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5-HT RECEPTOR-MEDIATED MODULATION OF GLUTAMATE TRANSMISSION IN THE HIPPOCAMPUS AND PREFRONTAL CORTEX AND ITS RELATION TO COGNITION AND DEPRESSION

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About the cover: The illustration, a 3D rendered fractal, resembles the shape of a human brain in longitudinal section and depicts the author's personal interpretation of brain regions interaction during cognition and memory processing.

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5-HT receptor-mediated modulation of glutamate transmission in the hippocampus and prefrontal cortex and its relation to cognition and depression

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ABSTRACT

Depression is a multifaceted disease which is marked by cognitive and emotional memory impairments. It is characterised by an imbalacement of serotonergic and glutamatergic system in brain areas such as hippocampus and prefontal cortex. Current antidepressant treatments available on the market and newer candidates need a better understanding of their mechanism of action, both at behavioral and molecular level.

During my PhD thesis I have tested several hypothesis:

In **paper I** – *5-HT_7 receptors are involved in emotional memory caused by a direct or indirect activation of the receptor.* This was tested in the passive avoidance (PA) task where systemic administration of the dual 5-HT_{1A}R/5-HT_7R agonist, 8-OH-DPAT, was combined with 5-HT_{1A}R and 5-HT_7R antagonists administration in mice. Local infusion of 8-OH-DPAT was administered into the dorsal hippocampus in order to delineate that 5-HT_{1A}R and 5-HT_7Rs potentially crosstalk in hippocampal processing of emotional memory.

In **paper II** – *lurasidone, a second generation antipsychotic, posesses antidepressive properties, similar to the well studied fluoxetine, a selective serotonin reuptake inhibitor (SSRI).* Chronic effects of lurasidone and fluoxetine were tested using behavioral approaches, such as novelty induced hyponeophagia (NIH), as well as immunobloting measurements in hippocampal and prefrontal cortex areas.

In **paper III** – *5-HT_{1B}R and p11 interaction affects the hippocampal neurotransmission.* This was tested using biosensors to measure *in vivo* glutamate release with fast analytical sensing technology (FAST). Immunoblotting measurements were also performed, to determine glutamate receptor phosphorylation. Moreover, neurochemical events associated with p11-mediated regulation of 5-HT_{1B}R function were tested by a non-invasive methodology, *in vivo* proton magnetic resonance spectroscopy (^{1}H-MRS) recordings.

In **paper IV** – *N methyl-D-aspartate receptor (NMDAR) antagonists, ketamine and Ro25-6981, compunds with rapid antidepressive properties, modulate glutamate release.* This was tested with FAST-subsecond glutamate release mesurements in hippocampal-prefrontal cortex circuitry.

In conclusion, the work conducted in this thesis has contributed in understanding the interaction between serotonergic and glutamatergic systems in hippocampal and prefrontal cortex areas, providing novel insights upon mechanism of action of different classes of antidepressants reflected both at the behavioral and molecular level.

LIST OF SCIENTIFIC PAPERS

Eriksson TM, Holst S, **Stan TL**, Hager T, Sjögren B, Ögren SO, Svenningsson P, Stiedl O.

5-HT_{1A} and 5-HT_7 receptor crosstalk in the regulation of emotional memory: implications for effects of selective serotonin reuptake inhibitors. Neuropharmacology, 2012; 63(6):1150-60.

Stan TL, Sousa VC, Zhang X, Ono M, Svenningsson P.

Similar effects of lurasidone and fluoxetine on novelty-induced hypophagia and regulation of NMDA receptor-subunits. Manuscript draft, 2014.

Eriksson TM , Alvarsson A, **Stan TL**, Zhang X, Hascup KN, Hascup ER, Kehr J, Gerhardt GA, Warner-Schmidt J, Arango-Lievano M, Kaplitt MG, Ögren SO, Greengard P, Svenningsson P.

Bidirectional regulation of emotional memory by 5-HT_{1B} receptors involves hippocampal p11. Molecular Psychiatry, 2013;18: 1096–1105.

Stan TL, Alvarsson A, Branzell N, Sousa VC, Svenningsson P.

NMDA receptor antagonists ketamine and Ro25-6981inhibit evoked release of glutamate in vivo.Translational Psychiatry, 2014; 4, e395.

LIST OF ABBREVIATIONS

aCSF	Artificial cerebro-spinal fluid
AMPAR	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APV	D-(-)-2-amino-5-phosphonopentanoic acid
Arc	Activity-regulated cytoskeletal-associated protein
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BD	Bipolar disorder
BS	Bursting spiking
Ca2+	Calcium ions
Cr	Creatine
CAMKII	Calcium2+/calmodulin-dependent protein kinase II
EEAT1/EEAT2	Excitatory amino acid transporter 1/2
EEG	Electroencephalography
et al	*et alibi*, and others
etc	*et cetera*, and other things
DAG	Diacylglycerol
DG	Dentate gyrus
DRN	Dorsal raphe nuclei
DSM- IV	Diagnostic and Statistical Manual of Mental Disorders, 4^{th} edition
F20	Fluoxetine 20 mg/kg
GABA	γ-aminobutyric acid

Glu	Glutamate
GluR1	Glutamate receptor subunit 1
Gln	Glutamine
GPC	GlyceroPhosphocholine
Gua	Guanidine
1HMRS	Proton magnetic resonance spectroscopy
IL	Infra limbic
Ins	Inositol
IP3	Inositol triphosphate
KCL	Potassium chloride
KO	Knock-out
Lur3	Lurasidone 3mg/kg
Lur10	Lurasidone 10mg/kg
Lac	Lactose
LTP	Long term potentiation
LTD	Long term depression
MAGUK	Membrane-associated guanylate kinase
MAO	Monoamine oxidase
MAPK	Mitogen activated protein kinase
MDD	Major depressive disorder
mGluR	Metabotropic glutamate receptor
mPFC	Medial prefrontal cortex
MRN	Medial raphe nuclei
MRI	Magnetic resonance imaging

m-TOR	Mammalian target of rapamycin
NAA	N-Acetylaspartate
NAAG	N-Acetylaspartatylglutamate
NIH	Novelty induced hyponeophagia
NMDAR	N-methyl-D-aspartate receptor
NR1	NMDA receptor subunit 1
NTDs	N-terminal domains
NH_4^+	Ammonium cation
OF	Open field
PA	Passive avoidance
PCr	Phospocreatine
PCh	Phosphocholine
PET	Positron emission tomography
PFC	Prefrontal cortex
P_i	Phosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PreL	Prelimbic
preNMDAR	Presynaptic NMDA receptor
PSD-95	Post synapstic density-95
po	*per os,* oral administration
P11	Also called S100A10, annexin II light chain
P11KO	P11 knock-out

RS	Regular spiking
SERT	Serotonin transporter
SNARE	Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SUB	Subiculum
SSRI	Selective serotonin reuptake inhibitor
Syn-I	Synapsin I
Tau	Taurine
TCA	Tricyclic antidepressant
TrKB	Tyrosine kinase or BDNF/NT-3 growth factors receptor
VEH	Vehicle
VOI	Voxel of interest
WB	Western blot
WT	Wild type
5-HT	5-hydroxytryptamine; serotonin, serotonergic
5-HTR	Serotonin receptor
5-HIAA	5-hydroxyindole-3-acetic acid

CONTENTS

1 INTRODUCTION

1.1 DEPRESSION

Depression, the predominant form of affective or mood disorders (Kessler et al., 2010), is one of the leading causes of disease burden worldwide, with a great impact on the health status, affecting all genders, ages and backgrounds (Schlaepfer et al., 2012). Diagnostic criteria for depression include symptoms such as sadness or low mood, cognitive dysfunctions, loss of interest or pleasure, disturbed sleep, poor concentration, guilt or low self-worth, disturbed appetite, poor energy, decreased interest in and enjoyment of sex, physical agitation or slowing, and thoughts or acts of suicide, according to Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (American Psychiatric Association, 2000). Depression could be triggered by several factors such as stress, genetic factors, inflammation, seasonal affective disorders (SAD), etc. (Caspi et al., 2003; Raison et al., 2006; Duman, 2014). Defects in the serotonergic (5-HT) system, which is involved in emotion, learning and memory processes, have long been implicated in the pathophysiology of depressive disorders (Hoyer et al., 2002). Most of the available antidepressants on the market accomplish their effect by increasing the levels of serotonergic neurotransmission but, overall, there is a certain delay of the therapeutic onset, making it difficult to fully delineate their action (Heninger et al., 1996; Barnes & Sharp, 1999). The pioneering experiments of Heninger et al. (1996) were the basis of the monoamine theory of depression, referring to the "depletion in the levels of serotonin, noradrenaline, and/or dopamine in the central nervous system" (see Figure 1). Apart from the mentioned serotonin (5-HT) dysfunction in depressed patients (Hoyer et al., 2002), the noradrenergic and dopaminergic system are also affected, since studies have shown alterations in β-adrenoceptor binding in suicide victims (Zanko & Beigon, 1983) and a lower

dopamine transporter binding in the striatum of depressed patients (Meyer et al., 2001).

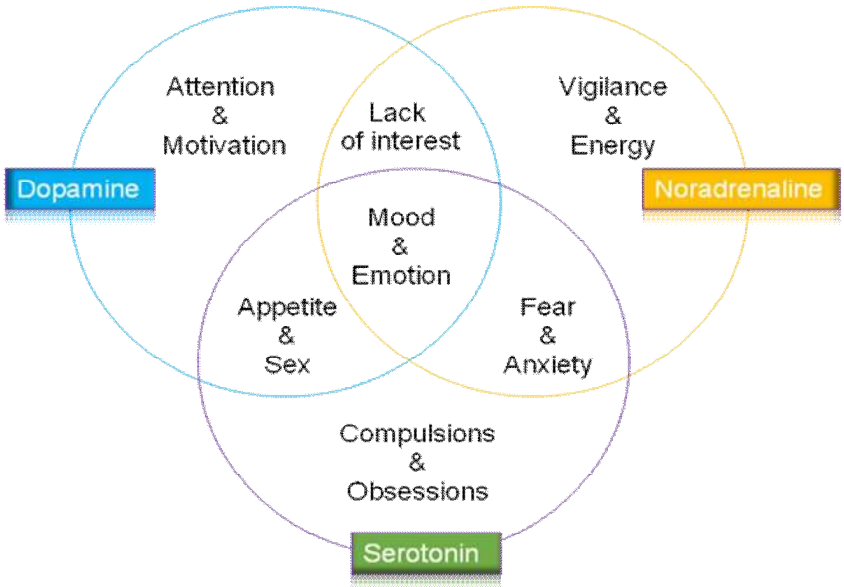


Figure 1. Involvement of monoamine neurotransmitters in the modulation of different symptoms in depression (adapted from Nutt et al., 2007).

However, there is clinical evidence that challenges the monoamine theory, namely the reported decrease of γ-aminobutyric acid (GABA) level in the cortex (Sanacora et al., 2002) or alterations of glutamatergic system of the depressed patients (Sanacora et al., 2008). Glutamate has been shown to be a key neurotransmitter in depressive pathology (Trullas & Skolnick, 1990; Skolnick et al., 2009). Clinical studies have found evidence for glutamatergic dysfunction using neuroimaging and analyses of plasma, serum, cerebrospinal fluid and *postmortem* brain tissue of depressed patients (Sanacora et al., 2011). Newer treatment with compounds that target the glutamate receptors, such as ketamine, a nonspecific NMDAR antagonist, after an acute, sub anesthetic

dose, indicate rapid and sustained antidepressive effects in humans (Zarate et al., 2006).

During my PhD I have been involved in projects focused on 5-HT receptor-mediated modulation of glutamate transmission in two main brain regions: hippocampus and prefrontal cortex. I tried to understand more about this interaction and its implications for cognition and depression. Previous studies performed in humans have identified that the excitatory neural circuits within the hippocampal-prefrontal cortical system, which regulate stress-responsiveness and mood, are over-activated in patients with major depressive disorder (MDD) (Ressler & Mayberg, 2003). Likewise, studies in rodent models have shown that different types of chronic stress induce depression-like changes on behavioral, morphological (eg. synaptogenesis) and signal transduction parameters (Maeng et al., 2008; Autry et al., 2011; Li et al., 2011; Müller et al., 2013; Duman et al., 2012) within the glutamatergic hippocampal-prefrontal cortical circuitry (Qi et al., 2009; Duman & Li, 2012; Schloesser et al., 2012).

1.2 HIPPOCAMPAL –PREFRONTAL CORTEX CIRCUITRY

1.2.1 HIPPOCAMPUS

In the mamalian brain, the hippocampus is part of the limbic system and consists of several anatomical subregions including the entorhinal cortex, Cornu Ammonis (CA) subfields, CA1, CA2 and CA3, dentate gyrus and subiculum (O'Mara et al., 2000), with an intrinsic excitatory network between them (Tsien et al., 1996) (Figure 2).

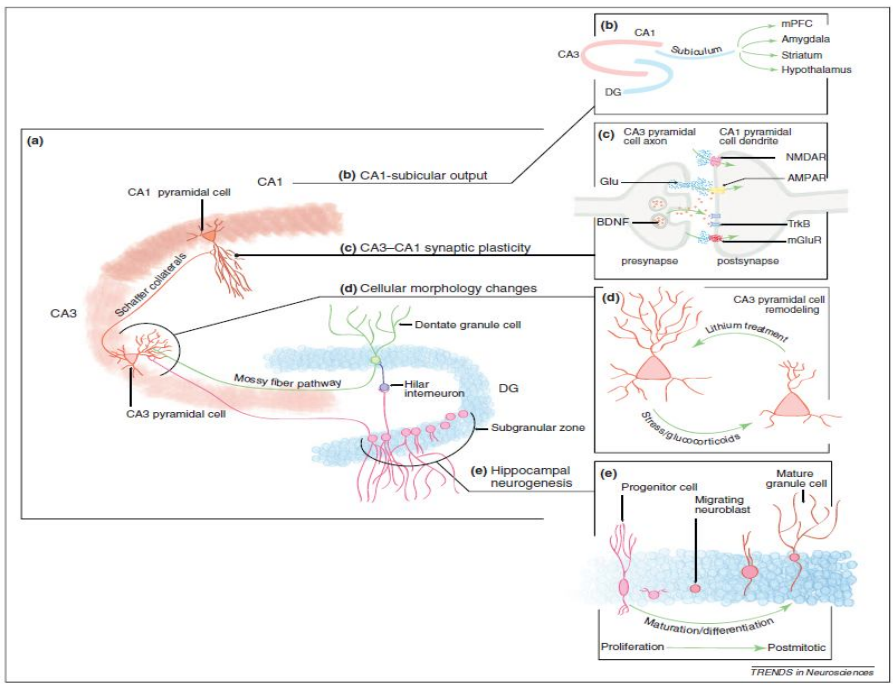


Figure 2. Hippocampal-prefrontal cortex circuitry involvement in depression. Hippocampal network a); CA1-Subiculum projections b); hippocampal synaptic plasticity c); cellular morphology changes d); hippocampal neurogenesis e). mPFC- medial prefrontal cortex; NMDAR-Nmethyl-D-aspartate receptor; AMPAR-alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; Glu-glutamate; BDNF- brain-derived neurotrophic factor; TrkB-TrkB tyrosine kinase or BDNF/NT-3 growth factors receptor; mGluR-metabotropic glutamate receptor. Printed with permission from the editor. From Schloesser et al., 2012.

In terms of hippocampal cell types, the majority is represented by glutamatergic pyramidal and granule cells whereas a small fraction includes GABA- releasing interneurons (Fritschy et al., 1998). The hippocampus receives serotonergic projections from medial and dorsal raphe nuclei (MRN & DRN) (Jans et al., 2007; Vertes, 2010). A plethora of electrophysiological and behavioral studies identified hippocampus as a critical region in forming new memories (Tsien et al., 1996; Vertes, 2010). In rodents, hippocampal oscillatory patterns, such as theta (4–12 Hz) (Kramis et al., 1975) and gamma (30–80 Hz) (Bragin et al., 1995; Wang & Buzsaki, 1996) have been intensively linked with learning and memory processes. Different serotonin receptors (5HTRs) such 5-$HT_{1A/B}$, 5-HT_4 and 5-HT_7R are well-represented in the CA1 region, indicating the involvement of the serotonergic system in learning processes (Barnes & Sharp, 1999; Hoyer et al., 2002; Jans et al., 2007; Roberts & Hedlund, 2012). Also, the serotonergic projections from MRN & DRN interfere with the theta/hippocampal EEG, altering memory-processing functions in hippocampus (Vertes, 2010). Systemic administration of ketamine in mice, at a sub-anaesthetic dose, produced a decrease in theta and increase in gamma power (Lazarewicz et al., 2010). These experiments were reproduced in human studies, where ketamine decreased the amplitude of low-frequency oscillations (delta 1–5 Hz, theta-alpha 5–12 Hz) and increased the amplitude of gamma oscillations (Hong et al., 2009). Interestingly, a recent study performed in rodents (Muller et al., 2013) revealed that acute ketamine treatment regulates the presynaptic release machinery in the hippocampus by a similar mechanism to chronic antidepressant treatment (Bonanno et al., 2005; Musazzi et al., 2010). Magnetic resonance imaging (MRI) studies indicated a reduction in hippocampal volume in depressed patients and individuals suffering posttraumatic stress disorder (PTSD) (Zubenko et al., 1990; Gilbertson et al., 2002; Campbell et al., 2004).

Factors such as severe and chronic stress alter the hippocampal formation in animals (Sapolsky et al., 1990).

1.2.2 SUBICULUM

Pyramidal neurons of the CA1 area send direct projections to the subiculum (O'Mara et al., 2000). This pathway is known to be involved in both short- and long term plasticity (O'Mara et al., 2000; Behr et al., 2009), whereas exposure to an acute stressor disrupts this plasticity (MacDougall et al., 2013). Subiculum, the main output of the hippocampus, sends glutamatergic projections to the amygdala, nucleus accumbens, hypothalamus and prefrontal cortex, creating neuroanatomic circuits known to be involved in mood regulation (see Figure 2b). If dysfunctionality may appear in any of the regions involved in these circuits, the subject/patient is more prone to develop a mood disorder (Soares & Mann, 1997).

1.2.3 PREFRONTAL CORTEX

Similarly to the hippocampus, the prefrontal cortex (PFC) receives projections from MRN & DRN (Jans et al., 2007; Vertes, 2010). It has been reported from *postmortem* and imaging studies on patients with bipolar disorder (BD), a decreased neuronal size and altered neuronal and glial cells density in PFC region (Savitz et al., 2014; Campbell & Macqueen, 2004). Furthermore, analyses of the PFC from patients with mood disorders have shown increased glutamate levels and decreased gray matter volume (Savitz et al., 2014; Hashimoto et al., 2007).

1.3 NEUROTRANSMISSION

1.3.1 Serotonin transmission

The serotonin neurotransmitter system plays major roles in the regulation of mood, sex, sleep, cognition and endocrine effects (Nichols & Nichols, 2008). Serotonin (5-hydroxytryptamine; 5-HT) is synthesized from the amino acid, tryptophan (Figure 3). The highest concentration of 5-HT (aprox. 90%) is found in the gut (enterochromaffin cells) whereas the rest of 5-HT is found in platelets and in the CNS (Azmitia & Gannon, 1986). In the brain, the serotonergic neurons are located in the caudal and rostral raphe nuclei of the brainstem, thereafter projecting to different brain areas such as the hippocampus, prefrontal cortex, amygdala, hypothalamus, basal ganglia and cingulate cortex (Jans et al., 2007). A significant body of data suggests an important role of 5-HT in hippocampal -dependent learning and memory processes (Ögren et al., 2008). Rodent studies indicate that changes in 5-HT neurotransmission can facilitate or impair learning and memory in various hippocampal tasks reflecting actions on multiple 5-HTRs (Ögren et al., 2008). Changes in serotonergic transmission implicated in affective disorders appear also to underlie the cognitive dysfunction observed in psychiatric disorders (Millan et al., 2012).

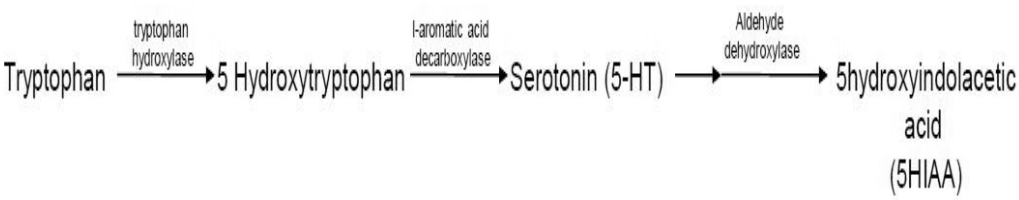


Figure 3. Serotonin (5-HT) synthesis and metabolism.

After its release from the synaptic terminals, 5-HT is cleared from synaptic cleft by the serotonin reuptake transporters (SERT) and re-packed in vesicles, whereas the free cytoplasmic 5-HT is inactivated by monoamine oxidase (MAO) to 5-hydroxyindole-3-acetic acid (5- HIAA) (Figure 4).

1.3.1.1 Serotonin receptors

Serotonin transmission is mediated by fourteen different 5-HT receptor (5-HTR) subtypes, divided in seven subclasses: 5-HT_{1A-F}, 5-HT_{2A-C}, 5-HT_3, 5-HT_4, 5-HT_{5A-B} 5-HT_6 and 5-HT_7 (Hoyer et al., 2002) (Figure 4). They have a diverse signaling pathway (Nichols & Nichols, 2008) and can be grouped in: a) 5-HTRs that decrease cAMP formation (5-HT_{1-} and HT_{5-}); b) 5-HTRs that increase cAMP formation (5-HT_4, 5-HT_6 and 5-HT_7); c) 5-HTRs that increase inositol triphosphate (IP3) and diacylglycerol (DAG) formation (5-HT2); d) 5-HTRs that play a major role in increasing Na^+ and Ca^{2+} influx (5-HT_3Rs). All the 5-HTRs are involved in a range of adaptive behaviors (Hoyer et al., 2002).

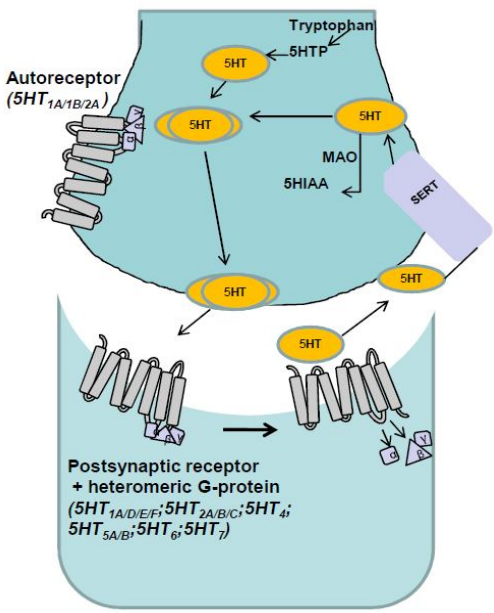


Figure 4. Serotonergic synapse and distribution of 5-HTRs (adapted from Nichols & Nichols, 2008). Following synthesis from tryptophan and in presence of action potential, 5-HT is released and activates the 5-HTRs and synaptic cascades, leading to conformational changes of G protein and its subunits. 5-HT is taken up by SERT into presynaptic site and degraded by enzymes. 5-HT- serotonin; α-,β-, γ- G-protein subunits, SERT- serotonin transport; 5HIAA- 5hydroxyindoacetic acid; 5HTP- 5hydroxytryptophan; MAO- monoamine oxidase.

In the course of my PhD, I was mostly interested in the following 5-HTR subtypes:

5-HT_{1A} receptors (Paper I & II):

5-HT_{1A}Rs are widely expressed in CNS, located either presynaptically (somatodendritic autoreceptors) in raphe, or postsynaptically, in other regions of the brain (Jans et al., 2007). 5-HT_{1A}Rs constitute a therapeutic target for multiple neuropsychiatric diseases such as anxiety, depression or schizophrenia (Ohno., 2011). Also, it has been indicated that 5-HT_{1A}R agonist treatment impaired memory function (Ögren et al., 2008).

5-HT_{1B} receptors (Paper III):

5-HT_{1B}Rs are auto- and heteroreceptors and are expressed in different brain regions such as basal ganglia, striatum and hippocampus, playing a major role in modulation of emotional memory performance (Eriksson et al., 2008). The expression levels of 5-HT_{1B}R is decreased in p11 knock-out (KO) mice, an animal model of depression (Svenningsson et al., 2006). Positron emission tomography (PET) studies performed in ventral striatal/ventral pallidal areas of the patients with MDD exhibit a reduced binding potential for 5-HT_{1B}R (Murrough et al., 2011), indicating the importance of 5-HT_{1B}R in depression.

5-HT_{2A} receptors (Paper II):

5-HT_{2A}Rs are mostly localized at the postsynaptic site, on dendritic shafts and in dendritic spines (Miner et al., 2003). 5-HT_{2A}Rs are also found on the presynaptic site suggesting that 5-HT_{2A}Rs can modulate excitatory neurotransmission (Jakab & Goldman-Rakic, 1998). A small fraction of 5-HT_{2A}Rs is expressed in glial processes (Miner et al., 2003). 5-HT_{2A}Rs are widely localized, including

prefrontal cortex (Miner et al., 2003). 5-HT_{2A}Rs undergo down-regulation in response to either agonist or antagonist treatment (Gray & Roth, 2001).

5-HT_4 receptors (Paper III):

The 5-HT_4R was cloned in 1995 (Gerald et al., 1995) and is expressed both peripherally and centrally (Nichols & Nichols, 2008). In humans, 5-HT_4Rs are expressed in cortex, hippocampus, basal ganglia and substantia nigra (Bonaventure et al., 2000). Electrophysiological studies performed in the CA1 region of the hippocampus, indicated that activation of 5-HT_4Rs do not influence long term potentiation (LTP) but only long term depression (LTD) (Kemp & Manahan-Vaughan, 2005). 5-HT_4Rs modulate the release of various neurotransmitters and play a major role in learning and memory (Chapin et al., 2002).

5-HT_7 receptors (Paper I & II):

The 5-HT_7Rs are the newest addition to the 5-HTRs family and several groups (Bard et al., 1993; Shen et al., 1993; Lovenberg et at., 1993; Plassat et al., 1993; Ruat et al., 1993) reported in parallel the cloning of the human, rat and mouse 5-HT_7 receptor. 5-HT_7Rs are widely expressed in the brain, including limbic system, thalamus, hypothalamus and cortical areas (Vanhoenacker et al., 2000; Neumaier et al., 2001). 5-HT_7Rs modulate hippocampus-dependent learning performance (Roberts & Hedlund, 2012), are involved in circadian rhythmicity and thermoregulation (Middlemiss et al., 2002). In particular, sleep studies in rats with 5HT_7R antagonist, SB269970, indicated a reduction of paradoxical phase, as similar to the effect after a treatment with selective serotonin reuptake inhibitors (SSRIs) in clinical trials (Hagan et al., 2000).

1.3.2 Glutamatergic transmission

L-Glutamate (Glu), the major excitatory neurotransmitter in the mammalian central nervous system (CNS), is synthesized from glutamine in the presynaptic compartment (Figure 5).

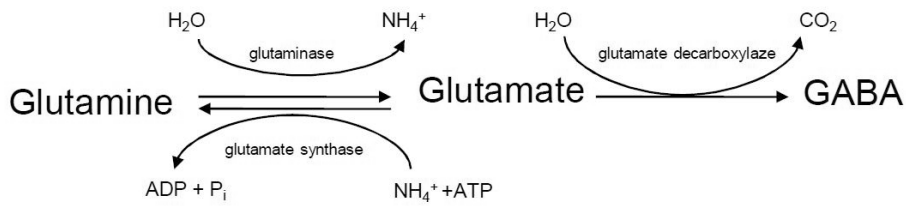


Figure 5. Conversion of Glutamate and GABA from Glutamine. H_2O- water; CO_2- carbon dioxide; NH_4^+- ammonium cation; P_i -phosphate; ADP- adenosine diphosphate; ATP- adenosine triphosphate; GABA- gamma-amino butyric acid.

Upon arrival of an action potential at the presynaptic site, Glu vesicles, located in terminal buttons, fusion in the presence of Ca^{2+}. This mechanism leads to changes in conformation of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex (Südhof & Rothman, 2009) and leads to a fast release of Glu and activation of glutamate receptors located on the postsynaptic site (Figure 6). Glu also activates the presynaptic NMDAR, a mechanism that causes reinforcement of Ca^{2+} dependent Glu release (McGuiness et al., 2010). From synaptic cleft, Glu in excess is taken up by surrounding glial cells, with high-affinity transporters. The activity of the glial transporters, such as the excitatory amino acid transporter 1/2 (EAAT1/2), help in prevention of Glu toxicity (Danbolt, 2001). In the glial cells, Glu is converted to glutamine (Gln).

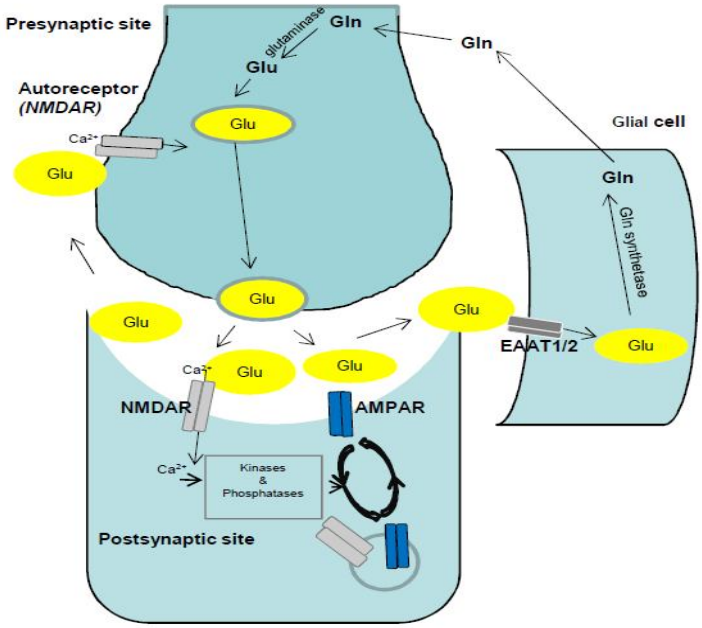


Figure 6. The glutamatergic tripartite synapse (adapted from Popoli et al., 2011). Following synthesis from glutamine and in presence of action potential, glutamate is released and activates the NMDAR, AMPAR and postsynaptic cascades. From the synaptic cleft, glutamate uptake occurs through EAAT1/2 dependent-transport into glial cells, converted into glutamine and furthermore converted back to glutamate, in the presynaptic site. Glu- glutamate; Ca^{2+} - calcium ion; NMDAR-N-methyl-D-aspartate receptor; AMPAR- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; EAA1/2- excitatory amino acid transporters 1/2.

1.3.2.1 Glutamate receptors

Glu acts on two major classes of synaptic receptors: ionotropic and metabotropic glutamate receptors. The ionotropic glutamate receptors include: AMPAR (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor), NMDAR and kainate receptors. The metabotropic glutamate receptors (mGluRs) are part of the G-protein coupled receptor family. NMDAR receptors (NMDARs) and AMPAR receptors (AMPARs) are directly involved in two forms of synaptic plasticity, LTP and LTD. Both physiological processes have

been thoroughly characterized in the hippocampus (Bliss & Lomo, 1973; Lomo, 2003; Bliss & Collingridge, 2013).

My current PhD thesis consisted mostly in biochemical measurements and behavior readouts from animals experiments. Therefore, it was vital to understand how different classes of antidepressants exert their function and regulate the molecular mechanism of synaptic transmission, such as subunit composition of NMDAR and AMPAR, additional synaptic proteins and intracellular signaling pathways.

1.3.2.1.1 NMDA receptors (NMDARs)

NMDARs are heterotetrametric ion channels and include three subtypes: NR1 (with 8 subunits), NR2 (with subunits- A, B, C, D) and NR3 (with 2 subunits- A and B) (Ogden & Traynelis, 2011) (Figure 7). In order to be functional, the NMDARs contain a mandatory NR1 subunit (Monyer et al., 1992; Ogden & Traynelis, 2011). Normally, Mg^{2+} blocks NMDAR due to binding at the channel pore (Paoletti & Neyton, 2007). The activation of NMDARs requires the simultaneous presence of Glu and glicine (Lerma et al., 1990). The NR2 subunits form the binding sites for Glu whereas the NR1 and NR3 form the binding sites for glycine (Furukawa et al., 2005). NMDAR activation triggers a cascade of biochemical processes (Bliss & Lomo, 1973).

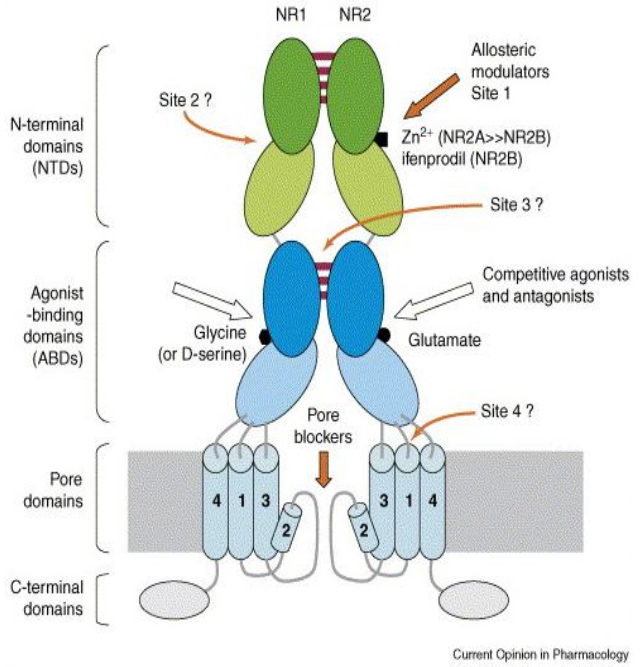


Figure 7. NMDAR subunit composition and the binding sites for agonists and antagonists. Printed with permission from the editor. From Paoletti & Neyton, 2007

In rodents, the NMDAR are located in different brain regions, including entorhinal cortex, hippocampus and prefrontal cortex (Sjöström et al., 2003; McGuinness et al., 2010; Rossi et al., 2012). NMDARs are expressed postsynaptically, in pyramidal cells (with the NR2A and NR2B subunits (Skolnick et al., 2009), GABA interneurons (Bagley & Moghaddam, 1997) and astrocytes (Krebs et al., 2003). NMDARs are also located at the presynaptic site (preNMDARs), as autoreceptors (McGuinness et al., 2010; Rossi et al., 2012; Buchanan et al., 2012). It is known that axonal compartments contain preNMDARs (McGuinness et al., 2010; Rossi et al., 2012; Buchanan et al., 2012) and CA1-subicular axonal projections possess the preNMDAR-containing NR1 and NR2B subunits (Behr et al., 2009). In rodent brain, subicular pyramidal cells posses a particular feature, similar to humans, namely that the cells are

classified as bursting spiking (BS) and regular-spiking (RS) cells (Behr et al., 2009). The BS cells are in a greater number than the RS cells and require presynaptic Ca^{2+} influx but not an increase of postsynaptic Ca^{2+} for induction of LTP, suggesting that LTP is induced via activation of presynaptic NMDAR (Behr et al., 2009). This aspect is very important in respect to synaptic neurotransmission, since it provides a rapid control over the action potential-driven, Ca^{2+}-dependent, glutamate release (Sjöström et al., 2003; Chamberlain et al., 2008; McGuinness et al., 2010). The CA1 and subicular cells possess another particular feature: immunohistochemical experiments performed in rodents indicated the expression NR2B subunits only at the neuronal cells, whereas, in a normal brain, astrocytes do not express it (Krebs et al., 2003). However, factors such as ischemia *in vivo* and anoxia *in vitro* trigger the expression of NMDAR-containing NR2B subunit in astrocytes (Krebs et al., 2003). Pharmacological studies have focused in developing compounds that target the Glu-blinding sites, ion-channel pore or alosteric site of the N-terminal domain (NTDs) (Figure 7). Over the past decades, new compounds that specifically target the NR2 subunits have been developed (the affinity ranking typically NR2A > NR2B > NR2C > NR2D) (Paoletti & Neyton, 2007). These studies indicate that NMDARs play a major role in the etiology of depression (Ogden & Traynelis, 2011). In mice, genetic inactivation of NMDAR-NR2A subunit leads to an anxiolytic and antidepressant-like effect, as measured by performance in the forced-swim test and tail suspension test (Boyce-Rustay & Holmes, 2006). Animal studies with ketamine (a non-selective NMDAR antagonist) and Ro25-6981 (a selective NMDAR NR2B-subunit antagonist) have shown that their fast-acting antidepressant effects involve the activation of the mammalian target of rapamycin (mTOR) signaling, as well as other pathways and proteins: mitogen activated protein kinase (MAPK), protein kinase

B (PKB), activity-regulated cytoskeletal-associated protein (Arc), post synaptic proteins, such as post synaptic density 95 (PSD-95) and GluR1 (Li et al., 2010). Both *in vivo* and *in vitro* experiments with D-(-)-2-amino-5-phosphonopentanoic acid (APV), an NMDAR selective antagonist, highlighted the involvement of this class of glutamate receptors in emotional memory (Stiedl et al., 2000) and dendritic arborization (Rocha & Sur, 1995; McAllister et al., 1996). Clinical trials performed in depressed patients challenged with a single intravenous ketamine injection, at a subanesthetic dose, showed a rapid and sustained antidepressant effect (Zarate et al., 2006).

1.3.2.1.2 AMPA receptors (AMPARs)

AMPARs are heterotetrametric ion channels and mediate the fast synaptic transmission in the CNS (Traynelis et al., 2010). AMPAR include four GluR subunits (GluR1-GluR4) which are distributed, in a functional AMPAR, as dimers of GluR2 and as dimers from GluR1, GluR3, or GluR4 (Traynelis et al., 2010). At rest, the majority of AMPARs at hippocampal synapses contain a higher ratio of GluR1/2 heteromer rather than GluR2/3 heteromer or GluR1 homomers (Lu et al., 2009). The GluR2 subunit regulates the permeability of AMPARs to Ca^{2+}, whereas the GluR2 inclusion or GluR2 genetic deletion alters the transmission at the synaptic level (Lu et al., 2009). The AMPARs function is regulated by antidepressants: acute and chronic treatment with fluoxetine, leads to increase in AMPAR phosphorylation (Svennigsson et al., 2002). The NMDARs regulate AMPA trafficking by either promoting AMPAR-containing GluR1 subunit insertion onto synapses (Hayashi et al., 2000) or removal of AMPAR-containing GluR1 and GluR2 subunits from the synapses (Beattie et

al., 2000). GluR1 double phosphomutant mice exibit an altered anxious and depressive-like behaviour (Kiselycznyk et al., 2013).

1.3.3 GABA transmission (Paper III)

GABA is the main inhibitor neurotransmitter in the brain. GABA regulates the activity of glutamatergic synapses and plays a major role in the generation of gamma oscillations and learning and memory processes (Buzsáki & Wang, 2012). It is worth noting that glutamine is the precursor for both Glu and GABA (Figure 5). In clinical trials, proton magnetic resonance spectroscopy (^{1}H-MRS) investigations have linked the cognitive impairments of schizophrenia with decreased GABAergic concentration in visual cortex (Yoon et al., 2010). Other studies have indicated that treatment with SSRIs in depressed patients lead to an increase of GABA levels in the occipital cortex area (Sanacora et al., 2002).

1.3.4 Synaptic proteins (Paper II & III)

1.3.4.1 P11 (Paper II)

P11 (S100A10, annexin II light chain) is an adapter protein which, at the cellular level regulates 5-HT_{1B}R functions and recruits both 5-HT_{1B}R and 5-HT_{4}R to the cell surface (Svenningsson et al., 2006; Svenningsson et al., 2007; Warner-Schmidt et al., 2009; Svenningsson et al., 2013). Reduced levels of p11 have been found both in *postmortem* human brain tissue from depressed individuals and suicide victims (Svenningsson et al., 2006; Anisman et al., 2008; Alexander et al., 2010), and in a rodent model of depression (Svenningsson et al., 2006). P11 knock-out (KO) mice exhibit a depressive-like phenotype and have reduced responsiveness to 5-HT_{1B}R agonists and antidepressants (Svenningsson et al.,

2006; Egeland et al., 2010). Chronic treatment with imipramine, a tryciclic antidepressant (TCA) and electroconvulsive therapy increased p11 protein level in cortex (Svenningsson et al., 2006). Recently, clinical trials in patients with MDD have indicated that chronic treatment with citalopram, an antidepressant from SSRI family, has been associated with decreased p11 levels in white blood cells (Svenningsson et al., 2014).

1.3.4.2 Post Synaptic Densisty 95 (PSD-95) (Paper III)

PSD-95 protein is a member of membrane-associated guanylate kinase (MAGUK) scaffolding protein family and is involved in the regulation of glutamatergic signaling at the postsynaptic site (Zhang et al., 2013), by interacting with NMDAR and downstream signaling proteins (Aoki et al., 2001). As previously mentioned, PSD-95 is also an indirect target of fast-acting antidepressants (the non-selective NMDAR antagonist, ketamine, and Ro25-6981 (Li et al., 2010). PSD-95 plays a critical role by mediating the actions of hallucinogens and atypical antipsychotic drugs which target the 5-HT_{2A}R and 5-HT_{2C}R (Zhang et al., 2013).

1.4.AIMS

Based on the need to develop novel and effective therapies for depression, studies were undertaken to identify pharmacological targets of improved antidepressive medication.

Paper I

(1) to examine the role of 5-HT_7Rs in emotional memory using direct or indirect activation of the receptor by combining systemic administration of the dual 5-HT_{1A}R/5-HT_7R agonist 8-OH-DPAT, together with 5-HT_{1A}R and 5-HT_7R antagonists

(2) to assess the involvement of hippocampal processing in emotional memory by local infusion of the 5-HT_{1A}R/5-HT_7R agonists into the dorsal hippocampus of mice

Paper II

(1) to investigate and compare the antidepressive properties of chronically administered lurasidone and fluoxetine

(2) to measure protein expression differences in the hippocampus and prefrontal cortex, associated with antidepressive effects of lurasidone and fluoxetine

Paper III

(1) to investigate the mechanism(s) whereby 5-HT_{1B}Rs and p11 regulate hippocampal neurotransmission

(2) to study neurochemical events associated with p11-mediated regulation of 5-HT_{1B}R function

Paper IV

(1) to investigate how local application of the NMDARs antagonists ketamine or Ro25-6981 affect tonic and evoked glutamate release in different brain regions relevant to depression

(2) to investigate if, in a time-course of 2 hours following acute systemic administration of ketamine, tonic and evoked glutamate release would be affected in the subiculum

2 MATERIALS AND METHODS

Various methodological approaches have been used in the current thesis, as they are enumerated below, with a full description in the corresponding papers. Additional methods that have been only used by the collaborators and not by the author of the thesis are described in detail in the corresponding paper. Pharmacological compounds (agonists and antagonists) used in the current thesis have been listed below (Table 1).

2.1 MOUSE MODELS

2.1.1 C57BL/6J mice (I, II, IV)

2.1.2 S100A10 knockout (P11 KO) mice (III)

2.2 PHARMACOLOGICAL TREATMENTS

2.2.1 Acute and repeated systemic administration (I-IV)

2.2.2 Chronic antidepressant drug administration (II)

2.2.3 Local brain infusion (I, III, IV)

2.3 BEHAVIORAL METHODS

2.3.1 Passive Avoidance Test (I)

2.3.2 Novelty Induced Hypophagia (II)

2.3.3 Open Field (II)

2.3.4 Nest Building Test (II)

2.4 BRAIN NEUROCHEMICAL MEASUREMENTS

2.4.1 Magnetic Resonance Spectroscopy (III)

2.4.2 Fast Analytical Sensing Technology (FAST) (III & IV)

2.5 STEREOTACTIC APPLICATIONS

2.5.1 Chronic Cannulae Implantation (I)

2.5.2 Intrahippocampal drug injection (I, III, IV)

2.5.3 Methylene Blue intracranial infusion (I, III, IV)

2.5.4 Enzyme-based Micro-Electrode Array (III & IV)

2.6 HISTOLOGY

2.6.1 Nuclear Fast Red Counterstaining (I, III, IV).

2.7 BIOCHEMICAL TECHNIQUES

2.7.1 Immunoblotting (II, III)

Table 1. Agonists and antagonists used in the present work

NAME	**MODE OF ACTION**
8-OH-DPAT	5-HT_{1A}R/5-HT_7R agonist
NAD-299	HT_{1A}R antagonist
SB269970	5-HT_7R antagonist
CP-94253	5-HT_{1B}R agonist
Lurasidone	D_2, 5-HT_{2A}, 5-HT_7R antagonist; 5-HT_{1A}R partial agonist
Fluoxetine	selective serotonin reuptake inhibitor (SSRI)
Ketamine	non-selective NMDAR antagonist
Ro25-6981	selective NMDAR-NR2B-subunit antagonist

3 RESULTS

In this section are indicated main findings from **Paper I-IV**.

3.1. *In vivo* 5-HT$_{1A}$Rs and 5-HT$_7$Rs interaction in the modulation of emotional memory function (Paper I)

We have found that pretraining (15 minutes before training) injections with 8-OH-DPAT, a dual 5-HT$_{1A}$R/5-HT$_7$R agonist, significantly affected training latencies both at 0.3mg/kg and 1 mg/kg (Figure 1a) in the passive avoidance (PA) test. Sytemic administration of 8-OH-DPAT, together with blocking the 5-HT$_{1A}$R activity by NAD-299, lead to a marked facilitation of PA retention due to the 5-HT$_7$R stimulation (Figure 1b).

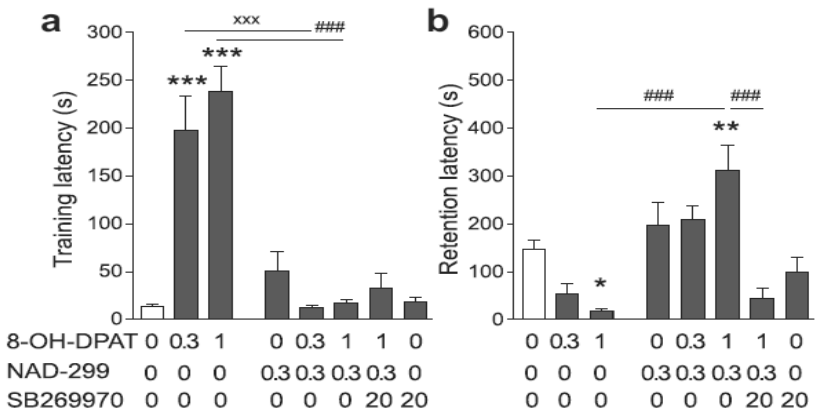


Figure 1. **Effects of pharmacological manipulation by the dual 5-HT$_{1A}$R/5-HT$_7$R agonist 8-OH-DPAT combined with selective 5-HT$_{1A}$R (NAD-299) and 5-HT$_7$R (SB269970) antagonists on PA performance.** Step-through latency at training after pre-training treatment (a). PA retention performance assessed by step-through latency performed 24 h after training (b). Drugs or vehicles were injected 30 min before training (NAD-299 s.c. and/or SB269970 i.p.) and 15 min before training (8-OH-DPAT s.c.). Data represent means ±S.E.M; numbers refer to systemic drug dosages in mg/kg; n = 8-13 mice/group and 59 controls. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. corresponding vehicle control group (white bar); ###$p < 0.001$ between indicated treatments. Statistical analysis included one way ANOVA or, in cases of pairwise comparison of two groups, by unpaired t-test. In cases of significant overall effect on the ANOVA, Neuman-Keuls was used for post-hoc analysis.

Furthermore, to assess the involvement of hippocampal processing in emotional memory, 8-OH-DPAT was locally infused into dorsal hippocampus of mice. 8-OH-DPAT impaired the PA performance similar to the effects of systemic administration (Figure 2a). Intrahippocampal administration of 8-OH-DPAT, together with blocking the 5-HT_{1A}R activity by NAD-299, lead to a marked facilitation of PA retention due to the 5-HT_7R stimulation (Figure 2b).

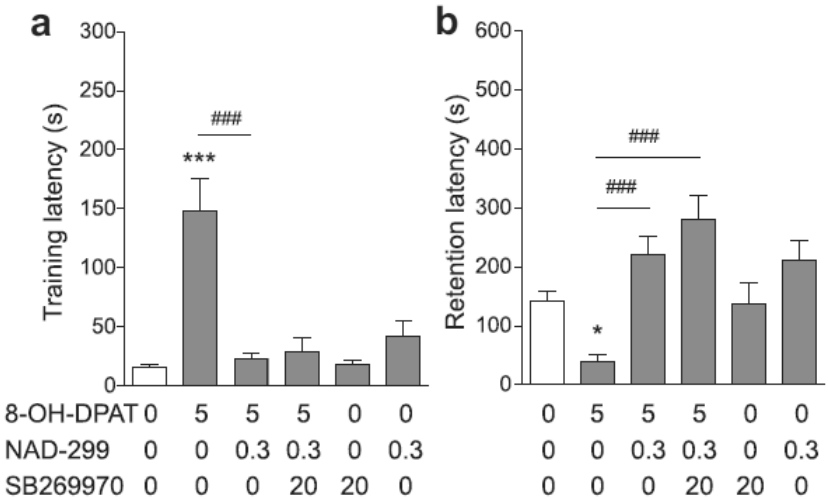


Figure 2. Effects of intrahippocampal infusion of the dual 5-HT_{1A}R/5-HT_7R agonist 8-OH-DPAT co-administered with selective 5-HT_{1A}R and 5-HT_7R antagonist on PA performance. Step-through latency at training after pre-training treatment (a). PA retention was determined by step-through latency 24 h after training (b). Drugs were injected 30 min before training (NAD-299 s.c. and/or SB269970 i.p.) and intrahippocampal infusions of 8-OH-DPAT or aCSF occurred 15 min before training. Data represent means ± S.E.M; numbers refer to systemic drug dosages in mg/kg or total dose of drug in mg/mouse infused into both dorsal hippocampi; n = 11-25 mice/group and 76 controls. *$p < 0.05$; ***$p < 0.001$ vs. corresponding vehicle control group (white bar); ###$p < 0.001$ between indicated treatments. Statistical analysis included one way ANOVA or, in cases of pairwise comparison of two groups, by unpaired t-test. In cases of significant overall effect on the ANOVA, Neuman-Keuls was used for post-hoc analysis.

3.2 Effects of chronic treatment with lurasidone and fluoxetine in mice (Paper II)

3.2.1 Lurasidone and fluoxetine decrease the latency to feed in the NIH test

We found that lurasidone, at both doses (3mg/kg and 10mg/kg) and fluoxetine (20mg/kg) were able to decrease latency to feed in the novelty-induced hyponeophagia (NIH) test, a behavioral paradigm sensitive to chronic antidepressive treatment (Figure 3):

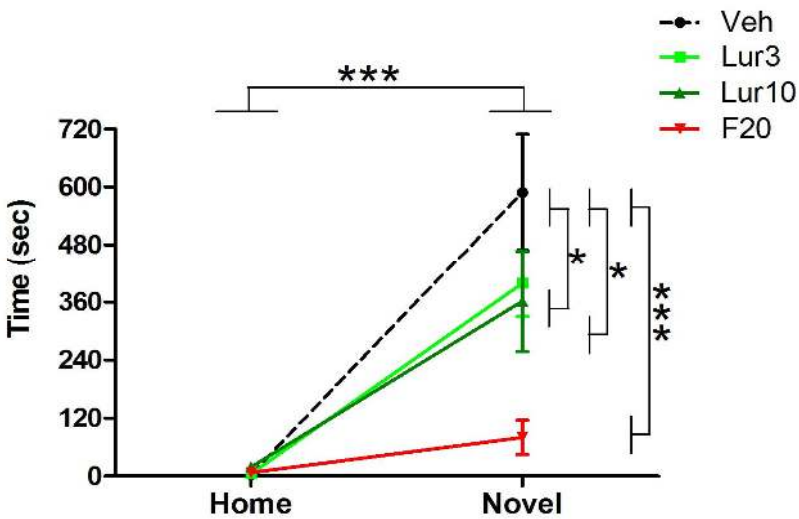


Figure 3. Chronic lurasidone and fluoxetine decrease the latency to feed in the novelty-induced hyponeophagia test. Graph showing home versus novel cage difference in the latency to consume milk reward. Chronic treatment with both 3mg/kg and 10mg/kg lurasidone as well as fluoxetine significantly decreased the latency to feed in the NIH test. The data are presented as mean values ± S.E.M. from 6-9 animals. *p<0.05; **p<0.01; ***p<0.001, Two-way repeated measures ANOVA followed by Fisher's LSD test, versus vehicle group. Legend: Veh: vehicle/control group (Methyl cellulose 0.5%) 10ml/kg; Lur3 - lurasidone (3mg/kg); (Lur10) - lurasidone (10mg/kg); F20 -fluoxetine (20mg/kg). All treatments were administered *per os* (p.o.).

3.2.2 Lurasidone and fluoxetine decrease total NMDAR subunit levels

Protein expression differences in hippocampus and prefrontal cortex, associated with antidepressive effects of lurasidone and fluoxetine were measured by Western blot (WB). Lurasidone and fluoxetine shared the ability to decrease the NR1 and NR2A subunit levels in hippocampus (Figure 4A), with similar effects on NR2A and NR2B subunits in prefrontal cortex (Figure 4B). In addition, fluoxetine decreased the NR1 subunit levels in prefrontal cortex (Figure 4B).

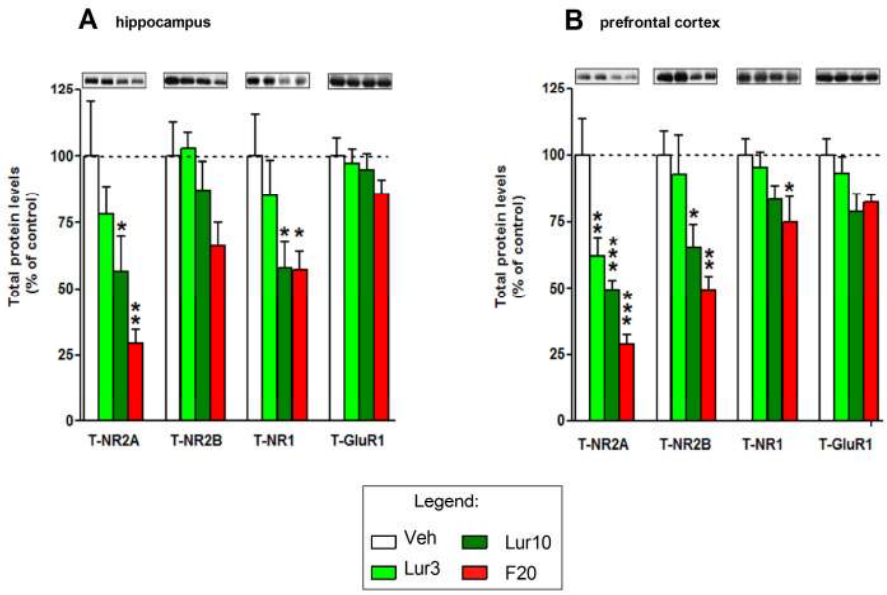


Figure 4: Chronic lurasidone and fluoxetine decrease the total NMDA receptor subunit levels 10mg/kg lurasidone and 20mg/kg fluoxetine decreased the levels of NMDA receptor subunits in hippocampus (A) and prefrontal cortex (B). The data are normalized by β-actin and presented as mean percentage of vehicle control ± S.E.M from 7-9 animals. *p<0.05; **p<0.01; ***p<0.001, One-way ANOVA followed by Fisher's LSD test, versus vehicle group. NMDAR subunits: T-NR2A, T-NR2B, T-NR1; AMPAR subunit: T-GluR1. Legend: Veh: vehicle/control group (Methyl cellulose 0.5%) 10ml/kg; Lur3 - lurasidone (3mg/kg); (Lur10) - lurasidone (10mg/kg); F20 -fluoxetine (20mg/kg).

3.2.3 Lurasidone and fluoxetine modulate NMDAR phosphorylation

Fluoxetine increased the P-Ser^{896}-NR1 in hippocampus and prefrontal cortex (Figure 5 A, B). Both compounds decreased the P-Ser^{1303}-NR2B in prefrontal cortex (Figure 5B). Also, fluoxetine increased the P-Ser^{897}-NR1 in prefrontal cortex (Figure 5B).

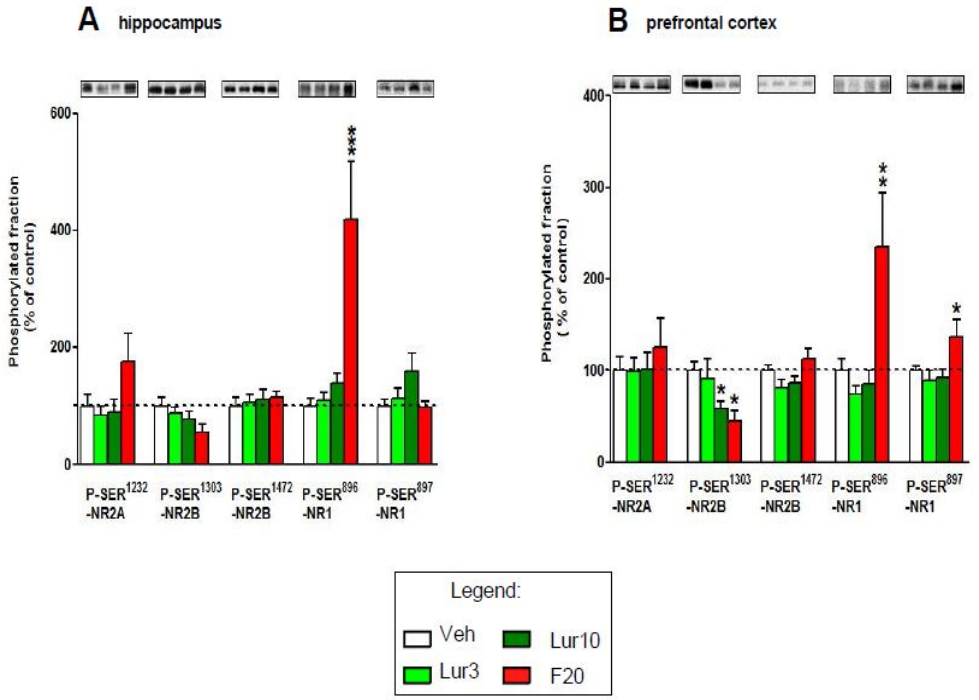


Figure 5: Chronic lurasidone and fluoxetine modulate the phosphorylation state of NMDAR subunits. Fluoxetine (20mg/kg) exerts post-translational modification on the phosporylation states of NMDAR subunits in hippocampus (**A).** Lurasidone (10mg/kg) and fluoxetine (20mg/kg) affects NMDAR phosphorylation in prefrontal cortex **B**). NMDAR phosphorylation states: P-Ser^{1303}-NR2B, P-Ser^{1472}-NR2B, P-Ser^{896}-NR1, P-Ser^{897}-NR1. The data are normalized by β-actin and presented as mean percentage of vehicle control ± S.E.M from 7-9 animals. *p<0.05; **p<0.01, ***p<0.001, One-way ANOVA followed by Fisher's LSD test, versus vehicle group. Legend: Veh: vehicle/control group (Methyl cellulose 0.5%) 10ml/kg; Lur3 - lurasidone (3mg/kg); (Lur10) - lurasidone (10mg/kg); F20 -fluoxetine (20mg/kg).

3.2.4 Lurasidone and fluoxetine decrease PSD-95 levels

Moreover, lurasidone and fluoxetine shared the ability to decrease the PSD-95 levels both in hippocampus (Figure 6A) and prefrontal cortex (Figure 6B).

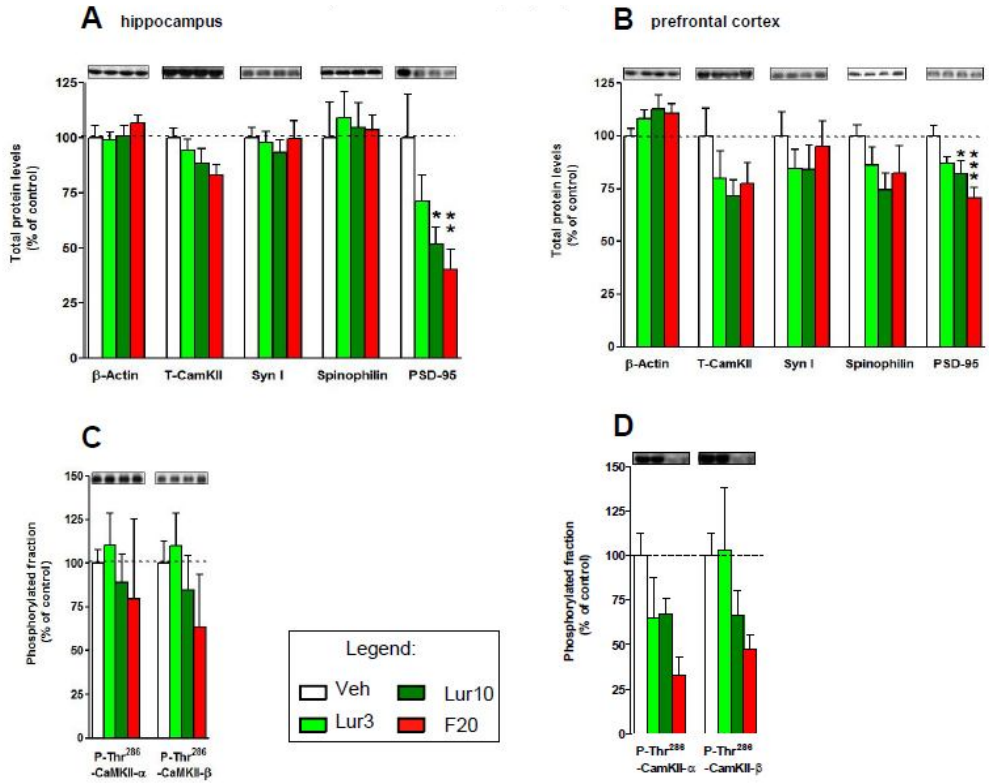


Figure 6: Chronic lurasidone and fluoxetine decrease synaptic protein levels Lurasidone (10mg/kg) and fluoxetine (20mg/kg) decreased the PSD-95 levels both in hippocampus (**A**) and prefrontal cortex (**B**). Lurasidone and fluoxetine did not affect the P-Thr^{286} -CamKII-α/β levels in hippocampus (**C**) and prefrontal cortex (**D**). syn I: synapsin I; PSD-95: post synaptic density 95. The data are normalized by β-actin (see methods) and presented as mean percentage of vehicle control ± S.E.M from 7-9 animals. *p<0.05; **p<0.01, ***p<0.001, One-way ANOVA followed by Fisher's LSD test, versus vehicle group. Legend: Veh: vehicle/control group (Methyl cellulose 0.5%) 10ml/kg; Lur3 - lurasidone (3mg/kg); (Lur10) - lurasidone (10mg/kg); F20 - fluoxetine (20mg/kg).

3.3 HT_{1B}Rs and p11 regulate hippocampal neurotransmission (Paper III)

3.3.1 5-HT_{1B}R agonist, CP-94253, increase presynaptic release of Glu in hippocampus of p11KO mice

Using *in vivo* amperometric recordings with fast analytical sensing technology (FAST), we found that 5-HT_{1B}R agonist, CP-94253, increased presynaptic glutamate release both in dentate gyrus and CA1 regions in p11KO mice (Figure 7a). The FAST is able to record, in real-time, sub-second potassium chloride (KCl) depolarization- evoked Glu release (Figure 7b).

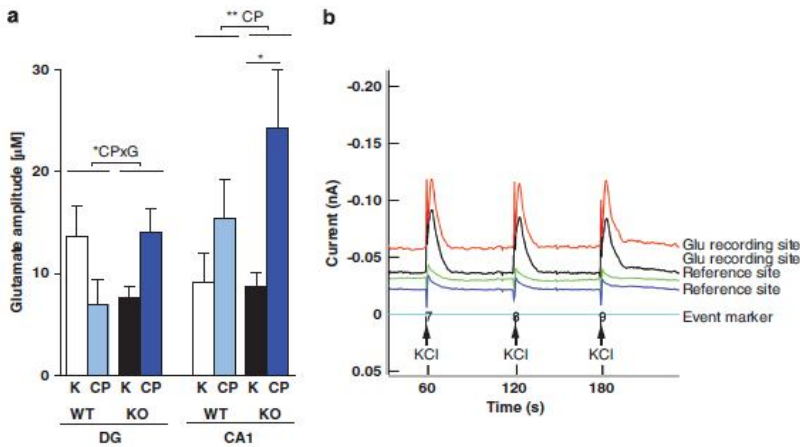


Figure 7. **Increased presynaptic hippocampal glutamate neurotransmission by 5-HT_{1B}R stimulation in p11KO mice.** Glutamate-oxidase enzyme based MEA recordings of potassium-evoked glutamate release amplitudes in hippocampal CA1 and DG subregions of anesthetized mice (a). Real-time in vivo amperometric responses of reproducible glutamate dynamics recorded at 2Hz, with glutamate recording sites (corresponding to responses in red and black) and sentinel or reference sites (corresponding to responses in blue and green) (b). Event markers indicated by arrows mark depolarization-induced responses evoked by 120mM KCl with and without co-administration of 10 mM of CP94253 (b), with 60 s between each local application of KCl. For glutamate release amplitudes in the DG, an interaction was found between CP94253 and genotype (a). In the CA1, CP94253 resulted in a higher KCl-evoked glutamate release amplitude in P11KO mice compared to baseline depolarization-evoked release of glutamate in P11KO mice (a). Data are presented as means ±s.e.m. (a) 3–5 reproducible peaks with n=10–14 (DG) and 8–10 (CA1) recordings per group. CA1: cornu ammonis 1 of hippocampus, DG: dentate gyrus of hippocampus, CP: CP94253 (5-HT1BR agonist), G: genotype, P11KO: p11 knock-out mice, WT: wild type mice, K: KCL (potassium chloride, 120mM), MEA: microelectrode array. *p<0.05; **p<0.01 between indicated treatments, Two-way ANOVA followed by Newman-Keuls test.

3.3.2 CP-94253 increases phosphorylation at P-Ser^{831}-GluR1 and P-Ser^{845}-GluR1 in the hippocampus of p11KO mice

The effects of 5-HT$_{1B}$R agonist, CP-94253, upon postsynaptic glutamatergic neurotransmission in the hippocampus of p11 KO mice were investigated by WB measurements. CP-94253 did not produce significant changes in total AMPAR and NMDAR levels (Figure 8c), but increased the phosphorylation of AMPAR, in particular the Ser^{831} and Ser^{845} of the GluR1 subunit (Figure 8d).

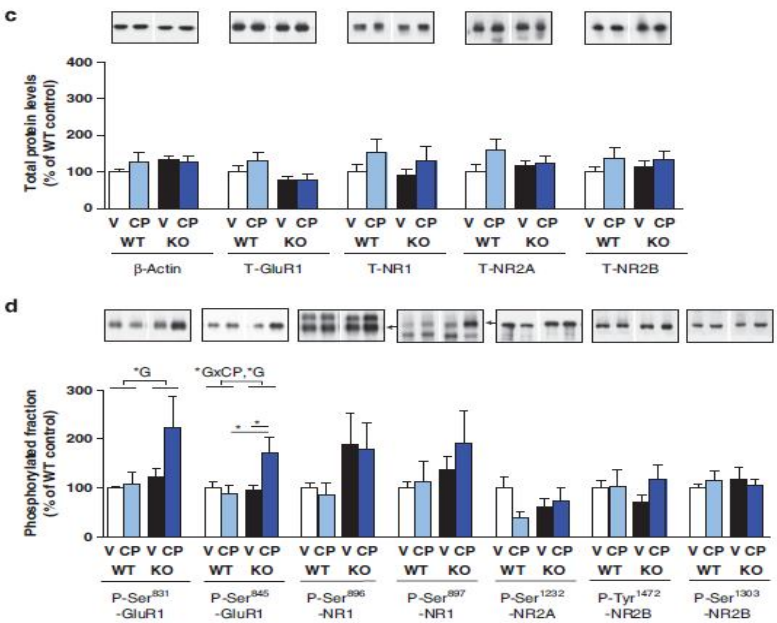
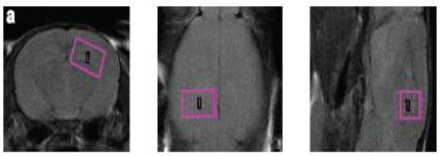


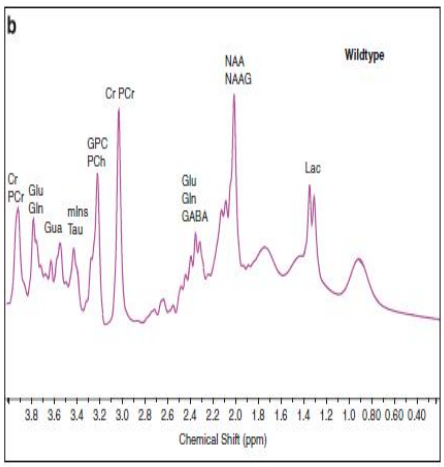
Figure 8. Increased postsynaptic hippocampal glutamate neurotransmission by 5-HT$_{1B}$R stimulation in p11KO mice. Histograms quantifying total protein levels and phosphorylated form of the protein normalized to the total level of the protein (c–d) in the hippocampus. Representative western blots are shown above each histogram. Genotype-dependent effects were found for phosphorylation at Ser^{831} of the GluR1 subunit and increased phosphorylation at Ser^{845}-GluR1 by CP94253 in p11KO mice (d). n=5–6 per group. Data are presented as means±S.E.M; V: vehicle (saline), CP: CP94253 (5-HT$_{1B}$R agonist), G: genotype, P11KO: p11 knock-out mice, WT: wild type mice, *p<0.05; **p<0.01 between indicated treatments, Two-way ANOVA followed by Newman-Keuls test.

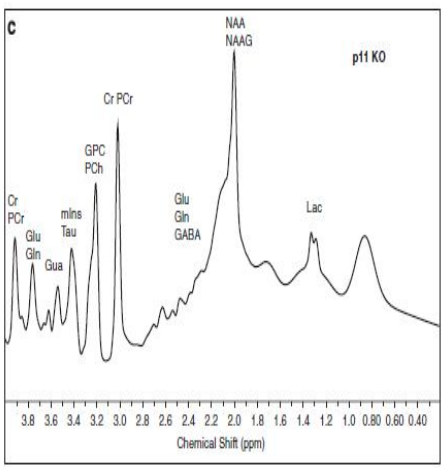
3.3.3 Decreased GABA and Gln upon (^{1}H-MRS) measures in hippocampus of p11KO mice

Upon in vivo proton magnetic resonance spectroscopy (^{1}H-MRS) measurements of neurochemical events, it was found that in the hippocampus of p11KO mice there is a decrease in GABA and Gln level (Figure 9d).

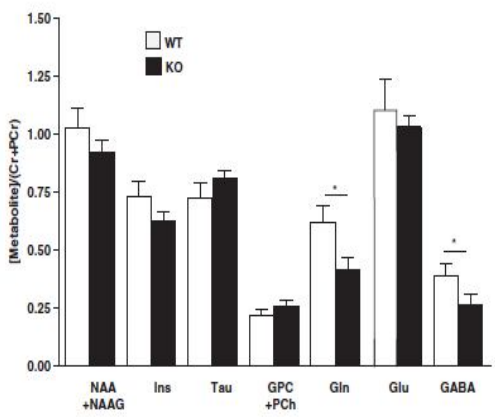
Figure 9. Reduced hippocampal inhibitory transmitters detected by in vivo proton magnetic resonance spectroscopy (1H-MRS).







Representative MRI (magnetic resonance image) featuring coronal, axial and sagittal slices through a mouse brain (a). Placement of the voxel, the volume of interest (VOI) sized 3.0x1.8x1.8 mm^3, for spectroscopy in the hippocampus 1H-MR spectra acquired from the voxel centered in the hippocampus of WT (b) and p11KO mouse (c). Mean neurochemical concentration in WT (white bars) and p11KO mice (filled bars) (d). Relative concentrations of glutamine and GABA were reduced in the hippocampus of p11KO mice when compared to WT mice. P11KO (n=8). NAA+NAAG:N-Acetylaspartate+N-Acetylaspartatylglutamate, Glu:Glutamate,Gln: Glutamine,GABA;gamma-amino butyric acid, GPC+PCh:GlyceroPhosphocholine+Phosphocholine, WT: wild type mice, P11KO: p11 knock-out mice. Data are presented as means±S.E.M for WT (n=7); *p<0.05 between indicated groups, Student's t test.



3.4 Effects of NMDARs antagonists, ketamine and Ro25-6981, upon glutamate release in hippocampal prefrontal cortex circutry (Paper IV)

3.4.1 Local application of ketamine or Ro25-6981 decreases the evoked glutamate release in subiculum

Local application of either ketamine or Ro25-6981 (100µM) altered the evoked glutamate release in the hippocampal-prefrontal cortical circuitry, in particular subiculum (Figure 10c, e), with no effect on tonic level of glutamate (Figure 10d, f).

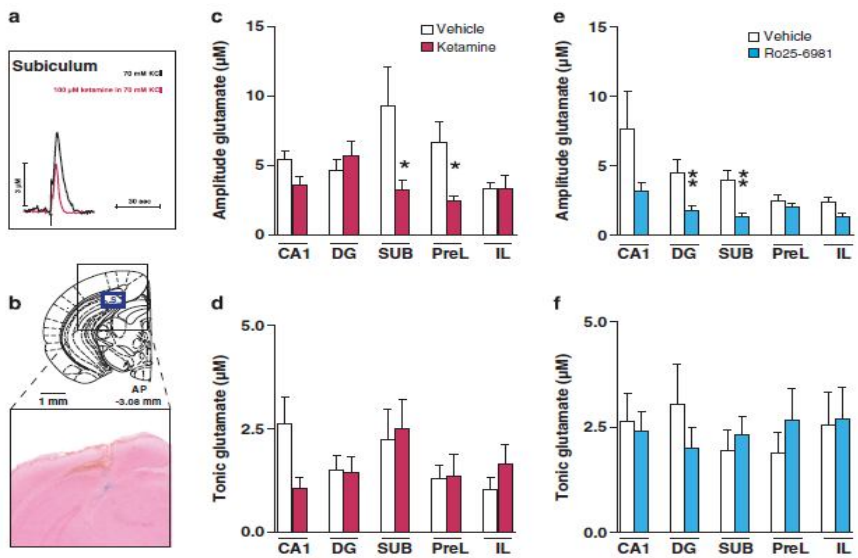


Figure 10. Evoked and tonic glutamate release after local application of ketamine and Ro25-6981. Representative glutamate peaks from the subiculum (a). Photomicrograph showing histological verification of the MEA recording site by local methylene blue injection in a 50-µm coronal brain section of the subiculum (indicated on the right hemisphere), after counterstaining with Nuclear Fast Red (b). The depolarizing solution (70mM KCl) was pressure-ejected (↑) in the absence (vehicle) or presence of ketamine or Ro25-6981 (100 µM) for 1-s duration with 1-min interval between each application. The evoked glutamate (c, e) and the tonic levels (d, f) are depicted per studied region. Note that local application of ketamine caused a significant decrease in presynaptic glutamate release in the subiculum and prelimbic area of the prefrontal cortex (c), whereas local application of Ro25-6981 caused a significant decrease in the subiculum and dentate gyrus (e). Tonic extracellular glutamate levels did not change after local application of either ketamine (d) or Ro25-6981 (f) in any of the regions analyzed. Data represent mean±S.E.M from five glutamate peaks from 8–14 animals. *$p<0.05$; **$p<0.01$, paired Student's t-test versus vehicle within region. CA1: cornu ammonis 1, DG: dentate gyrus, SUB: subiculum, PreL: prelimbic region of the prefrontal cortex, IL: infralimbic region of the prefrontal cortex.

3.4.2 Acute, systemic, subanesthetic dose of ketamine decreases the evoked Glu release in subiculum

We have also found that, an acute, systemic administration of ketamine, at an antidepressant-like dose (10mg/kg), led to a decrease in evoked glutamate release (Figure 11a) but not in tonic Glu levels in subiculum (Figure 11b).

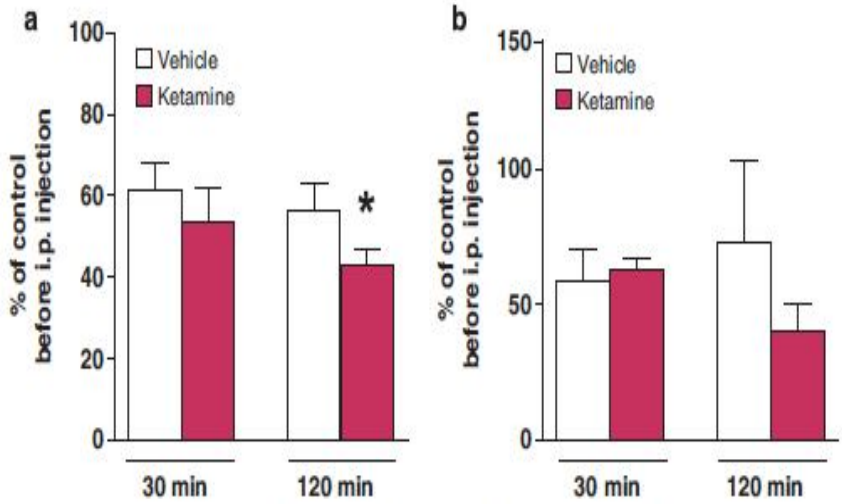


Figure 11. Evoked and tonic glutamate release in the subiculum after subanesthetic injection of ketamine. The depolarizing solution (70mM KCl) was pressure ejected for 1-s duration with 1-min interval between each application. The evoked glutamate (a) and the tonic levels (b) are depicted as a percentage of control before the intraperitoneal (i.p.) injection of either S-ketamine (15 mg kg− 1) or saline. Note that the subanesthetic dose of S-ketamine caused a significant decrease in presynaptic glutamate release in the subiculum 120 min after the administration but not after 30 min (a). Tonic extracellular glutamate levels did not significantly change at any time post injection. Data represent mean ±S.E.M from five glutamate peaks from six to nine animals. *$p<0.05$, unpaired Student's t-test versus vehicle within region.

4 GENERAL DISCUSSION

In **paper I**, by using systemic and intrahippocampal administration, we have shown PA impairment by hippocampal 8-OH-DPAT in mice. The PA facilitation was due to activation of 5-HT_7R through 8-OH-DPAT and blockade of 5-HT_{1A}R by NAD-299; this effect was blocked by the 5-HT_7R antagonist, SB269970. These results indicate the crosstalk between 5-HT_{1A}Rs and 5-HT_7Rs, in particular modulation of PA retention in this hippocampus-dependent memory task.

In **paper II**, by using behavioral and biochemical approaches, we have investigated and confirmed the antidepressant properties of lurasidone and fluoxetine. Lurasidone exerts its effects upon D_2, 5-HT_{2A} and 5-HT_7, as a receptor antagonist, and upon 5-HT_{1A}, as a receptor agonist (Huang et al., 2014). Lurasidone has been recently approved for treatment of bipolar depression by the food and drug administration (FDA). Clinical trials indicated that lurasidone alleviated both positive and negative symptoms of schizophrenia (Meltzer et al., 2011). As previously studied, the homozigous SERT knockout ($SERT^{-/-}$) rats show an anxious and depressive-like behavior when tested in paradigms such as the forced swim test and sucrose consumption (Olivier et al., 2008). In this particular rat model of depression, lurasidone restored the neurotrophin deficits in prefrontal cortex region (Luoni et al., 2012). We have shown that chronic lurasidone and fluoxetine treatment shares not only similarities upon effects in the NIH (test sensitive to chronic antidepressants exposure in mice, eg. Dulawa et al., 2004), but also at molecular levels. Both compounds decreased the NMDAR expression, in particular, the NR1, NR2A and NR2B-subunits in hippocampus and prefrontal cortex.

In line with these results, in **paper IV** we have demonstrated that in the subiculum, local administration of either ketamine or Ro25-6981 decreases the depolarization-evoked Glu release. Moreover, we have shown that an acute, systemic administration of ketamine, at subanesthetic and antidepressive dose, decreases the evoked Glu-release in the subiculum, after 2h of administration. Based on previous studies (Behr et al., 2009; McGuiness et al., 2010; Buchanan et al., 2012) and our data, we hypothesized that this effect is upon preNMDAR blocking. Both acute ketamine treatment (sub anaesthetic dose) (Muller et al., 2013) and chronic antidepressant treatment with three classic antidepressants-SSRI, tricyclic antidepressant and norepinephrine reuptake inhibitor, regulates the presynaptic release machinery in the hippocampus (Bonanno et al., 2005). Ketamine has a rapid and sustained antidepressive effect on clinical and pre-clinical trials (Zarate et al., 2006; Maeng et al., 2008; Li et al., 2010; Autry et al., 2011; Duman & Aghajanian., 2012; Muller et al., 2013).

In **paper II**, fluoxetine enhanced the expression of P-Ser^{896}-NR1, both in hippocampus and prefrontal cortex, and of P-Ser^{897}-NR1 in prefrontal cortex. The phosphorylation of NR1 subunits at Ser^{896} (PKC site) and at Ser^{897} (cyclic AMP-dependent protein kinase, PKA site) are involved in enhancement of synaptic efficacy (Gao et al., 2005). Moreover, it has been indicated that impairment of P-Ser^{897}-NR1 leads to glutamatergic alterations that can contribute to behavioral deficits in psychiatric disorders (Li et al., 2009). Importantly, recent studies indicate that fluoxetine selectively blocks the NMDAR-containing NR2B subunit (Vizi et al., 2013).

Furthermore, in **paper II** lurasidone and fluoxetine decreased PSD-95 levels in hippocampus and prefrontal cortex. PSD-95 interacts with NMDAR and downstream signaling proteins (Aoki et al., 2001) and is associated with the regulation of glutamatergic signaling at the postsynaptic site (Zhang et al., 2013).

A single dose of either ketamine or Ro25-6981 increases the PSD-95 levels in PFC, which was shown to be accompanied by behavioral antidepressive effects (Li et al., 2010). On the other hand, chronic treatment with desipramine, a TCA compund, at daily dose of 15 mg/kg for three weeks, reduced PSD-95 levels in rat hippocampus (Martinez-Turrillas et al., 2005). *Postmortem* studies from depressed patients reported that PSD-95 levels are either increased in amygdala (Karolewicz et al., 2009) or decreased in the PFC, together with NR2A and NR2B subunit levels (Feyissa et al., 2009). It has been indicated that these differences in *postmortem* studies might have appeared due to experimental techniques and also subject's characteristics (medication exposure) (Feyissa et al., 2009). Preclinical studies performed with PSD-95 constitutive knockout (KO) mouse indicated an increased stress reactivity in elevated plus maze test and anxiety-related responses in stress induced hypothermia test (Feyder et al., 2010). However, the PSD-95 KO mice show an antidepressive-like phenotype in the forced swim-test (Kiselycznyk et al., 2012).

Thus, together with these previous findings linking decreased NMDARs function with antidepressive phenotype, our observations that both chronic fluoxetine and lurasidone are able to downregulate NMDAR subunits, modulate their phosphorylation, and decrease PSD-95 levels, indicate that lurasidone shares common antidepressant mechanisms with the classical antidepressants.

In **paper III**, stimulation of 5-HT_{1B}R by CP-94253 regulated neurotransmission in the hippocampus of p11KO mice, a mouse model with genetic predisposition for depressive-like symptoms (Svenningsson et al., 2006). Similar to **paper IV**, we employed FAST methodology and found increased Glu release in hippocampus of p11KO mice, upon stimulation of 5-HT_{1B}Rs. It has

been previously shown that acute and chronic treatment with fluoxetine increase AMPAR phosphorylation (Svennigsson et al., 2002).
Postsynaptic measurements in hippocampus of p11KO mice revealed that CP-94253 increases AMPAR- GluR1 subunit phosporylation at Ser^{831} and Ser^{845}.
In line with increased excitability in p11KO mice upon 5-HT_{1B}R stimulation, ^{1}H-MRS recordings showed decreased hippocampal levels of inhibitory neurotransmitter, GABA, in p11KO mice. Our data are in line with clinical studies: decreased GABA concentrations have been linked with depression in humans (Sanacora et al., 2008), whereas treatment with SSRIs increase GABA levels (Sanacora et al., 2002).

In summary, the work performed in this thesis provided novel insights upon the interaction between serotonergic and glutamatergic systems in hippocampal and prefrontal cortex areas.

Four compunds with antidepressive-like properties, lurasidone, fluoxetine, ketamine and Ro26-6981 were able to downregulate/block NMDA receptors, in particular, the NR2 subunits.

It could be thus, suggested that NMDARs remain a valid target in developing innovative antidepressant compounds.

5 CONCLUSIONS

From the data presented in this thesis the following conclusions can be drawn:

- 5-HT_{1A} and 5-HT_7 receptors interacted in the modulation of hippocampal emotional learning.
- Chronic treatment with lurasidone or fluoxetine reduced anxiety-like behaviour in the NIH test. This antidepressive effect was followed by a decreassed NMDAR expression, in particular NR1, NR2A and NR2B-subunit levels in the hippocampus and prefrontal cortex. Moreover, both compunds modulated NMDAR phosphorylation and decreased PSD-95 levels in the studied regions.
- 5-HT_{1B}R agonist, CP-94253, increased hippocampal neurotransmission of p11KO mice.
- P11KO mice shown a decreased GABAergic inhibition in the hippocampus as compared with controls.
- Local intervention with the non-selective NMDAR antagonist, ketamine, or with selective NMDAR NR2B-subunit antagonist, Ro25-6981, reduced Glu evoked release in subiculum, with no significant effects over tonic glutamate levels in the studied regions. Moreover, an acute, systemic, subanesthetic and antidepressive-like dose of ketamine, decreased depolarization-evoked, but not the tonic glutamate release in subiculum.

6 POTENTIAL FURTHER STUDIES

The results of this thesis provided new insights into the interrelation of serotonergic and glutamatergic system in depression in general, and antidepressant medication in particular. Although studies presented here were primary focused on hippocampal-prefrontal cortex areas, further studies are needed; for example, to investigate regions associated with hippocampal-prefrontal cortex circuitry or/and afflicted by depression.

In brief, a few immediate points can be suggested:

- Continue to investigate NMDA receptor subunit-specific antagonist.
- Exploit FAST capabilities and perform *in vivo* experiments in freely moving mice.
- Combine FAST experiments with simple exploratory test or paradigms sensitive to depression.
- Perform longitudinal studies upon chronic antidepressant treatment combined with ^{1}H-MRS measurements. These measurements are non-invasive, could be region-specific oriented and could follow the treatment efficacy over time.
- Perform western-blot measurements upon acute/chronic antidepressant treatment and investigate other signaling pathways.

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