and exploratory behavior (rearing) were studied in rats treated with FLX, DES or SAL (control) in locomotor cages. A significant effect of antidepressant treatment on locomotor activity and rearing was detected in the first 10 min of test (Figure 8). This indicates that reduced immobility in FLX- or DES-treated rats is not a consequence of increased locomotor activity and/or exploratory behavior. The fact that both the NA reuptake blocker DES and the 5-HT reuptake blocker FLX produced similar effects in locomotion suggests that their differential effects on climbing or active coping are not simply a matter of changes in locomotion.

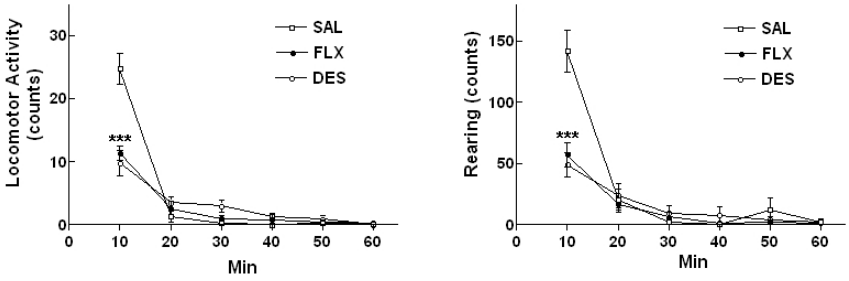

Figure 8. Locomotor activity and rearing in rats treated (i.p.) 23, 5, and 1 h before test (60 min) with either FLX (10 mg/kg)(n=8), or DES (10mg/kg)(n=8), or SAL (1ml/kg)(n=8). (□)SAL; (●)FLX; (○) DES. Data represent mean±S.E.M. *Significant difference as compared to the SAL-treated group, $p<0.05$ (Fisher's PLSD).

4.4.4 Effects of Numbers and Time of Fluoxetine Injections on Anxiety-Like and Social Behaviors

Subchronic treatment with FLX (given 23, 5, and 1 h before the OF and SI tests) significantly decreased SI time as compared to the SAL-treated controls. Treatment with two i.p. injections given 25 and 1 h before the tests reduced SI time, but this reduction did not reach statistical significance. However, FLX injection given twice significantly reduced time in center in the OF test. This is consistent with previous studies reporting an anxiogenic-like effect of acute SSRI treatment in animal models of anxiety, including the SI test (Kent *et al*, 1998; File *et al*, 1999; To *et al*, 1999; Salchner and Singewald, 2002). However, FLX is used for the treatment of a wide spectrum of anxiety disorders including social phobia (Kent *et al*, 1998). Taken together, our data point to an anxiogenic-like effect of FLX when given acutely to control rats. But it is important to note that the rats in this experiment were not subjected to swim stress.

4.4.5 Effects of Swim Stress and Fluoxetine Treatment on Anxiety-Like and Social Behavior

Rats subjected to acute inescapable swim stress 24 h before testing in OF and SI tests displayed reduced social behavior in comparison to the naïve group (neither swimmed, nor injected), the SAL-treated control group, and the FLX control group (not swimmed) (Figure 9). This demonstrates that swim stress in the rat can induce deficits in social behavior lasting at least 24 h. The SAL-treated control group did not differ from the naïve group, indicating that the stress of the injection procedure had no long-term effects on social behavior in the rats. These findings agree with previous studies that reported social deficits after different chronic mild stressors in young and adult rats (Willner, 2005; Toth *et al*, 2008). Importantly, FLX given to rats prior to the exposure to inescapable swim stress significantly attenuated the decrease in social interaction time. This suggests that FLX can reduce the adverse consequences of inescapable swim stress on social behavior. These results further confirm that 5-HT plays an important role in rodent social behavior, as shown in previous studies (Lucki, 1998).

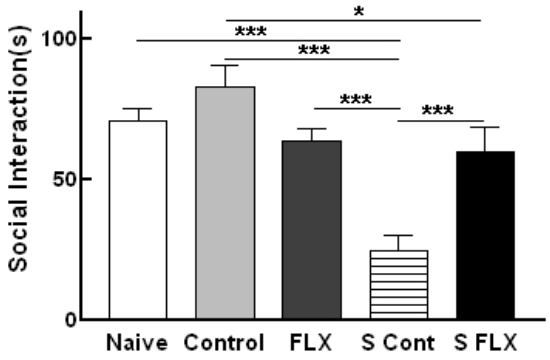

Figure 9. Social interaction time in the SI test (3 min). Rats in the naïve group (n=5) were neither treated nor subjected to swim stress. Rats were treated (i.p.) with SAL (1ml/kg) or FLX (10 mg/kg) 49 and 25 h before the SI test in the control group (n=6), or in the FLX group (n=5) respectively. Rats were treated with SAL in the stress-control group (S-Cont) (n=6) or with FLX in the stress-FLX (S-FLX) (n=6) 25 and 1 h before subjecting them to a 10 min swim stress session, and 24 h later rats were examined in the SI test. *Significant difference as compared to the SAL-treated group, $p<0.05$ (Fisher's PLSD).

4.5 Potential Mechanisms Underlying Antidepressant-Like Effects of Galanin Receptor 2 Stimulation in the Rodent Forced Swim Test

The pathophysiology of mood disorders involves several genetic, social and predisposing factors, as well as a dysregulated response to chronic stress. Accumulated results have indicated that several neuropeptides, including galanin and their receptors, are potential targets for the development of novel antidepressant treatment. In this context, it should be mentioned that neuropeptides appear to be particularly involved during situations of high neuronal activity, e.g. stressful or aversive events, resulting in up-regulation of peptidergic signaling (Lundberg and Hökfelt, 1986).

The present thesis has focused on a potential role of galanin and its receptors in the regulation of depression-like behavior following exposure to a stressful event, i.e. the FST. The results strongly support an involvement of galanin and galanin receptors in rodent depression-like behavior and a differential role of galanin receptor subtypes.

In addition to galanin and its receptors modulating the monoaminergic systems at the postsynaptic level, i.e. LC and DRN (based on **paper II**) (Kuteeva et al, 2008), this thesis points to additional sites that might contribute to stress modulation and consequently depression-like behavior in rodents. The results obtained and our hypothesis must be interpreted with caution, since it is partly based on data obtained from transgenic mice. In the GalR2OE mice, there may be compensatory, developmental effects of the GalR2 overexpression that explain our results.

In the subiculum of GalR2OE mice, GalR2-positive cells mainly represent pyramidal neurons, and the receptor protein was also detected in the dendritic processes, i.e. at possible postsynaptic receptor sites. The subiculum is implicated in stress integration through its indirect connections, GABAergic neurons in the BNST, to the HPA axis (see Herman and Mueller, 2006). Thus, it seems possible that a glutamatergic GalR2-expressing, subiculo-BNST pathway can regulate the HPA axis via a BNST-PVN GABA projection (Cullinan *et al*, 1993; Bowers *et al*, 1998). Studies have demonstrated that lesions restricted to the VS can enhance CRH mRNA and peptide expression in the PVN, and increase corticosterone (CORT) responses following restraint stress (Herman *et al*, 1995; 1998). Thus, galanin released in the subiculum from noradrenergic (and non-noradrenergic) nerve terminals in our FST experiments is in the position to activate $G_{q/11}$ protein-coupled GalR2s in glutamatergic subiculo-BNST neurons, causing increased intracellular Ca^{2+}concentrations and increased glutamate release in the BNST. This in turn could activate the inhibitory GABAergic projection to the PVN, resulting in reduced CORT levels and subsequently an antidepressant-like effect (Figure 10).

The study also shows an overexpression of GalR2 in the medial prefrontal cortex (MpFC), i.e. the prelimbic and infralimbic cortices. Previous studies have emphasized abnormalities in the neuronal circuits involving several interconnected limbic regions, including the prefrontal cortex (PFC) in the regulation of mood and the pathophysiology of MDD (Botteron *et al*, 2002; Drevets *et al*, 2008).

There is considerable evidence from rodent studies for a psychogenic stress (restraint)-specific HPA inhibitory role for the MpFC (Diorio *et al*, 1993; Figueiredo *et al*, 2003). Lesions of the MpFC in rats significantly increased plasma levels of both ACTH and CORT in response to a 20 min restraint stress (Diorio *et al*, 1993).

Like the VS, an overexpression of GalR2 in the MpFC may lead to an inhibition of PVN through the BNST and subsequently decreased levels of CORT. Hence, overexpressed GalR2 both in VS and PFC could in parallel exert antidepressant-like effects. Very few MpFC neurons directly project to the PVN (Canteras *et al*, 1992; Cullinan *et al*, 1993), but relays may, again, occur through GABAergic regions such as the BNST (Hurley *et al*, 1991). Importantly, the subiculum has also rich projections to the prelimbic and infralimbic cortices (Jay and Witter, 1991; Canteras *et al*, 1992; Naber and Witter,

1998), making it possible that stress-related information from subiculum in addition is conveyed through these structures to the PVN (Figure 10).

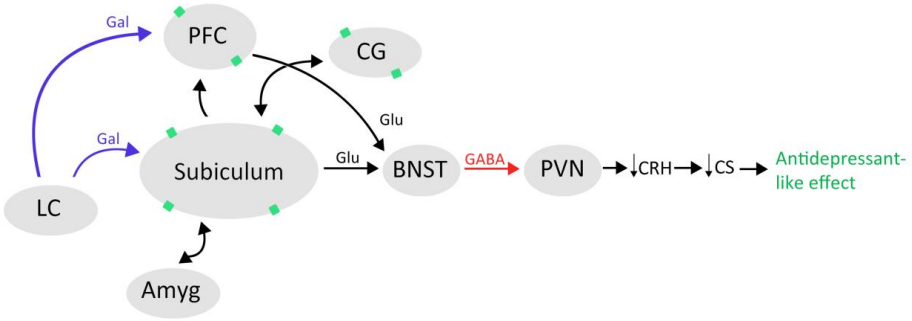

Figure 10: Hypothetical mechanisms underlying the involvement of subicular GalR2 in depression-like behavior. During swim stress, galanin might be released from LC NA neurons to activate GalR2 in ventral subiculum. There may also be other sources of galanin. Subiculum then activates BNST through glutamatergic relays. The released glutamate might in turn activate the GABAergic neurons of the BNST, finally inhibiting PVN, leading to decreased levels of CRH and eventually corticosterone in plasma. The information on the anatomy and connectivity between brain structures is based on studies by Herman et al. (2006), Canteras et al. (1992), Cullinan et al. (1993), Perez et al. (2001) and Loy et al. (1980).
The green boxes indicate overexpression of GalR2 subtype; Amyg, Amygdala; BNST, Bed nucleus of stria terminalis; CG, cingulate cortex; CRH, Corticotropin releasing hormone; CS, corticosterone; DG, Dentate gyrus; EC, Entorhinal cortex; GABA, Gamma-aminobutyric acid; LC, Locus Coeruleus; PFC, Prefrontal cortex; PVN, Paraventricular nucleus.

5. CONCLUSIONS

5.1 Paper I

Mood disorders such as depression involve dysfunctions in brain NA and 5-HT systems, as well as impaired coping with traumatic events.

I.c.v galanin increased time of immobility compared to the saline-treated rats indicative of depression-like behavior. Co-infusion of the unspecific galanin receptor agonist M35 blocked the effect of galanin. Importantly, M35 given alone decreased immobility time to the same extent as the antidepressants DES and FLX. These results support an involvement of brain galanin systems in depression-like behaviour in rodents, and that galanin might be involved in maladaptive reactions to inescapable stress, e.g. an impairment of coping behaviour.

5.2 Paper II

Like galanin, i.c.v. administration of the GalR1 agonist (M617) increased immobility time in the rat FST. Similarly, the GalR2 antagonist (M871) increased immobility time in the FST, while the GalR2 agonist (M1896) decreased immobility time, supporting an antidepressant-like effect of GalR2 stimulation. The in situ results showed that stimulation of galanin receptors and swim stress altered the levels of TH, TPH2 as well as 5-HT_{1A} mRNA, giving further evidence for the involvement of galanin in monoamine mechanisms linked to depression-like behavior.

Taken together, the present results indicate that exposure to stress results in release of galanin, a neuropeptide expressed both in NA LC and 5-HT DR neurons, and stimulation of multiple galanin receptor subtypes. These receptors appear to play differential roles in stress coping mechanisms, involving changes in gene expression of molecules important for NA and/or 5-HT transmission in the LC and the DRN. In view of the present findings, it is suggested that GalR1 and/or GalR3 receptor antagonists, or GalR2 receptor agonists may represent new therapeutic principles for the development of drugs for treatment of mood disorders.

5.3 Paper III

In the brain of GalR2OE mice, a strong signal for GalR2 mRNA and receptor protein was detected in the olfactory bulb, cingulated, retrosplenial granular and agranular cortices, subiculum, PrS and PaS, and subregions of the prefrontal cortex. Additionally, GalR2 immunoreactivity was detected in fibers in both the olfactory bulb, lateral olfactory tract and subiculum. Unexpectedly, the galanin fibers innervating the cerebral cortex were markedly lower in the GalR2OE mice than in the WT controls.

The GalR2OE mice, tested at different ages, displayed a significant decrease in immobility time in the FST compared to controls. However, there were no differences between the genotypes regarding anxiety-like behavior, learning and memory (emotional learning), or locomotor activity. These results suggest that GalR2 may be involved in neuronal mechanisms underlying depression-like behavior in the GalR2OE mice, most likely through its expression/overexpression in the subiculum and/or limbic areas like the medial prefrontal cortex, brain systems involved in psychogenic stress and stress regulation in the HPA-axis.

5.4 Paper IV

A single (i.p.) injection of the SSRI FLX, irrespective of time of administration, did not alter immobility time in the FST. In contrast, when FLX was injected twice immobility time was significantly reduced as compared to the corresponding control rats. Antidepressant activity can be detected after two but not one, injections of the FLX in the FST. Further, treatment with either FLX or DES significantly reduced locomotor activity and rearing, indicating that reduced immobility in the FST caused by these two antidepressants is not a consequence of increased locomotor activity and/or exploratory behavior.

Finally, exposure to acute, inescapable swim stress caused a significant reduction in social behavior in the rat in comparison to the non-stressed rats. FLX given to rats prior to the exposure to inescapable swim stress significantly attenuated the decrease in SI time. This suggests that FLX can reduce the adverse consequences of inescapable swim stress on social behavior and that increases in 5-HT transmission can counteract the behavioral consequences of inescapable swim stress.

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