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Changes in synaptic proteins in hippocampal area CA1 of a rat learned helpless model of depression.

Oslo, June 2013

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Front page: Drawing of the dendritic spines of the human frontal cortex (Santiago Ramon y Cajal).
Serrano (2012), *Neuroscience*.

Abstract

Major depressive disorder (MDD) is one of the most common mood disorders in the world with a life-time prevalence of approximately 17%. MDD is characterized by emotional and cognitive disturbances, and is accompanied by volumetric changes of neural areas that likely arise from disturbed forms of neuroplasticity. The hippocampus has received a wealth of attention in MDD-related research not only because it is a prominent area to study forms of neuroplasticity, but also because it is engaged in cognitive as well as in emotional functions. In addition, the hippocampus displays a volumetric decrease in MDD. While it has been shown that stress associated with the development of MDD modulates forms of synaptic plasticity, investigations into the molecular correlates of these plastic changes are negligible. The present work investigates the immunolocalization and concentration of synaptic proteins involved in synaptic plasticity in the learned helpless model of depression. By means of immunogold electron microscopy, the relative concentration of the synaptic proteins syntaxin1, the NMDA receptor subunit NR2B, and Arc were examined in different region of the synapse. The synaptic regions of interest were the active zone, the postsynaptic density (PSD), and the presynaptic and postsynaptic cytoplasm in Schaffer collateral synapses of hippocampal area CA1. Comparing the learned helpless group to the wild-type group, I found a higher concentration of NR2B in the postsynaptic cytoplasm and in the PSD, as well as a higher concentration of Arc in the PSD of the learned helpless group. By contrasting the non-learned helpless group to the wild-type group, I found a lower concentration of syntaxin1 in both the presynaptic cytoplasm and in the PSD, as well as a greater concentration of NR2B in the postsynaptic cytoplasm and in the PSD in the non-learned helpless group. In addition, the learned helpless group displayed a higher concentration of syntaxin1 in the pre- and postsynaptic cytoplasm compared to the non-learned helpless group. The altered relative concentrations of these proteins are probably related to changes in synaptic plasticity in the learned helpless model of depression. One may speculate that the changed relative concentrations of the synaptic proteins contribute to the behavioral changes in the learned helpless model, and hence may be related to the pathology of MDD.

Abbreviations

ACTH	Adrenocorticotropin
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of variance
Arc	Activity-regulated cytoskeleton-associated protein
BDNF	Brain-derived neurotrophic factor
CaMKII	Calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CA1	Cornu Ammonis Area 1
CA2	Cornu Ammonis Area 2
CA3	Cornu Ammonis Area 3
CN	Calcineurin
CNS	Central nervous system
CRF	Corticotropin-releasing factor
DLPFC	Dorsolateral prefrontal cortex
DSM	Diagnostic and Statistical Manual of Mental Disorders
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
EC	Entorhinal cortex
EPSP	Excitatory postsynaptic potential
HF	Hippocampal formation
HPA	Hypothalamic-pituitary-adrenal axis
HSA	Human serum albumin
IEG	Immediate-early gene
IQR	Interquartile range
LH	Learned helpless
LTD	Long-term depression
LTP	Long-term potentiation
MDD	Major depressive disorder
NAc	Nucleus accumbens
NLH	non-learned helpless
NMDAR	N-methyl-D-aspartate receptor
PEG	Polyethylene glycol-electrolyte
PFC	Prefrontal cortex
PKA	Protein kinase A
PNS	Peripheral nervous system
PSD	Postsynaptic density
PSD-95	Postsynaptic density protein 95
SGZ	Subgranular zone
SNARE	Soluble NSF attachment protein receptor
SNAP-25	Synaptosomal-associated protein 25
SSRIs	Selective serotonin re-uptake inhibitors
SVZ	Subventricular zone
TBST	Tris-buffered saline tween 20
TEM	Transmission electron microscope
VAMP2	Vesicle-associated membrane protein 2
WT	Wild-type

Contents

1. Introduction

1.1 The brain as a substrate for diseases of the mind

Human beings have a great range of cognitive abilities such as the ability to learn, to make decisions or to produce and understand language. All of these functions arise from complex communication between cells in the brain. To understand the biological basis of behavior, cognition, and emotion, as well as determining the molecular events underlying pathological conditions in these human functions is one of today's greatest scientific challenges.

Despite the fact that the relationship between rigorous neural activity and the emergence of behavior and cognitive states has not been fully understood, the discipline of neuroscience is making constant progress in apprehending the working mechanisms of the brain. One of the most demanding questions neuroscientists try to find answers to is how consciousness evolves in terms of neurobiological processes. The current approach in trying to explain the commencement of consciousness focuses on the "*neural correlates of consciousness*". These neural correlates represent the idea that the firing of neural circuits might eventually terminate in conscious awareness. So far, knowledge on the neural mechanisms of consciousness is sparse (Chalmers, 1995).

Much of our current understanding of the brain derives from Santiago Ramon y Cayal's neuroanatomical investigations of the nervous system which lead to the foundation of the *neuron doctrine (Kandel and Squire, 2000).* The neuron doctrine postulates that the brain consists of a vast amount of discrete cells, the neurons, which pass on information through specialized contact points, the synapses. Neurons and synapses are subjected to constant experience-dependent modifications throughout life. These changes in neural pathways and synapses are the consequence of our daily experiences and shape our future thinking, feeling and behavior. Disturbed neural pathways and molecular processes within the synapse might lead to the development of *psychiatric disease*.

Given the advance of functional neuroanatomy and our increasing understanding of the molecular mechanisms underlying synaptic transmission, neuroscience has been contributing immensely to neurology and psychiatry. Though there exist many valuable treatment options for persons suffering from psychiatric disorders, it is of utmost importance for neuroscientific

research to further study neural areas whose activity is affected by psychiatric disorders in order to implement excellent treatment options (Kandel and Squire, 2000).

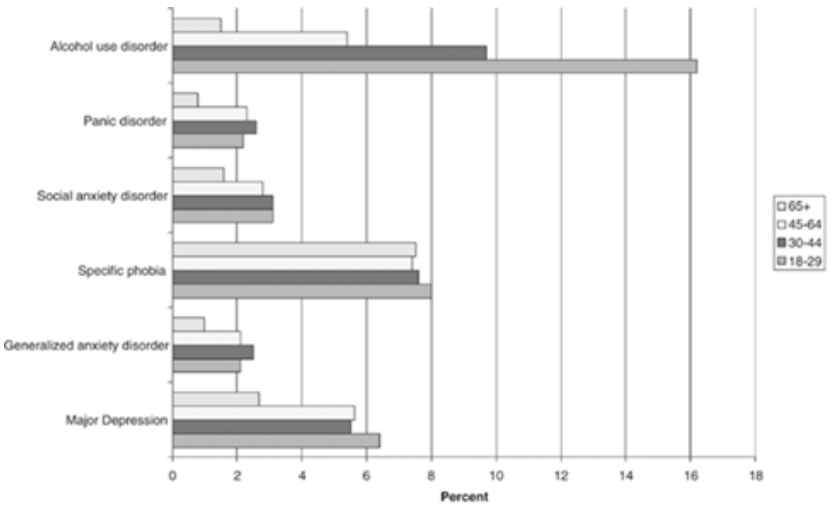
Understanding the neural mechanism underlying behavior and cognition, and hence psychiatric disorders, requires the ever increasing advancement in all fields of neuroscience to fully reveal an integrative concept about normal and abnormal human behavior. The purpose of this work is to examine the involvement of synaptic proteins in psychiatric disease; in this MDD.

1.2 Epidemiology of mood disorders

Mood disorders, such as MDD or substance-induced mood disorders are among the most prevalent mental disorders in the world (*figure 1)*. Furthermore, persons affected by mental illness show a high relapse rate. MDD is regarded as one of the top ten leading medical causes of disability in the world, being second to only ischaemic heart disease (Murray and Lopez, 1997). The lifetime prevalence of MDD of experiencing at least one major depressive episode is 17 %. In comparison to all psychiatric disorders captured by the DSM-V (*Diagnostic and Statistical Manual of Mental Disorders*, fifth edition), MDD has the highest projected lifetime risk with 23 % of the population suffering from MDD by the end of their lives. MDD affects all age ranges; however, it affects people above the age of sixty to a lesser extent. While MDD is equally affecting males and females during childhood, MDD is more common in women than in men after puberty (Seedat et al., 2009, Kessler et al., 2005). About 85 % of recovered patients relapse at some time in their lives, and only one out of nine persons that experienced

Figure 1

12-month prevalence of selected psychiatric disorders by age



Retrieved from "Kaplan and Sadock's comprehensive textbook of psychiatry", Blazer (2000): Mood disorders epidemiology.

one depressive episode will recover for more than 15 years without any relapse (Mueller et al., 1999).

There are many risk factors associated with the development of MDD. However, it is difficult to determine whether these risk factors are an association or causation of MDD. Nevertheless, studies have consistently demonstrated that there are four risk factors which are associated with MDD. These four risk factors are gender, stressful life events, aversive childhood experiences, and certain personality traits (Fava and Kendler, 2000).

1.3 Symptoms and classification of major depressive disorder

Everybody reacts with sadness in response to a painful stimulus because being sad is an evolutionary adaptive response forcing us into action. MDD is not necessarily adaptive and distinguishes itself from regular sadness by its severity, pervasiveness, duration, and symptoms. These symptoms comprise physiological, behavioural as well as cognitive disturbances. A depressive episode is marked in most cases by *anhedonia* (an inability to experience pleasure) and *depressed mood* (guilt, suicidality etc.). Some patients also predominantly express anger or irritability (Hyman and Cohen, 2013).

The *International Classification of Diseases (ICD), 10th edition, chapter V: Mental and Behavioural Disorders* by the World Health Organization and the *Diagnostic and Statistical Manual of Mental Disorders (DSM), fifth edition,* issued in May 2013 by the American Psychiatric Association are commonly applied diagnostic tools to classify abnormal behaviour as manifesting itself in a psychiatric disorder. Though there are different forms of depression, MDD is the most common type according to the DSM-V. The equivalent of MDD in the ICD-10 is *recurrent depression*.

As shown in *figure 2*, the classification of MDD by the DSM-V depends on the expression of five or more symptoms that must occur within a two-week period. One of the expressed symptoms must be anhedonia or depressed mood. The ICD-10 classifies a recurrent depression if one or more depressive episodes occur within a two-week time period, and if the patient expresses two out of three core symptoms (depressed mood, loss of interest and enjoyment, reduced energy leading to fatigability and diminished activity) and at least three common symptoms (Hyman and Cohen, 2013).

Because MDD (DSM-V) and recurrent depression (ICD-10) are the major types of depression, and are largely overlapping, I will refer to MDD throughout the text when writing about depression.

Figure 2

Criteria for a major depressive episode

A. Five (or more) of the following symptoms have been present during the same 2-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. **Note:** Do not include symptoms that are clearly due to a general medical condition, or mood-incongruent delusions or hallucinations.

- 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). **Note:** In children and adolescents, can be irritable mood.
- 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)
- 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. **Note:** In children, consider failure to make expected weight gains.
- insomnia or hypersomnia nearly every day
- psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down)
- fatigue or loss of energy nearly every day
- feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick)
- diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others)
- 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

B. The symptoms do not meet criteria for a Mixed Episode.
C. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.
D. The symptoms are not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition (e.g., hypothyroidism).
E. The symptoms are not better accounted for by Bereavement, i.e., after the loss of a loved one, the symptoms persist for longer than 2 months or are characterized by marked functional impairment, morbid preoccupation with worthlessness, suicidal ideation, psychotic symptoms, or psychomotor retardation.

Retrieved from http://dsm.psychiatryonline.org/content.aspx?bookid=22&sectionid=1890406#2132

To consider the experimental work in a broader context, I will give a brief overview of the anatomy of the nervous system, and will specifically highlight those regions which are involved in MDD.

1.4 The structure of the brain

1.4.1 The nervous system and signal transmission

The nervous system is subdivided into the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS comprises the spinal cord and the brain, which is again divided into six parts. These six parts are the medulla, the pons and the midbrain (brain stem), the cerebellum, the diencephalon (containing e.g. the thalamus and the hypothalamus), and the cerebrum (containing the cerebral hemispheres, the hippocampus, structures of the basal ganglia, and the amygdala).

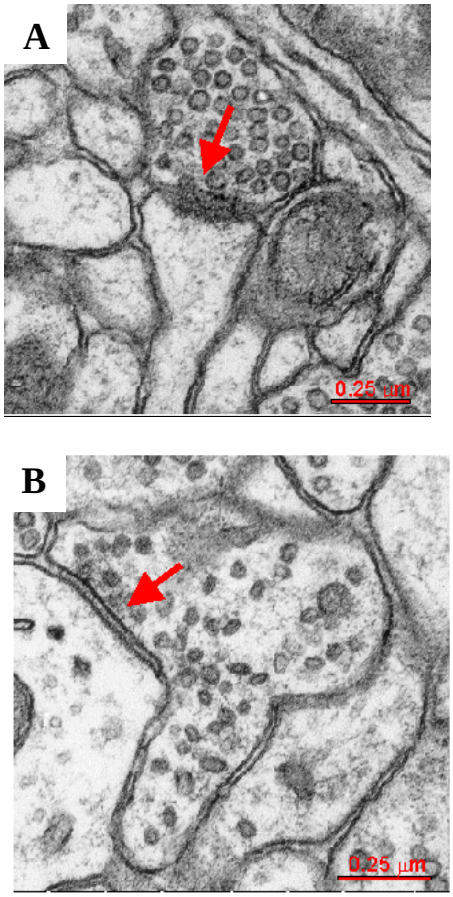
The cerebral hemispheres comprise the largest part of the brain and are divided into four different lobes. These are the frontal, the parietal, the temporal and the occipital lobes, each embodying a different set of functions such as the planning of actions, somatic sensation, vision or hearing. The CNS does not unite with the overlying bone of the skull. Instead, three membranes, the dura mater, the arachnoid membrane and the pia mater (collectively called the *meninges*), cover the CNS. The cavernous interior part of the brain, making up the ventricular system, is filled with cerebrospinal fluid (CSF). The PNS is defined as all the parts of the nervous system lying outside the brain and spinal cord, i.e. consisting of the spinal nerves and autonomic nervous system (Kandel and Hudspeth, 2013).

Brain tissue is made up by two different main cell types which are the neurons and neuroglia. A neuronal cell consists of a cell body, dendrites, axons and synaptic terminals. The intermediate filaments, microtubules and actin filaments lend the neuron its shape and stability, and are also involved in the delivery of pre- and postsynaptic supplies to the synaptic plasma membranes. There are about 50 different types of neurons each expressing different genes and synthesizing different proteins (Schwartz et al., 2013). Glia cells (astrocytes, oligodendrocytes, ependymal cells and microglia) support neuronal functioning. They are for example supplying neurons with nutrients and oxygen, or are involved in the recycling of neurotransmitters. There seems to be evidence that astrocytes also regulate synaptic strength and synaptogenesis (Nedergaard et al., 2003).

The contact point for information exchange is at chemical and electrical synapses. While in electrical synapses electrical current flows

Figure 3

Asymmetric and symmetric synapses



A = asymmetric synapse in the hippocampus. **B** = symmetric synapse in the hippocampus. Arrows point to the presynaptic site of the synapse. Compared to the symmetric synapse, the excitatory asymmetric synapse has a larger synaptic cleft, is located on dendritic shafts, and expresses a clearly visible PSD. Retrieved from http://synapses.clm.utexas.edu/anatomy/chemical/synapse.stm

passively through gap junctions, chemical synapses release neurotransmitters which act as messengers communicating between the presynaptic and postsynaptic membrane. There are two main types of chemical synapses: Excitatory and inhibitory synapses. These two types of synapses differ with respect to the kind of postsynaptic response, neurotransmitter types, and morphology (*figure 3*). The more common excitatory type I (asymmetric) synapse has a large synaptic cleft, is located on dendritic shafts, expresses a clearly visible PSD, and releases glutamate. The inhibitory type II (symmetric) synapse releases GABA (gamma-aminobutyric acid) and has a less pronounced, but more elongated, PSD. Though this so-called *functional Gray concept* is accepted and frequently applied, debate continues about the strict division between type I and type II synapses (Klemann and Roubos, 2011).

The information exchange at chemical synapses is mediated by action potentials arriving at the presynaptic terminal thereby depolarizing the presynaptic cell. This leads to the opening of voltage-gated Ca^{2+} channels. The increasing amount of [Ca^{2+}] into the cell triggers the actual exocytic fusion of the vesicle membrane with the presynaptic plasma membrane in order to deliver neurotransmitters into the synaptic cleft. A coding system of Rab proteins on the surface of the vesicle allows for the initial recognition between the vesicle and the target membrane. Once the vesicle is tethered to the target plasma membrane by holding onto the vesicles' Rab proteins, v-SNARE's (SNARE proteins on the vesicle) bind to complementary t-SNAREs (SNARE proteins on the target membrane) that ultimately fuse the vesicle to the target membrane through an interplay with SM (Sec1/Munc18-like) proteins (Sudhof and Rizo, 2011).

Neurotransmitters are generally divided into amino acids (such as glutamate or GABA), amines (acetylcholine, serotonin or dopamine amongst others) and peptides (for instance neuropeptides). Released neurotransmitters diffuse from a local high concentration to a region with a lower concentration, and in most cases bind to postsynaptic receptors. Whether postsynaptic channels open or close depends on the receptor onto which the neurotransmitters bind. The opening or closing of channels determines the postsynaptic current generating either an excitatory or inhibitory postsynaptic potential in the cell. Neurotransmitters in the synaptic cleft are eventually degraded, taken up by astrocytes or recycled and sent back to the presynaptic neuron (Bear, 2007).

While the section described above focused on the general anatomy and cell biology, specific brain regions are involved in MDD. These brains regions, specifically the hippocampus, will be highlighted below.

1.4.2 *Brain areas involved in major depressive disorder*

The first serious discussions and analyses of the idea that certain neural areas are involved in the pathology of mood disorders emerged with Papez's outline of the *system of emotion* (Papez, 1937). This emotion-processing circuit involves the cingulate gyrus, the hippocampus, the hypothalamus and the anterior thalamic nuclei. Today, major parts of this system such as the amygdala or the hippocampus are accepted structures involved in maintaining emotional stability. In addition, the PFC (prefrontal cortex) is now a recognized neural area of the emotion-processing system. As discussed below, certain neural areas involved in the processing of emotion show a volumetric reduction during MDD (*table 1*). These volumetric changes need to be considered with caution. It is not yet apparent whether (i) these volumetric changes are sole reversible neuroplastic abnormalities, and (ii) whether structural changes precede MDD or occur over the course of the disease. The amygdala, the PFC, and the hippocampus have received major attention in neuroplasticity-related research of MDD.

Table 1

Structural changes in selected brain areas

Brain region	Volume	Antidepressant treatment
VMPFC	↓	
ACC	(?)	↓ (Metabolic activity)
Hippocampus	↓	↑ (Volume)
Amygdala	↓ (?)	↓ (Metabolic activity)
DLPFC	↓	(?)

ACC = anterior cingulate cortex, DLPFC = dorsolateral prefrontal cortex, VMPFC = ventral medial prefrontal cortex. Retrieved from Palazidou (2012), *Br Med Bull*.

The amygdala plays a major role in the regulation of emotion due to its involvement in emotional learning as well as in coordinating cortical arousal and neuroendocrine responses. It is not agreed upon whether the amygdala increases or decreases in size during MDD. It seems that in depressed patients the size of the amygdala increases at first, but with iterative depressive episodes decreases in size (Hamilton et al., 2008, Lorenzetti et al., 2009).

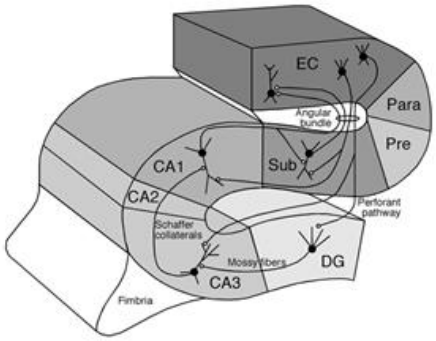
The PFC is located in front of the motor and premotor areas and is involved in integrating sensorimotor information with motivation and affect. The PFC is grossly divided into the

dorsolateral prefrontal cortex (DLPFC), the anterior cingulate cortex (ACC), and the orbitofrontal cortex. Each of these subdivisions controls different aspects of emotion regulation and processing, and shows a varying degree of neural activity during MDD. This varying neural activity seems to be correlated with some of the expressed symptoms of depressed persons. For example, decreased neural activity in the DLPFC is associated with psychomotor retardation and anhedonia. It has been shown that MDD is accompanied with a significant decrease in volume of this brain region (Palazidou, 2012).

The hippocampus proper is part of the hippocampal formation (HF) which in addition contains the dentate gyrus (DG), the subiculum, the presubiculum, the parasubiculum, and the entorhinal cortex (EC) as shown in *figure 4*. The hippocomapus is the most widely studied brain region in the field of MDD. The reasons for this are because i) it is involved in learning and memory that play a role in emotional responses, ii) it is enriched with corticosteroid receptors and a malfunctioning hypothalamic-pituitary-adrenal (HPA) axis is a prominent feature of MDD, iii) it is one of only two brain regions known today that exhibit neurogenesis which is disturbed during MDD, and iv) it has strong connections with the amygdala and PFC (Palazidou, 2012). A hippocampal volume loss is also a distinct feature of MDD (Lorenzetti et al., 2009).

Figure 4

The hippocampal formation and its internal projections



EC = entorhinal cortex, para = parasubiculum, pre = presubiculum, DG = dentate gyrus, CA1/CA2/CA3 = CA1/CA2/CA3 regions of the hippocampus. Retrieved from «The hippocampus book», Amaral & Lavenex (2006): *Hippocampal neuroanatomy*.

The hippocampus is divided into different parts and structural layers that will be briefly outlined in the next section.

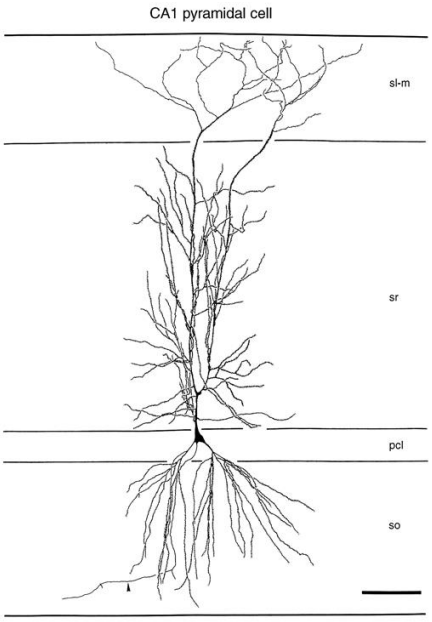
1.4.2 CA1 of the hippocampus

The hippocampus accommodates three distinct regions which are the *Cornu Ammonis Area 1* (CA1), *Cornu Ammonis Area 2* (CA2), and *Cornu Ammonis Area 3* (CA3) each consisting of several layers.

Figure 5 displays a CA1 pyramidal cell. The basal dendrites of the pyramidal cells project into the stratum oriens. Superior to the pyramidal cell layer in CA3 lays the stratum lucidum. Above the stratum lucidum in CA3 and directly above the pyramidal cells layer in CA1 and CA2 is the stratum radiatum. The most superficial layer of the hippocampus above the stratum radiatum is the lacunosum-moleculare (van Strien et al., 2009).

Figure 5

CA1 pyramidal neuron



Pcl = pyramidal cell layer, sl-m = stratum lacunosum-moleculare, so = stratum oriens, sr = stratum radiatum, Scale bar = 100 µm. Retrieved from «The hippocampus book», Amaral & Lavenex (2006): *Hippocampal neuroanatomy*.

During the experimental part of this work I focus on the apical dendrites of pyramidal cells which span the stratum radiatum of the CA1. Irrespective of their location in CA1, all pyramidal cells have approximately the same dendritic length (13.5 mm). It is known that almost 30000 excitatory and 1700 inhibitory neurons connect with a single CA1 pyramidal cell. Inhibitory input mainly targets the soma and the axon of the pyramidal cell. Excitatory input arrives through the dendrites of various external inputs. The distal tufts of CA1 pyramidal cell dendrites receive their main input from the EC through the *perforant pathway* and the thalamus, while more proximal dendrites receive the input from CA3 neurons through the *Schaffer collaterals*. The analyses of apical dendrites in CA1 during this work focus on spines containing Schaffer collateral synapses that constitute the postsynaptic sites of excitatory glutamatergic input. There are different morphological types of spines in the hippocampus. The morphology is possibly related to the spines' functions such as increasing the surface of dendrites and therefore the amount of synapses, restricting and controlling the diffusion of molecules, or regulating the electrical cell properties (Spruston, 2008).

The hippocampus can be regarded as the origin of our knowledge on various types of plasticity. Structural and synaptic plasticity have been immensely studied in this neural area and revealed many insights on the growth, loss and remodelling of neurons, dendritic spines and synapses. It is widely accepted that stress has a negative effect on the rate of neurogenesis

or the induction of LTP in the hippocampus. Neuroplastic changes are the consequence of our experiences, and are the basis of our future thinking, behaviour, and feeling. Malfunctioning forms of plasticity might therefore contribute to the pathology of MDD. Exploring disturbed forms of plasticity might reveal explanations why the hippocampus decreases in size during MDD. Prominent forms of plasticity will be highlighted below.

1.5 Forms of synaptic plasticity

Long-term molecular changes of synapses are due to experience-dependent modifications that underlie altered neural activity. This process is termed *synaptic plasticity*. Mechanisms of synaptic plasticity are acting at different levels including long-term potentiation (LTP) and long-term depression (LTD), as well as structural plasticity or the recently described form of homeostatic plasticity. It has recently been shown that plastic changes in the brain as measured by region-specific cognitive tasks can be used as a model to test the *neuroplasticity hypothesis* of MDD (Nissen et al., 2010). The neuroplasticity hypothesis assumes that the adaptation of neurons and synapses to external and internal stimuli plays a major role in the pathology of MDD. The study by Nissen et al. (2010) revealed that synaptic plasticity is decreased in the hippocampus and the PFC, and increased in the amygdala as measured by cognitive tasks involving one of the later three neural regions. In addition, a study by Hajszan et al. (2009) revealed that learned helpless behavior in rats is associated with a loss of synapses in the hippocampus. In sum, these studies indicate alterations in long-term synaptic plasticity are occurring during MDD.

1.5.1 Long-term potentiation and long-term depression

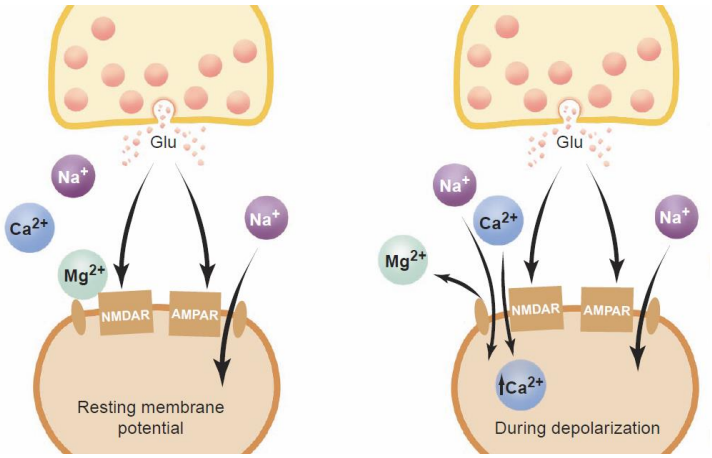
1.5.1.1 Long-term potentiation

The mechanisms of LTP and LTD demonstrate that individual synapses are capable of long-lasting experience-dependent modifications. LTP was initially discovered by Timothy Bliss and Terje Lømo who found that a high-frequency electrical stimulation (tetanus) of excitatory pathways to the hippocampus leads to a long-lasting amplitude increase of the excitatory postsynaptic potentials (EPSPs) in dentate granule neurons (Bliss and Collingridge, 1993).

Figure 6 shows how LTP is induced. The interplay between Schaffer collaterals and CA1 pyramidal neurons relies on *input-specificity* ([Ca^{2+}] increases only in a certain activated dendritic spine) and *associativity* (activation of one set of synapses can facilitate LTP in another set of synapses in the same cell given an appropriate time window). The release of glutamate from the Schaffer collaterals activates both AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazolepropanoic acid receptors) and NMDARs (*N*-Methyl-D-aspartate receptors) in the postsynaptic membrane. In contrast to the AMPARs, opening of the NMDARs requires that Mg^{2+}, which blocks the NMDA channel at the resting stage, is displaced. Removal of the Mg^{2+} block is achieved through a concurrent binding of glutamate, and a significant depolarization of the postsynaptic cell by means of a Na^{+} influx through the AMPA channel. Opening of the NMDARs ultimately allows the conductance of Ca^{2+} into the cell, and therefore signals the simultaneous activation of the presynaptic and postsynaptic cell. Due to this concurrent activation, NMDARs are said to act as *coincidence detectors*. The rise of [Ca^{2+}], the critical trigger of LTP, activates downstream signalling pathways such as the protein kinase C (PKC) and calcium calmodulin dependent protein kinase II (CaMKII) (Hu et al., 1987). Activation of the kinases leads to an increased effectiveness of the existing AMPARs either through enhanced ionic conductance of their channels or by the insertion of new AMPARs into the postsynaptic plasma membrane (Song and Huganir, 2002, Bredt and Nicoll, 2003). It has also been proposed that retrograde messengers are released in the postsynaptic cell, which activate protein kinases in the presynaptic cell leading to an increased neurotransmitter release (Malenka and Bear, 2004).

Figure 6

Induction of LTP



During normal synaptic transmission, glutamate (Glu) is released from the presynaptic cell and acts on AMPA and NMDA receptors. Na^{+} flows only through the AMPA receptor, because Mg^{2+} blocks the channel of the NMDA receptor. Depolarization of the postsynaptic cell relieves the Mg^{2+} block of the NMDA receptor, allowing Na^{+} and Ca^{2+} to flow into the dendritic spine. The resultant rise in Ca^{2+} is the critical trigger of LTP. Retrieved from Malenka (1999), *Science*.

The development of new synapses during late LTP requires the synthesis of new proteins.

This process is mediated by signalling molecules such as PKA (protein kinase A) or CaMKII that link the activity of LTP to the nucleus activating transcription factors such as CREB (cAMP response element-binding protein) and immediate-early genes (IEG) (Malenka and Bear, 2004). The maintenance of LTP eventually leads to structural changes such as an increased amount of synapses, growth of dendritic spines, and the remodelling of the cytoskeleton (Matsuzaki et al., 2004).

1.5.1.2 Long-term depression

LTD is induced by prolonged low-frequency stimulation leading to a rise in postsynaptic [Ca^{2+}]. In contrast to LTP, the lower but longer influx of Ca^{2+} due to the low-frequency tetanus leads only to a modest postsynaptic depolarization, and is thus less effective at removing the Mg^{2+} blockade of NMDARs. It is assumed that LTD involves the activation of NMDARs containing the NR2B subunit (Massey et al., 2004).

While a high influx of Ca^{2+} leads to the activation of kinases in LTP, lower but longer influx of Ca^{2+} during LTD leads to the activation of the calcium-dependent phosphatase calcineurin (CN). This again triggers signalling cascades that activate protein phosphatases dephosphorylating the AMPAR subunit GluA1 (Lee et al., 1998) or increased phosphorylation of AMPAR subunit GluA2 (Malenka and Bear, 2004). The combinatorial effect of the phosphorylation of GluA1 and the dephosphorylation of GluA2 leads to the endocytosis of AMPARs in the postsynaptic membrane diminishing the EPSP (Beattie et al., 2000). Because LTD involves the reduction of AMPARs, place holders, or *slot proteins*, are required to keep signal transmission in balance by substituting the internalized AMPARs. One of the slot proteins could be PSD-95. Overexpression of this protein is correlated with a reduction of AMPARs while a decrease of PSD-95 is associated with an increased amount of AMPARs (Malenka and Bear, 2004).

Though LTP and LTD are not the only contributors to the development or sustainment of volumetric reduction of the neural areas in MDD, there is evidence that these forms of synaptic plasticity are involved in MDD. As outlined above, LTP is associated with changes in the ultrastructural organization of synapses and spines. Perturbations of LTP in MDD are therefore likely to mediate regional volume differences of the brain. For example, rats that experienced stress by confining them into restraining tubes showed impaired LTP as measured in slices immediately after death (Foy et al., 1987).

The stress-induced negative impact on LTP is modulated by the degree of control the rat possesses over the stressor. It has been shown that the rats' ability to terminate a foot shock only diminished LTP slightly, whereas rats who lacked the control to terminate the foot shock displayed a significant impairment in the induction of LTP (Shors et al., 1989). This indicates that the controllability of aversive stimuli affects synaptic plasticity at the basic level. The impairment of LTP is due to the psychological factor of a lack of control and not the shock itself.

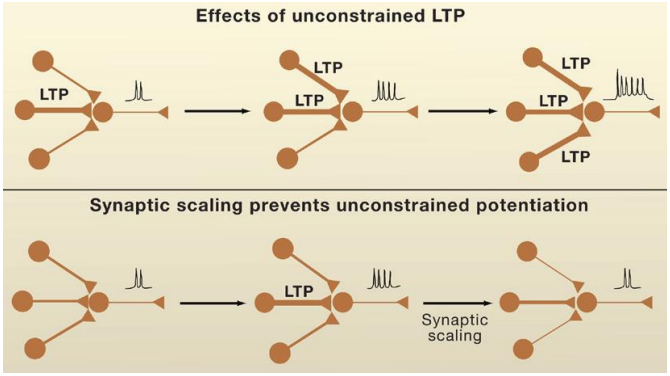
It is likely that LTP and LTD are involved in many aspects of the theoretical explanations dealing with the pathology of MDD.

1.5.2 Homeostatic plasticity

Activity-dependent forms of plasticity such as LTP and LTD tend to destabilize neural network activity in the long-run by constantly increasing or decreasing synaptic strength. Therefore, it has been suggested that neurons can regulate their activity with respect to the global network activity by either modulating synaptic activity or by changing the properties of ion channels (*figure 7*). This form of homeostatic plasticity, or synaptic scaling, was initially shown by lowering the inhibition of neurons. At first, inhibition increased firing rates of the postsynaptic cell, but later these firing levels fall back to the control levels despite not modifying the level of inhibition (Turrigiano et al., 1998).

Figure 7

Homeostatic synaptic scaling



Unconstrained LTP will lose synapse specificity. Homeostatic scaling prevents for instance runaway excitation by reducing the strength of other synaptic inputs until the firing rate returns to control levels. Retrieved from Turrigiano (2008), *Cell*.

It is assumed that neurons have the ability to detect their own firing rates by means of calcium-dependent sensors. According to the measured firing rates, the trafficking of glutamate receptors leads to a decreased or increased amount of AMPARS and/or NMDARS in the postsynaptic membrane (Perez-Otano and Ehlers, 2005). The release probability of

neurons demonstrates another homeostatic mechanism, because neurons have the ability to release a flexible amount of glutamate thereby regulating postsynaptic activity (Turrigiano, 2008).

1.5.3 Structural plasticity: Neurogenesis and synaptogenesis

1.5.3.1 Neurogenesis

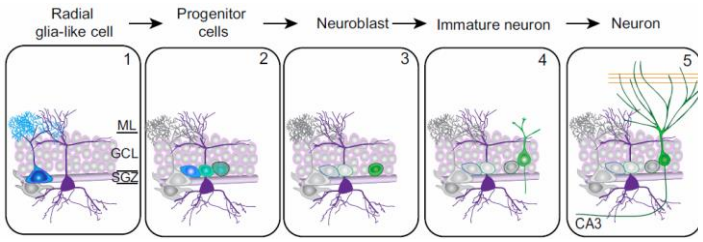
Neurogenesis is the process of generating functional neurons from precursors (Ming and Song, 2011). Though it has been thought that this process is restricted to embryonic and perinatal stages, there are two "neurogenic" regions, the subgranular zone (SGZ) in the DG and the subventricular zone (SVZ) in the lateral ventricles, that generate new neurons throughout life.

Stem cells in the brain are able to perform self-renewal process through cell division and are also able to differentiate into specific cell types. One hypothesis assumes that radial glia-like cells exhibiting glial fibrillary acidic protein (GFAP) and non-radial cells act as neural stem cells leading to the generation of new neurons in the SVZ and the SGZ (Ming and Song, 2011).

Neurogenesis largely resembles the sequence of generating new neurons during development as shown in *figure 8*. For instance, radial glia-like cells in the SGZ serve as progenitor cells that lead to the development of neuroblast. These immature neurons then migrate into the granule cell layer of the DG where they differentiate into granule cells. Within days, dendrites of the new neurons develop towards the molecular layer by projecting axons towards the CA3 (Ming and Song, 2011). The neurotransmitter GABA (Ge et al., 2006) and glutamatergic inputs via NMDARs (Tashiro et al., 2006) ensure the survival of new-born neurons.

Figure 8

Stages of neurogenesis in the dentate gyrus of the hippocampus



ML = molecular layer, GCL = granule cell layer, SGZ = subgranular zone. Retrieved from Mink (2011), *Neuron*.

During the maturation phase, newly generated neurons display a greater potential of synaptic plasticity (Schmidt-Hieber et al., 2004). This might give new-born neurons an advantage in

competing with mature neurons. Enhanced synaptic plasticity might also contribute to the stabilization of the afferent and efferent synaptic connections.

In contrast to LTD and LTD, the occurrence of neurogenesis is restricted to only two brain regions. A lack of neurogenesis might not be sufficient to describe the broad spectrum of volumetric alterations and associated cognitive disturbances as seen during MDD.

1.5.3.2 Synaptogenesis

The generation of new neurons during neurogenesis is naturally accompanied by the establishment of new synapses between cells. The process of generating new synapses is defined as the process involving the formation and precise alignment of a neurotransmitter release site in the presynaptic neuron, and a receptive field at the postsynaptic neuron (Jin, 2005). Synaptogenesis involves two steps. These are the selection of appropriate molecular partners to establish synapses in a certain subcellular compartment (*synaptic specificity*), and the process of forming the presynaptic structure and the postsynaptic signaling-rich specialization (*synaptic assembly*) (Colon-Ramos, 2009).

Synaptogenesis is not restricted to co-occur with neurogenesis. In fact, synapses within neural circuits of the cortex are formed or eliminated throughout life due to activity-dependent structural changes (Trachtenberg et al., 2002). It has been shown that the generation of spines is linked to the generation of synapses a few days after the spines appeared. In addition, these new synapses specifically connect with already existing boutons (Knott et al., 2006). As measured by *in vivo* imaging and serial-section electron microscopy (SSEM), 20 % of spines in the cortex generally disappear while 60 % of spines persist for a time period of at least eight days. The new spines are particularly responsible for the generation of new synapses leading to the experience-dependent rewiring of the adult brain (Trachtenberg et al., 2002).

It has been shown that transient and persistent spines belong to distinct populations (Holtmaat et al., 2005). Spines that persisted for more than eight days continued to persist for at least three weeks. Transient spines tended to disappear after a few days. In addition, persistent and transient spines displayed a different morphology. It might be possible, however, that transient spines that contained the developing synapses stabilize at a later stage and then enlarge due to synaptic stimulation into persistent spines.

The process of turning transient spines into persistent spines might involve the insertion of AMPARs into the postsynaptic membrane (Malinow and Malenka, 2002) and LTP (Matsuzaki et al., 2004). Interestingly, LTP leads to the generation of thin small spines which tend to be transient. These transient spines might grow into larger persistent spines resembling the strengthening of synaptic contacts. With respect to MDD, it has been shown that inescapable foot shocks in learned helpless rats eliminates synapses in hippocampal area CA1 and CA3 as well as in the dentate gyrus (Hajszan et al., 2009). In addition, it has been proposed that astrocytes regulate synaptogenesis. When neurons are co-cultured with astrocytes, the generation of new synapses is increased (Pfrieger and Barres, 1997). Therefore, glial cells induce synapses in the CNS and may contribute to synaptic plasticity.

Taken together, plasticity mechanisms such as LTP and LTD, homeostatic plasticity, and structural plasticity are major mechanisms that lead to long-lasting changes in synaptic activity. These forms of plasticity are therefore at the heart of the theories that relate MDD to plastic changes in the brain. These theories incorporating MDD-associated neuroplastic changes of the brain will be outlined in the next section.

1.6 Synaptic plasticity in major depressive disorder

There is strong evidence that uncontrollable aversive life events in human development are central to the aetiology of MDD (Pryce et al., 2011). These uncontrollable aversive life events most likely correlate with findings of morphological studies showing that MDD is associated with alterations in the shape and number of neurons and glia cells (Palazidou, 2012). Though there seems to be a strong link between aversive life events and neuroplastic changes leading to the onset of MDD, this relationship needs to be considered with caution. First, the exact cause-effect needs to be further elucidated, i.e. is a volumetric change of neural areas the cause or the result of the pathology of MDD? Second, hippocampal volume loss also occurs in other stress related disorders and in schizophrenia (Bremner et al., 1995, Heckers, 2001).

The volumetric decrease of the hippocampus is crucial to investigate not at least because this structure is involved in cognition and emotion regulation – processes that are severely disturbed during MDD. The following hypotheses of the development of MDD will focus on the structural alterations in the hippocampus.

1.6.1 Neurotrophic hypothesis of major depressive disorder

The hippocampus displays a large potential of plastic modifications and, as outlined above, accommodates one of the two neurogenic niches in the brain. Due to the observed volumetric change of this neural area during MDD, the *neurotrophic hypothesis* of depression postulates that the cause of neuronal cell loss is the result of a decrease in neurotrophic factors. While it was first assumed that neurotrophic factors only regulate cell growth and differentiation of cells during development, they are now also known to be involved in plasticity and survival of adult neurons. The most prevalent neurotrophic factor is brain-derived neurotrophic factor (BDNF) which regulates neurogenesis and synaptic plasticity (Duman and Monteggia, 2006). It is claimed that while stress reduces BDNF-mediated signalling, antidepressant treatment enhances BDNF-mediated signalling (Krishnan and Nestler, 2008). Malberg et al. (2000) highlight that only chronic antidepressant treatment leads to an improvement in mood. This suggests that the time interval between the beginning of antidepressant treatment and the improvement mood spans the time range of new-born neurons differentiating and maturing into functional neurons. So far, however, there has been little discussion about the mechanisms by which new neurons enhance mood. Kempermann (2008) proposed that neurogenesis increases neural activity allowing the hippocampus to adapt to new experiences.

The retraction of spines and the decrease in BDNF concentration during stress is mediated by an excessive amount of glucocorticoids. Glucocorticoids modulate transcriptional mechanisms controlling the concentration of BDNF. Antidepressant treatment increases BDNF concentration and the number of spines possibly through the activation of the transcriptional factor CREB. These changes might alleviate depressive symptoms by preventing actions of stress to influence the hippocampus (Nestler et al., 2002). The relationship between the concentration level of BNDF and corticosteroids has been demonstrated in a study by Mirescu et al. (2004), who showed that stress in early life suppresses neurogenesis later in life. The authors argued that rats separated from their mothers develop a hypersensitivity towards glucocorticoids, because they displayed a normal level of glucocorticoids but nevertheless display reduced cell proliferation.

There is also evidence that MDD cannot be attributed solely to a reduction in BDNF concentration. In contrast to the hippocampus, the nucleus accumbens (NAc) shows an increase of the BDNF concentration level in MDD, and direct infusion of BDNF into the NAc leads to depressive behaviour in rats (Krishnan and Nestler, 2008).

Overall, the reduced concentration of BDNF and the smaller size of brain regions in stressed rats add evidence to the correlation between stress, reduced concentration of growth factors and reduced sizes of brain regions. Nevertheless, the effect of other neurotrophic factors, and their influence on the development of MDD or antidepressant related changes, need to be examined in the future as well.

1.6.2 Monoamine hypothesis of major depressive disorder

The *monoamine hypothesis* most likely has been the most prevalent theory of MDD over the last decades. Today, the monoamine hypothesis is being regarded as an oversimplified view of the pathology of MDD. The popularity of the monoaminergic system in MDD is owed to two chemical compounds. These are imipramine and iproniazid, which display antidepressant effects by amplifying serotonin and noradrenaline transmission. The monoamine hypothesis postulates that a decreased amount of serotonin (5-HT), norepinephrine (NE) and/or dopamine (DA) lead to the development of MDD (Krishnan and Nestler, 2008). Today's prescribed antidepressants either inhibit the uptake (e.g. SSRIs, selective serotonin re-uptake inhibitors) or inhibit the degradation of monoamines (MAOIs, monoamine oxidase inhibitors).

However, MDD is not solely caused by a decrease in monoamines. Mood conditions in patients only improve after several weeks of treatment while, for example SSRIs, lead to an immediate extracellular increase in serotonin in the CNS. It is assumed that antidepressants enhance functional plasticity such as LTP (Stewart and Reid, 2000). Stewart and Reid (2000) have shown that the administration of SSRIs enhances LTP. This was possibly due to the generation of new proteins that alter the responsiveness of neurons, or allow the remodelling of connections that assist in alleviating depressive behaviour. It could be speculated that this enhanced plasticity is might be due to the new-born cells which exhibit a greater potential of synaptic plasticity (Schmidt-Hieber et al., 2004). Electron microscopic analyses at the ultrastructural level revealed the formation on new synapses during SSRI treatment (Hajszan et al., 2009).

1.6.3 Neuroendocrine functions

The activity of the HPA axis is controlled by many neural pathways. One of them is the input

from the hippocampus that inhibits the release of cortisol by the hypothalamic CRF-containing neurons. It widely accepted that excessive cortisol impairs cell growth and enhances cell death during MDD.

The HPA axis controls reactions to stress through a mechanism which will be briefly outlined below. Corticotropin-releasing factor (CRF) is released from the paraventricular nucleus (PVN) of the hypothalamus upon inputs from its anatomical connections with the amygdala or the hippocampus. This release of CRF initiates the production of adrenocorticotropin (ACTH) from the anterior pituitary. ACTH will then enter the bloodstream and reaches the adrenal cortex leading to the release of glucocorticoids (*cortisol* in humans, *corticosterone* in rodents). In chronically depressed patients, the stress hormone cortisol as well as CRF and ACTH are elevated due to the absent feedback inhibition of CRF from the hypothalamus. The missing feedback inhibition of CRF from the hypothalamus is a result of absent inhibitory input from the hippocampus on hypothalamic CRF-containing neurons. Under normal conditions, cortisol enhances the inhibition of the hippocampus and may also enhance functioning of the hippocampus during cognitive processes. In at least half of the patients suffering from MDD, the HPA axis is resistant to the suppression of excess glucocorticoids (Palazidou, 2012). It is assumed that a dysregulation of the HPA axis does not provoke depressive episodes itself, but rather is the manifestation of neurobiological abnormalities that predispose someone to develop MDD (Pariante and Lightman, 2008).

It has been shown that an increased corticosteroid levels in rats leads to the down-regulation of BDNF, and possibly is related to the retraction of spines and cell death (Schaaf et al., 1998). In addition, it has been shown in rats that stressors in early-life attenuate cell proliferation and immature neuron production later in life (Mirescu et al., 2004). The study by Mirescu et al. (2004) suggests that aversive experiences in early-life inhibit structural plasticity later in life due to a hypersensitivity to corticosteroids. This conceals the possible link between stress, a malfunctioning HPA axis and neurogenesis.

1.6.4 Epigenetics

With a risk of 40-50 % MDD is just as heritable as are other diseases such as type II diabetes or hypertension that are generally being considered as genetic diseases (Nestler et al., 2002). However, until this point no depression vulnerability genes have been found. It appears that

genes alone do not cause MDD but interact with developmental and environmental factors leading to depressive behaviour. This relationship between genes and the environment is evident due to two reasons. First, the temperament of persons determines which situations they place themselves into and second, genetic factors might mediate the stress response of individuals towards adverse life events. One finding is that environmental experiences are able to change gene functions without changing the DNA sequence, also known as *epigenetic modifications*. Epigenetic modifications such as post-translational modifications of histone N-terminals or DNA methylation are acquainted through experience in development or might also be partly inherited (Krishnan and Nestler, 2008).

Chromatin of the nucleus is either present as heterochromatin inhibiting the transcription of genes or as euchromatin allowing gene expression. Modification of histone proteins, proteins of the nucleosome repeat unit of chromatin, enables a mechanism that can switch between these two states of chromatin by means of acetylation, methylation or phosphorylation. For instance, acetylation of histone acetylases (HAT) opens promotor regions of target genes which enables RNA polymerase II to bind, and then increase the transcription rate of, e.g., BDNF. On the other side, activation of histone deacetylase (HDAC) leads to a lower transcription rate. The modification of histone proteins has been shown to slow down neurogenesis in a rat model of social defeat. HDAT inhibitors have an antidepressant effects (Paslakis et al., 2011).

The second form of chromatin modulation, DNA methylation, is regulated by environmental influences and has an influence on stress responses. For instance, DNA methylation is affected by the maternal treatment in rats. Offspring avoided by their mothers expressed a higher rate of anxiety and a lower transcription of glucocorticoid receptors in the hippocampus than offspring that received maternal care. The greater concentration of anxiety and lower transcription of glucocorticoid receptors is due to the increased methylation of the glucocorticoid receptor gene promotor repressing gene expression (Szyf et al., 2005).

Epigenetics is a relatively new field in depression-related research but there seems to be a promising link between environmental factors influencing gene functions, neurogenesis and the functioning of the HPA axis.

1.6.5 Resilience-related research

In most cases, people react adversely to painful life events. However, some people do not necessarily react with depressive behaviour to situations which seem to be uncontrollable for others. Resilience-related research informs us about the two different behavioural responses, i.e. whether individuals can adapt well to unfortunate life events or whether they react with depressive behaviour (Krishnan and Nestler, 2008).

It has been shown in an animal model of learned helpless that the level of the transcription factor ΔFOSB in substance-P neurons in the periaqueductal gray (PAG) is up-regulated in rats that are prone to develop depressive behaviour upon shock stimulation (Krishnan and Nestler, 2008). Plasticity mechanisms such as LTP and LTD are also differently mediated by stress. LTP was significantly more decreased in rats that did not have the ability to terminate an inescapable foot shock, i.e. have a lack of control, in the learned helpless paradigm (Shors et al., 1989). Furthermore, stressed rats display an increased excitability of dopaminergic neurons in the ventral tegmental area (VTA) which release BDNF onto neurons in the NAc. Resilient rats on the other side show an up-regulation of potassium channels as a homeostatic mechanism to restore normal excitability and low levels of BDNF in the NAc (Krishnan et al., 2007).

Though findings are scarce until this moment, it seems that resiliency is an active neurobiological process of allostasis that needs to be further explored to advance the development of successful treatment strategies against MDD.

1.7 Synaptic proteins involved in major depressive disorder

It is acknowledged that symptoms of MDD originate from long-term changes in various neural areas that mediate cognitive-emotional functioning. Morphological changes affect various levels from the ultrastructural level of the synapse to the level of an entire neuron. Morphological changes might be associated with the established finding of volumetric changes in the neural areas during MDD. The next section of this work will briefly discuss the role of synaptic proteins involved in synaptic plasticity. Insights on the involvement of these proteins in normal and abnormal brain functioning will inform us how ultrastructural changes of the synapse might be related to neuroplastic changes in the brain during MDD. Given that previous work in our group has revealed an altered concentration of the proteins syntaxin1,

the NMDAR2B subunit and Arc in the whole brain as measured by western-blotting (Daaland, 2012), I will pay a particular focus on these proteins.

1.7.1 SNARE proteins

Short- or long-term changes of neurotransmitter release are the underlying mechanism of synaptic plasticity. Synaptic plasticity, or the modification of synaptic strength, can be induced through (i) an altered neurotransmitter release presynaptically (including changes in the protein network that regulates synaptic vesicle exocytosis and endocytosis controlling the release of neurotransmitter), or (ii) changes in the amount of receptors postsynaptically that sense the release of neurotransmitters. Some of the core proteins that mediate endocytosis and exocytosis are the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins.

The SNARE complex includes the fusion proteins of syntaxin1, SNAP-25 (synaptosomal-associated protein 25) and VAMP2 (vesicle-associated membrane protein 2) which is also termed synaptobrevin-2. The SNARE motif of syntaxin interacts with motifs of others syntaxins and SNAP-25 thereby shaping the target-membrane SNARE (t-SNARE) complex. The t-SNARE complex is located at the target membrane which again interacts with the vesicle-membrane SNAREs (v-SNAREs) of the vesicle. The target membrane proteins syntaxin and SNAP-25 interact with the vesicle-associated synaptobrevin-2 forming the alpha-helical trans-SNARE complex which moves the two membranes together leading the opening of the fusion pore and the release of neurotransmitter (Sudhof and Rizo, 2011).

The focus of this work is on the target membrane protein syntaxin with its distinguished forms of syntaxin 1A and syntaxin 1B. In approximately 84 % of the cases the two proteins have the same amino acids and interact with the membrane trafficking protein synaptotagmin. Syntaxin 1A, the first identified syntaxin, is composed of 288 amino acids and is most commonly found in neurons and neuroendocrine cells making up approximately 1 % of the total amount of brain proteins (Lang and Jahn, 2008). It is assumed that syntaxin1 is primarily expressed in excitatory synapses (Koh et al., 1993). Though the common assumption is that SNARE proteins are expressed primarily in the presynaptic cell, in particular the active zone, SNARE concentration is also found postsynaptically (Jurado et al., 2013; personal communication, Suleman Hussain, 2013). For instance, synatxin3 and SNAP-47 are expressed in the

postsynaptic cell and are involved in the exocytosis of AMPARs during LTP (Jurado et al., 2013).

The involvement of some SNARE proteins in the pathology of a few psychiatric disorders, such as schizophrenia is known (Honer et al., 2002). However, the action of these proteins in MDD is much less determined. The lack of results is driven by two possible shortcomings in the study of SNARE proteins. First, it is problematic to pinpoint whether abnormalities of SNARE regulation is a cause or consequence of the given disease. Second, while SNARE proteins act cooperatively during neurotransmission, each SNARE protein may play an independent role in pathology. For instance, chronic restraint stress leads to an increase of VAMP2 in the hippocampus, while there is no difference between chronically stressed and non-stressed rats with respect to the concentration of SNAP-25 or syntaxin (Gao et al., 2006). On the other hand, Muller et al. (2011) have found that chronic stress down-regulates synaptobrevin in the hippocampus, while up-regulating VAMP2 mRNA levels. Furthermore, Fatemi et al. (2001) found an up-regulation of SNAP-25 in the presubiculum of depressed patients.

The role of syntaxin1 in the aetiology of MDD is largely unknown, but it seems that syntaxin1 is up-regulated in the brain of learned helpless rats (Daaland, 2012). Fujiwara et al. (2006) investigated the role of syntaxin 1A in knock-out mice and demonstrated that the lack of syntaxin 1A significantly impairs LTP. Furthermore, syntaxin 1A KO mice showed an enhanced LTP induction by increasing the presence of noradrenaline and dopamine. Depletion of these catecholamines reduced LTP. This shows that syntaxin1 might be crucial for the exocytosis of dense-core vesicles that regulate the catecholaminergic system.

1.7.2 Glutamate receptors

It is increasingly acknowledged that glutamate signalling mediated by NMDARs is disrupted in MDD and might also be involved in morphological changes of the brain. Glutamate receptors exist in two different categories: ligand gated ionotropic receptors and G-protein coupled metabotropic receptors. There are three different types of ionotropic glutamate receptors, which are AMPA, kainate, and NMDA receptors. While all the ionotropic receptors are excitatory, the action of metabotropic receptors can either be excitatory or inhibitory (Siegelbaum et al., 2013).

The NMDARs contain four subunits of several types (NR1, NR2, NR3, or GluN1, GluN2 and GluN3) where for instance the subunit NR2 contains another set of subunits (NR2A, NR2B, NR2C, NR2D). One receptor contains two obligatory NR1 subunits and two regulatory NR2A-D or NR3A-B units. The combination of subunits determines the function of the receptor (Cull-Candy and Leszkiewicz, 2004). NMDARs are able to interact with various other proteins initiating intracellular signalling cascades by linking NMDARs to downstream signalling molecules, kinases, phosphatases and adhesion proteins.

The functions of NMDARs are twofold. On the one side, these receptors are crucial for excitatory signalling, synaptic transmission and synaptic plasticity. On the other side, chronic overstimulation brings about the demise of cells. Ca^{2+} influx is essential for synaptogenesis, experience-dependent synaptic remodelling and changes in synaptic efficacy on the basis of LTP and LTD. In contrast, an excessive amount of glutamate leads to a great influx of deleterious Ca^{2+} into the postsynaptic cell. This high level of Ca^{2+} eventually leads to the generation of free radicals that again lead to a retraction of spines (Duman, 2009). Therefore NMDARs play an important role in psychiatric disorders with underlying perturbations in synaptic plasticity.

It has been shown that acute stress involving NMDAR activation impairs LTP and enhances LTD (Kim et al., 1996). In addition, the concentration of the NMDA subunit NR1 is increased (Karolewicz et al., 2009), while the subunit NR2A is decreased in patients of MDD (Beneyto et al., 2007, Feyissa et al., 2009). Altering the action of glutamate by means of the NMDAR antagonist ketamine leads to antidepressant effects (Zarate et al., 2006). It is believed that chronic stress leads to the retraction of spines which is mediated by NMDAR activation, i.e. the selective deletion of the NMDARs in the hippocampus does not lead to the retraction of spines during stress (Christian et al., 2011). Because the glutamatergic system comprises the all excitatory pathways in the brain, variances of this system could be linked to morphological changes as seen in the pathology of MDD.

1.7.3 Activity-regulated cytoskeletal gene Arc

The protein Arc (activity-regulated cytoskeleton-associated protein) is an ideal protein to study synaptic changes in MDD. As shown briefly below, this protein is involved in many forms of plasticity. In addition, it plays a role in several neurological diseases. However, also

the involvement of this protein in MDD has not – according to my best knowledge – been explored before.

Arc, also named Arc/Arg3.1, is an IEG that is rapidly activated upon strong synaptic, neurotransmitter or growth factor stimulation and integrates the expression of various genes which encode synaptic proteins (Tzingounis and Nicoll, 2006). As revealed by immunogold electron microscopy, Arc is mainly present in the PSD of excitatory synapses that recently underwent LTP (Moga et al., 2004). The signaling mechanisms of Arc transcription are still unknown. It seems that PKA and mitogen-activated protein kinase (MAPK) cascades are involved in the induction of Arc (Waltereit et al., 2001). The involvement of Arc in molecular aspects of synaptic functions was established when researchers found that Arc regulates *endophilin 3* and *dynamin 2*, which are components of the protein network involved in endocytosis (Korb and Finkbeiner, 2011).

As mentioned above, the role of Arc in synaptic plasticity is diverse. First, it has been shown that Arc modulates the trafficking and endocytosis of AMPARs (Chowdhury et al., 2006, Shepherd and Bear, 2011). Arc either concentrates the endocytic proteins to sites where AMPAR endocytosis is possible (Shepherd and Bear, 2011) or it has been suggested that Arc indirectly moves AMPARs out of the PSD through an interaction with endophilin and dynamin (Chowdhury et al., 2006) thereby mediating forms of plasticity such as LTD (Waung et al., 2008).

Due to its involvement in AMPAR endocytosis it is assumed that Arc has a mediating effect on LTP, LTD and homeostatic plasticity. Currently, it is known that Arc has a strong influential effect on the late phase of LTP (Guzowski et al., 2000). By applying Arc antisense oligonucleotides (AOD) to the dentate gyrus and hippocampal area CA1 the authors were able to block the transient increase of Arc mRNA/protein production after high-frequency stimulation. The effect of this blockade was not significant during the first 4 hours, but decayed LTP to a large extent after 4 hours and decreased to baseline after day five.

Arc might also be a regulator of homeostatic plasticity fine tuning neuronal activity through the endocytosis of AMPARs (Beique et al., 2011). It is accepted that neurons scale up AMPARs during low network activity and scale down AMPARs during increased network excitability. Similarly, during on-going neural activity AMPARs might be scaled down by an increase in Arc and scaled up by a decrease of Arc (Shepherd et al., 2006).

Arc additionally modifies cellular responses by controlling the size and type of spines. A high concentration of Arc increases the amount of thin plastic spines while it decreases the amount of stable stubby spines (Peebles et al., 2010). Notably, LTP increases the amount of thin spines which might be related to the increased concentration of Arc (Holtmaat et al., 2005)

Whether Arc has a direct effect on synaptic plasticity in MDD is unknown. The main finding of Arc's involvement in mood disorders resolves around the fact that the relative concentration level of Arc is mediated by antipsychotic drugs, and an up-regulation of Arc in the CA1 due to antidepressant treatment might be one mechanism how long-term changes in synaptic function are achieved (Pei et al., 2003). Our group has previously found that the concentration level of Arc is up-regulated in brains of learned helpless rats (Daaland, 2012).

1.7.4 Other proteins

Besides the previously mentioned proteins, various other proteins regulating synaptic plasticity play a central role in the pathology of MDD. The concentration levels of synaptophysin and synaptotagmin, proteins required for the fusion of the vesicle with the plasma membrane, are altered after stress exposure. While chronic and acute stress lead to an increase in synapotagmin, synaptophysin is significantly decreased (Thome et al., 2001). Further support that MDD might have a presynaptic basis and that malfunctioning of synaptotagmin correlates with depressive behavior stems from the finding that synaptotagmin KO mice show decreased depressive behavior in a forced swim test. The normal behavior in synaptotagmin KO mice is expressed instead of an assumed depressive reaction (Ferguson et al., 2004).

The PSD, a dense complex of proteins to assure the presence of sufficient receptors in the postsynaptic membrane once neurotransmitters are released from the active zone, is also altered upon exposure to stress. Rats implemented with corticosterone pellets have a reduced concentration level of the scaffolding protein PSD-95 and the glutamate subunit NR1 in the hippocampus. It is assumed that a reduction of PSD-95 leads to a reduction of the insertion of NMDARs and therefore leads to spine mortality (Cohen et al., 2011). However, it has also been shown that the scaffolding protein PSD-95 is up-regulated in the amygdala of post-mortem brain of depressed patients (Karolewicz et al., 2009). The same study also found an up-regulation of NMDAR2A subunit in the amygdala, suggesting that glutamate signaling in

depression is disrupted. In contrast, western-blot analyses revealed that the NMDAR subunits NR2A and NR2B, and PSD-95 levels are reduced in the PFC in post-mortem brains of depressed patients (Feyissa et al., 2009).

It has conclusively been shown that genes associated with synaptic functions are down-regulated during MDD (Kang et al., 2012). The down-regulation of these synapse-related genes most likely occurred due to the exposure to chronic unpredictable stress. This down-regulated might contribute to depressive behaviour. A microarray analysis revealed that the mRNA concentration of GATA1, a transcription factor, is increased in the PFC during MDD possible leading to the decreased concentration of synapse-related genes and the atrophy of dendritic processes.

Having established a framework of the neuroplasticity hypothesis of depression and possibly involved synaptic proteins, I will now turn to the purpose and aim of this project.

1.8 Purpose and aim

One reoccurring finding is that certain neural areas decrease in volume during MDD. Theoretical explanations describing the origins of MDD have shifted in the last decades from solely regarding the cause in a deficiency of monoamines to a more complex, and more ambiguous, *neuroplasticity hypothesis* of MDD. The neuroplasticity hypothesis postulates that stress alters mechanisms of synaptic plasticity through neuroendocrine, neurotropic and epigenetic factors leading to an alteration in the structure and amount of synapses and neurons. However, far too little attention has been paid to the molecular correlates of the plastic changes in MDD. A way to shed light on possible interactions between forms of plasticity, their molecular basis, and how they relate to the pathology of MDD is to explore functionally important synaptic proteins involved in synaptic plasticity. The involvement of the SNARE protein syntaxin1, the NMDAR subunit NR2B, and Arc in MDD are for the most part unknown, or literature has emerged that offers contradictory findings. However, given that we know far more about how these proteins act in synaptic plasticity in normal conditions, we may be able to describe general synaptic alterations in MDD by gaining insights on how these proteins are involved in MDD. Previous studies in our group have revealed an increase in the concentration level of syntaxin and Arc, and a decrease in the concentration level of NMDARs in the whole brain (Daaland, 2012).

In this work, I will compare animals of the learned helpless model of depression to explore the relative concentration of the three functionally important synaptic proteins syntaxin 1, the NMDAR2B subunit, and Arc by means of their immunoreactivity in Schaffer collateral synapses of the stratum radiatum in hippocampal area CA1. In particular, I compare the learned helpless group, the non-learned helpless group and the wild-type group with each other to detect differences in the synaptic concentration of the proteins of interest. For each group, I investigate the proteins of interest using immunogold electron microscopy. Based on previous work in our group (Daaland, 2012) and the involvement of these proteins in mechanism of synaptic plasticity as outlined above, I propose the following hypotheses that will be explored in the remainder of this work:

Hypothesis 1: Synaptic concentrations of syntaxin1 in Schaffer collateral synapses in CA1 are different between the learned helpless, non-learned helpless and wild-type group.

Hypothesis 2: Synaptic concentrations of the NMDAR subunit NR2B in Schaffer collateral synapses in CA1 are different between the learned helpless, non-learned helpless and wild-type group.

Hypothesis 3: Synaptic concentrations of Arc in Schaffer collateral synapses in CA1 are different between the learned helpless, non-learned helpless and wild-type group.

2. Materials and Methods

2.1 Background of the methods

2.1.1 Learned helpless model

During this work I made use of the learned helpless model of depression. The initial concept of learned helpless was put forward by Seligman and Overmier (Overmier and Seligman, 1967). The concept of learned helpless assumes that misinterpreting cognitive information as being more negative than it is will lead to a feeling of helplessness or loss of control. The negative interpretation of information and the uncontrollability of aversive events terminate in MDD as mentioned above. The learned helpless model is one of the most valid animal models of MDD. It displays *construct validity* (depressive behavior is caused by the same etiologies that cause MDD in humans), *face validity* (profile of symptoms in the model is akin to symptoms seen during MDD) and *predictive validity* (effects of antidepressant treatment is similar in the learned helpless model and in MDD) (Vollmayr et al., 2007).

The use of animal models, specifically related to the validity of those, is associated with limitations as outlined in the discussion part of this work. The term *model* implies that there exist deviations from reality. The study of human psychiatric disease can therefore only be regarded as an approximation. MDD is a mental disorder that is not defined by the presence or absence of only a single feature. Rather, a complex interplay between behavioral, cognitive and physiological functions terminates in MDD.

2.1.1.1 Induction of the learned helpless state

Applying uncontrollable and unpredictable aversive stimuli to a rat induces a depressive behavioral state in the learned helpless model. In 10-20 % of the rats, a helpless state is induced by applying a foot shock of 0.8-mA for a total time of 40 to 60 min thereby exposing the animal to a total of 20 min of electric shock. Following this procedure, the rat is placed in the same cage 24 hr later, and is exposed to electric foot shocks. However, this time with the possibility to terminate the electric shock by pressing a lever. The degree of failure (i.e. not terminating the shock) is related to the degree of helplessness. A rat is being regarded as helpless if it fails in about 10 out of 15 cases to press the lever and terminate the foot shock, while a failure rate of less than 5 out of 15 the animal is regarded as a non-helpless animal.

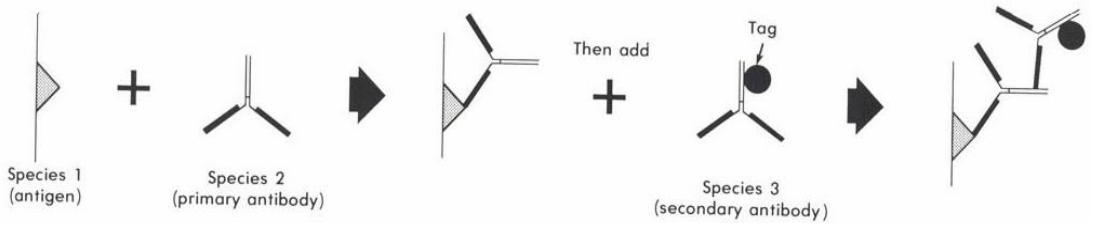
Applying this criterion, 15-20% of the trained Sprague-Dawley rats are identified as learned helpless (Vollmayr and Henn, 2003).

2.1.2 Immunocytochemistry

In order to detect the antigens of the proteins syntaxin1, NR2B, and Arc I made use of the immunolabeling technique. An antibody (or *immunoglobulin*), which consists of two identical heavy chains and two identical light chains forming a Y shape, has an antigen binding sit at the tip of the Y where it binds to the antigens of the proteins of interest. However, in order to quantify these proteins in the synapse they require an attached molecule which is visible in the electron microscope. Therefore, I made use of *indirect labeling*, i.e. I first incubated the tissue in the primary antibody which bound to the antigen of the protein and thereafter incubated the tissue in a secondary antibody that binds to the primary antibody (f*igure 9*). In case of electron microscopic analyses, the secondary antibody has a gold-tag. The gold particles are electron-dense (i.e. reflect the electron beam of the microscope) and therefore visualize the original protein in the microscope.

Figure 9

Indirect method for antibody labeling



A tissue antigen (species 1, rat) is exposed to a aprimary antibody (second species, rabbit) that has been made to bind the antigen. After binding of the primary antibody, a tagged secondary antibody (third species, goat) is exposed to the bound antigen-antibody complex. The secondary antibody was produced to react against all IgGs of the second species and contains a tag. The result is a two-layered antibody sequence with an attached tag. Retrieved from "Electron Microscopy", Bozzola & Russell (1992*), Immunocytochemistry*.

2.1.3 Transmission electron microscopy

Following the labeling procedure, I investigated the presence of the proteins in synapses with the transmission electron microscope (TEM). The TEM offers two advantages in comparison to the western-blot technique applied previously (Daaland, 2012) when exploring the relative concentration level of proteins. First, this microscopic technique allowed me to identify changes at the ultrastructural level of an individual synapse. Second, by using the TEM I

could target and visualize specific regions of the brain; in this case the stratum radiatum of hippocampal area CA1.

The make-up of a TEM is similar to a light-microscope with the main difference being the illumination source. In case of the TEM, the illumination source is an electron gun with a tungsten filament. Once the tungsten filament produces heat, an electron cloud is established emitting electrons. The beam of emitted electrons is focused by electromagnetic lenses. Depending on how many electrons hit the fluorescent screen, it sends out light that is captured by an electronic imaging device such as a CCD (charge-coupled device) camera. The whole procedure progresses at high-vacuum because air molecules would interfere with the electrons and disturb the image-forming process. While the human eye can resolve between two points which are located up to 0.1 mm apart, by use of the TEM points as less as 0.127 nm can be distinguished. For biological tissue a maximum resolution of 0.344 nm is excellent as the conventionally prepared material does not allow for greater resolution. The magnification of a conventional TEM reaches up to 200.000x. At normal conditions, the TEM operates at 100-120 kV, while 80 kV is seen as appropriate for biological tissue because it gives a better contrast than at higher kV (Dykstra and Reuss, 2003).

2.2 Materials

I obtained sample tissue blocks of a rat inbred helpless line established in the laboratory of Prof. F. Henn, Cold Spring Harbor Laboratory, United States. This material contained neural tissue of four LH, four NLH rats and two WT rats (a*ppendix 1*). As seen in *table 2*, the primary antibodies used for immunolabeling were Anti-Syntaxin 1, 1:30 (*AN-002, Alamone Labs, Jerusalem, Israel*), Anti-NMDAR2B, 1:600 (*ab65783, Abcam, Cambridge, UK*), and Anti-Arc, 1:400 (*156003, Synaptic Systems, Göttingen, Germany*). *Table 3* displays the secondary antibody goat anti-rabbit (10nm gold) (*ab27234, Abcam, Cambridge, UK*) that was used in this work. Further information about the solutions described in the following paragraphs is depicted in *appendix 2* and *appendix 3*.

Table 2

Primary antibodies

	Anti-Syntaxin1 (Alomone Labs)	Anti-NMDAR2B (Abcam)	Anti-Arc (Synaptic systems)
Current Lot #	AN-002	GR98904	156003/33
Host	Rabbit	Rabbit	Rabbit
Type	Polyclonal	Polyclonal	Polyclonal

Table 3

Secondary antibodies

	Goat anti-rabbit IgG H&L antibody (Abcam)	Goat anti-rabbit IgG H&L antibody (Abcam)
Current Lot #	GR73433	GR104536-1
Size	10nm	10nm
Type	Polyclonal	Polyclonal

2.3 Perfusion fixation

The primary goal of perfusion fixation is to eradicate the tissue of the animal, but at the same time preserve the fine structure of the tissue for later electron microscopic analyses. The rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with 2 % dextran in 0.1 M phosphate buffer (pH 7.4). Thereafter they were fixated with a combination of 0.1 % glutaraldehyde (GA) and 4 % paraformaldehyde (PA) in the same buffer. The brains were stored *in situ* at 4°C.

2.4 Region of interest

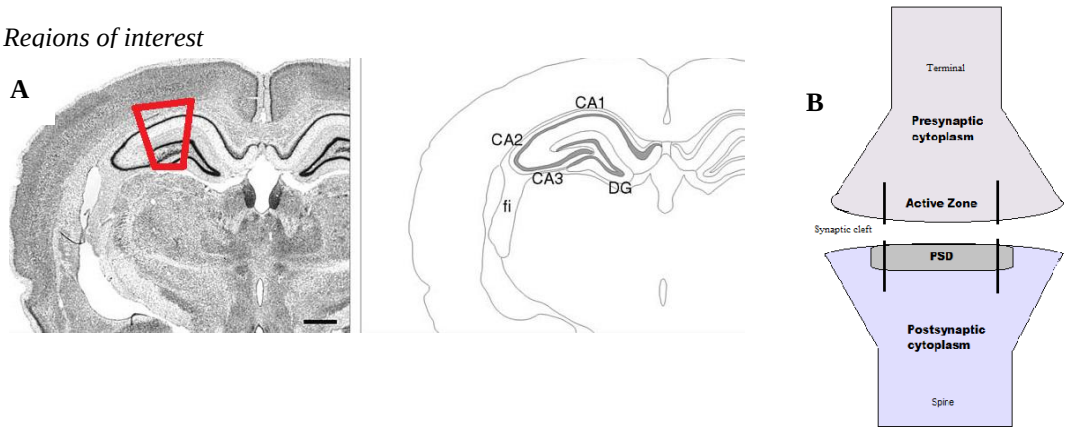
The region of interest to quantify the concentration level of the proteins of interest was the hippocampal area CA1. Having retrieved the brains from the skull 24 hr after perfusion fixation, the brain was sliced until structures of the HF were clearly visible. Thereafter, pieces with the size of approximately 1.0x0.5 mm were dissected from the slices of the hippocampus

as shown in *figure 9A*. Parts of the cortex and dentate gyrus were kept attached to the dissected piece for later identification of the striatum radiatum in CA1.

Immunolabeling was performed on sliced sections of the CA1, and the relative concentration levels of the proteins of interest were obtained in selected areas (active zone, PSD, presynaptic and postsynaptic cytoplasm) of Schaffer collateral synapses in the stratum radiatum (f*igure 9B*).

Figure 9

Regions of interest



A: Red drawing displays the dissected region of interest, hippocampal area CA1. Scale bar = 1mm. Retrieved from "The hippocampus book", Amaral & Lavenex (2006), *Hippocampal neuroanatomy*. **B**: Schematic drawing showing regions of interest (bold) of the synapse in immonogold electron microscopical material of hippocampal area CA1.

2.5 Tissue preparation

Once the tissue was dissected, cryofixation was performed in order to stabilize the tissue of hippocampal CA1 for later ultramicrotomy and electron-microscopic analyses. One subtype of cryofixation is the freeze-substitution method. During this procedure, the biological tissue is being frozen and the developing ice crystals are replaced by polymerized resin.

The embedding procedure (freeze-substitution method) was carried out by Bjørg Riber, University of Oslo.

The dissected tissue pieces of the CA1 region were cryoprotected by immersing them in increasing concentrations of glycerol (10 %, 20 % and 30 %) buffered in sodium phosphate buffer. The tissues were immersed for 30 min in concentrations of 10 % and 20 % of glycerol and were immersed overnight in the concentration of 30 % at 4°C. The tissue was then moved it into a quick-freeze device filled with propane cooled down with liquid nitrogen to -170°C

in a cryfixation unit (*Reichert KF80, Vienna, Austria*).

Afterwards, the tissue was transferred to 1.5 % uranyl acetate diluted in anhydrous methanol into the pre-cooled chamber (-90°C overnight). This solution was filled into Reichert capsules which were placed in a cryosubstitution unit (*AFS, Reichert*). Following 30 hr in -90°C, the temperature was raised with 4°C increments per hour from -90°C to -45°C. Thereafter, the samples were washed with anhydrous methanol to remove water and excess uranyl acetate.

Once this has been performed, the samples were infiltrated to replace the dehydrated fluid with lowicryl HM20 resin (*Polysciences, Inc*., Warrington, PA 18976. Cat#15924). This embedding was carried out at a temperature of -45°C while the tissue was placed in different concentrations of lowicryl/methanol. The infiltration lasted for 2 hr at a concentration of lowicryl/methanol of 1:1, another subsequent 2 hr at a concentration of 2:1, then 2 hr in pure lowicryl and once again overnight in pure lowicryl. The Reichert capsules with the specimen were then moved to the lowicryl-filled gelatin capsules in the G-chamber. The capsules were transferred to another container filled with ethanol. At last, the resin was polymerized with UV-light at -45°C for 24 hr, and then increased the temperature by 5°C increments per hour until 0°C was reached. At 0°C the resin was polymerized for further 35 hr.

2.6 Ultramicrotomy

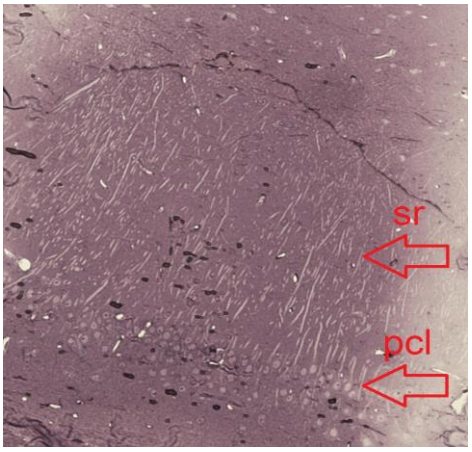
I obtained ultra-thin slices of the embedded tissue block for immunolabeling. At first I rough trimmed the 44 blocks (4 blocks per animal) removing abundant plastic, and then fine trimmed the blocks to achieve a trapezoid form of 1 mm square at the tip of the block. I then cut semi-thin sections (500 nm) with a glass knife where the shorter length of the trapezoid was in contact with the glass knife first, i.e. was cut first. The semi-thin sections were removed from the water basin of the glass knife and put on a drop of distilled water on a glass slide. Once the drops were dried up, the semi-thin sections were stained with toluidine blue for 90 sec. After the removal of excess stain, I explored the semi-thin sections as exemplified in *figure 10* with a light microscope to i) determine the quality of fixation procedure for each block, ii) the quality of the tissue section, iii) the clarity of the CA1 and the stratum radiatum, and iv) to further trim of excess plastic of the block. Given the criteria a-c, I selected 10 blocks out of the total 44 blocks (one block per animal). I did not consider blocks of animal ID-6006 any further, as these were not well fixated. The semi-thin sections were later used as

guidance to find the area of the stratum radiatum.

After obtaining semi-thin sections, I cut ultra-thin sections (90 nm) with an ultratome diamond knife (*Diatome*, ultra 45°) of each block. I selected optimal sections free of gaps and fractures and placed them on 300 lines nickel mesh grids (*Electron microscopy sciences*, G300-Ni) which were mounted with a grid coating pen beforehand (*Coat-Quick "G"*). I collected 20 ultra-thin sections of each animal (a total of 200 sections).

Figure 10

Semi-thin section of CA1



The semi-thin (500 nm) section was used to localize the stratum radiatum in the ultra-thin (90 nm) section. Pcl = pyramidal cell layer, sr = stratum radiatum.

2.7 Immunolabeling

In order to localize the proteins in the ultra-thin sections with the TEM, I made use of postembedding immunogold labeling. In the postembedding procedure, each antigen molecule at the surface has the same chance of being immunodetected which is of great importance for the quantification of proteins. The relative labeling intensity (number of gold particles/number of antigen molecules) is always below 100% in this procedure. The labeling efficacy needs to be determined by modifying the labeling protocol for each antibody detecting the protein of interest.

I followed the postembedding procedure of van Lookeren Campagne et al. (1991) with some modifications. *Table 4* displays the modified protocol I followed for the various proteins. *Appendix 4* shows entire protocol by van Lookeren Campagne et al. (1991).

Table 4

Protocol immunoincubation

	Anti-Syntaxin 1	*Anti-NMDAR2B*	*Anti-Arc*
Concentration of primary antibody	*1:30*	*1:400*	*1:600*
Concentration NaCl	*0.5%*	*0.3%*	*0.3%*
Concentration Triton	*0.01%*	*0.1%*	*0.1%*
Incubation time primary antibody	*30mins in 37.5 degrees*	*4hrs*	*Overnight*
Secondary antibody (10nm) LOT#	*GR73433*	*GR104536-1*	*GR104536-1*
Concentration secondary antibody	*1:20*	*1:20*	*1:20*
Incubation time secondary antibody	*90mins*	*90mins*	*90mins*

All sections were labeled blinded on a grid support plate at room temperature if not otherwise specified. First, free aldehyde groups were neutralized by incubating the tissue in TBST (Tris buffered saline with Tween 20) buffer containing 50 mM of glycine for 10 min. Afterwards I incubated the tissue in TBST containing 2 % human serum albumin (HSA) for 10 min in order to block nonspecific antibody binding sites before incubation with the primary antibody. In the next step, I incubated the tissue in drops of TBST containing 2 % HSA and the primary antibody with respect to its optimal concentration and incubation time as shown in *table 4*.

After incubating with the primary antibody, the tissue was rotationally rinsed and immersed in TBST, before it was incubated in colloidal gold-secondary antibody for 90 min. The TBST containing the secondary antibody included PEG (polyethelyne glycol-electrolyte) solution to avoid clustering of gold particles on the tissue section. After incubation of 90 min in the secondary antibody, I rinsed the sections with distilled water and post-stained them with 2 % uranyl acetate and 0.3 % lead citrate staining (90 sec each) to enhance tissue contrast. Uranyl acetate and lead citrate were removed with distilled water, and sections were left to dry completely before use in the electron microscope.

During the optimization processes in which I determined the correct concentration of antibodies and solutions, I applied controls such as leaving out the secondary antibody to determine if there exists non-specific binding (*negative control*), and using an antibody that I trust to work to ascertain that the labeling protocol is working properly (*positive control*).

2.8 Picture acquisition

I acquired the pictures of Schaffer collateral synapses in the stratum radiatum of the CA1 using a TEM (*Tecnai G^2 Spirit, TWIN/BioTWIN*, FEI Company, Hillsboro, U.S.) operating at high tension (80kV) with a magnification of 43 000x. For each group (WT = 2 animals, LH = 4 animals, NLH = 4 animals) I took a total of 180 pictures (WT *N* = 180, LH *N* = 180, NLH *N* = 180) of excitatory synapses. I identified excitatory (asymmetric) synapses by their specific morphology. I obtained a total of 540 pictures for each antibody.

I localized the regions of interests by comparing the structure of the ultra-thin slice under the TEM with the outline of the identical semi-thin section under the light microscope. In order to avoid collecting the identical synapse twofold and to assure that the pictures were taken from a large spread of the given area, pictures were acquired in a top-down/left-right fashion.

2.9 Quantification IMGAP and resolution

Following the acquisition of the pictures, I quantified the gold particles (and therefore the relative concentration each protein) of each section with the tailor made program IMGAP (*IMmumo-Gold-Analysis-Program*) (Haug et al., 1994). The regions of interest for quantification were the active zone, the PSD, the presynaptic cytoplasm, and the postsynaptic cytoplasm. I outlined freehand each region of interest in the cell and gold particles were counted either automatically, or manually in case a *type-I* or *type-II* error occurred, in the regions of interest of each section.

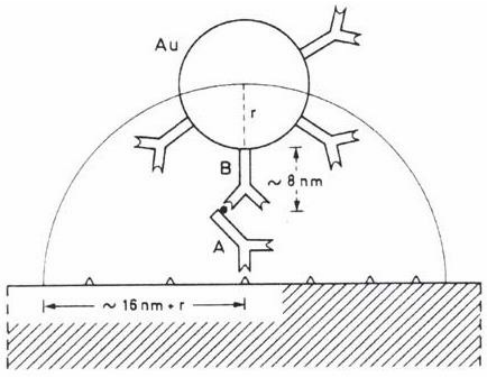


Figure 11

Two-step postembedding procedure and resolution

Triangles represent the antigen against which the primary antibody (A) was raised. The secondary antibody (B) is coupled to a colloidal gold particle (Au). The radius (r) of the gold particle is 5 nm. This adds up to a maximum distance of 21 nm between the epitope and the centre of the gold particle. Retrieved from Mathiisen (2005), *Molecules, neurons, systems*.

In order to distinguish the presynaptic and postsynaptic membranes and whether particles more likely attach to one membrane or the other, I determined the resolution of the post-

embedding technique and adjusted it in the analysis program. As depicted in *figure 11*, the resolution of the post-embedding immunogold technique is theoretically described as the distance between the epitope of the antigen and the centre of the gold particle. This equals the radius of the gold particle and the total diameter of the antibodies. In practice, however, many gold particles are located closer to the epitope of the antigen than as described by the theoretical distance (Mathiisen et al., 2005). The resolution (length of antibody bridge and the dimension of the colloidal particle) was accounted to be 21 nm (10 nm gold particles with a radius of 5 nm, primary and secondary antibody each with a diameter of 8 nm, in sum 21 nm).

2.10 Statistical analysis

I analyzed the data using the statistical software package SPSS (Statistical Product and Service Solution) (*IBM Corp., Armonk NY*). The obtained data of this work are presented as mean values with standard errors. A natural log-transformation was applied to non-normal data if possible and rendered a good Gaussian distribution. I performed a one-way ANOVA (analysis of variance) for independent samples with subsequent multiple comparison post-hoc testing for normally distributed data. If the data did not meet the assumption of normality I used the non-parametric Kruskal-Wallis test for independent samples, and in case an overall effect was found between the groups, I performed subsequent Mann-Whitney U post-hoc tests. Though there is no objective way to remove outliers from the data, I removed outliers which were further than three interquartile ranges (IQR) away from the end of a boxplot. These observations most likely have been caused by chance, or a different mechanism which does not reflect the true antigen-antibody binding. While data values being 1.5 IQRs away from a boxplot are generally considered as outliers, I kept them in the data, as I had no real evidence that these are erroneous values.

3. Results

I adopted a criterion for statistical confidence of $p < .05$. Only the test statistics of significant results are reported below. Therefore, not all regions of interest for each protein are mentioned. The dependent variables were *linear density* of gold particles per µm and *density* of gold particles per μm^2. The dependent variable linear density (µm) was measured in response to the independent variable group belonging (WT=wild-type, LH=learned helpless, NLH=non-learned helpless) in the regions interest of the synapse (active zone and PSD) (*figure 9B*). The dependent variable density (μm^2) was measured in response to the independent variable group belonging (WT, LH, NLH) in the regions interest of the synapse (presynaptic cytoplasm and postsynaptic cytoplasm) (*figure* 9B). If suitable, I applied a log-transformation in case the assumption of normality was violated. I performed a one-way ANOVA for data that followed a good Gaussian distribution. I used the non-parametric Kruskal-Wallis test if a transformation was not appropriate for the given data values. I applied a Bonferroni adjustment by multiplying the *p*-value by the number of groups (x3) for all the multiple comparisons I performed following an overall effect of the Kruskal-Wallis test. *Table 5* displays a summary of the significant findings between each group.

Table 5

Significant findings of between-group comparisons

Comparison between groups	Protein	Region of interest	Finding	Two-tailed *p*-value
LH vs. WT	NR2B	PostCy	LH > WT	.012
	NR2B	PSD	LH > WT	.000
	Arc	PSD	LH > WT	.048
NLH vs. WT	Syntaxin1	PreCy	NLH < WT	.002
	Syntaxin1	PSD	NLH < WT	.024
	NR2B	PostCy	NLH > WT	.000
	NR2B	PSD	NLH > WT	.003
LH vs. NLH	Syntaxin1	PreCy	LH > NLH	.001
	Syntaxin1	PostCy	LH > NLH	.001

LH = learned helpless, NLH = non-learned helpless, WT = wild-type, PreCy = presynaptic cytoplasm, PostCy = postsynaptic cytoplasm.

3.1 Synaptic concentration of syntaxin1

I tested the hypothesis that there is a significant difference in the synaptic concentration of syntaxin1 in Schaffer collateral synapses in CA1 between the learned helpless, the non-learned helpless and the wild-type groups. Because the localization of syntaxin1 is likely to occur in all regions of the presynaptic and postsynaptic synapse, values of zero, i.e. no presence of syntaxin1, were removed from the analysis.

I was interested in the regions of the synapse beforehand, and therefore I split the file according to the region of interest and then compared the groups. The assumptions of the ANOVA test, i.e. data values are independent from each other, display homogeneity and are normally distributed, were checked. I performed a log (x+1) transformation because a Shapiro-Wilk test revealed that the data does not follow normality ($p < 0.00$). The data followed a Gaussian distribution after the transformation. I made use of a one-way ANOVA to investigate the concentration of syntaxin1 in various regions of the synapse with respect to the dependent variable (linear density µm, density μm^2) and the independent variable group belonging (WT, LH, NLH).

3.1.1 Presynaptic and postsynaptic cytoplasm

An ANOVA with the dependent variable *density* (μm^2) and the independent variable *group belonging* (WT, LH, NLH) revealed an overall effect for the presynaptic cytoplasm ($F_{2,483} = 7.56$, $p = 0.001$). A Tukey post-hoc test revealed a significant difference between the learned helpless and the non-learned helpless group at $p < 0.00$. The mean of the learned helpless group (M = 75.28, SD = 43.21) was significantly greater than that of the non-learned helpless group (M = 53.54, SD = 37.77) in the presynaptic cytoplasm. A further Tukey post-hoc test revealed an effect for the presynaptic cytoplasm for the wild-type and the non-learned helpless group at $p = 0.002$, where the mean of the wild-type group (M= 65.70, SD=38.85) was significantly higher than the mean of the non-learned helpless group (M = 53.54, SD = 37.77) (*figure 12*). Syntaxin1 concentration in the presynaptic cytoplasm was therefore greater for both the learned helpless group and the wild-type group in comparison to the non-learned helpless group.

Another overall effect of the dependent variable density (μm^2) in relation to the independent variable group belonging was found for the postsynaptic cytoplasm ($F_{2,483} = 7.56$, $p = 0.01$).

As revealed by a Tukey post-hoc test a significant difference existed between the learned helpless group and the non-learned helpless group at $p < 0.00$. The mean of the learned helpless group (M = 44.61, SD = 36.80) was significantly greater than for the non-learned helpless group (M = 31.45, SD = 24.55) in the postsynaptic cytoplasm (*figure 12*). The concentration of syntaxin1 in the postsynaptic cytoplasm is therefore higher in the learned helpless group in contrast to the non-learned helpless group.

Additional independent *t*-tests with a Bonferroni correction revealed that syntaxin1 concentration is higher in the presynaptic cytoplasm compared to the postsynaptic cytoplasm within the wild-type ($t = 9.55$, $df = 358$, two-tailed $p < 0.00$), the learned helpless ($t = 8.78$, $df = 358$, two-tailed $p < 0.00$) as well as in the non-learned helpless group ($t = 7.96$, $df = 358$, two-tailed $p < 0.00$).

3.1.2 PSD

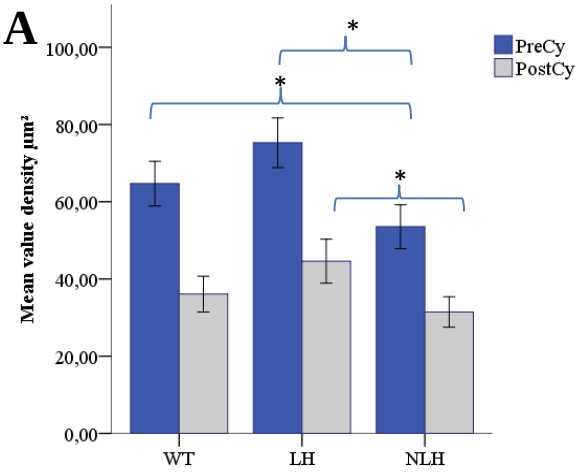
An ANOVA with the dependent variable *linear density* (µm) and the independent variable *group belonging* (WT, LH, NLH) revealed an overall effect for the PSD ($F_{2,325} = 3.5$, $p = 0.031$). Because the one-way ANOVA is an omnibus test and did not reveal between which groups the significant differences occurred, a Tukey post-hoc test revealed that syntaxin1 concentration is significantly lower in the non-learned helpless group compared to the wild-type group in the PSD at $p = 0.024$. (*figure 13*). The mean of the non-learned helpless group (M = 9.49, SD = 7.03) was significantly smaller than the mean of the wild-type group (M = 12.16, SD = 8.60). No significant differences between the groups were found in the active zone. Within the groups, there was no significant difference in the concentration of syntaxin1 between the active and the PSD.

Though we would expect syntaxin1 to be mainly located presynaptically, compared to the non-learned helpless group, syntaxin1 concentration was increased in the learned helpless and wild-type group in the pre- and postsynaptic cytoplasm as well as in the PSD and the presynaptic cytoplasm respectively. This finding is in line with the assumption that SNARE proteins are also active postsynaptically (Jurado et al., 2013; Suleman Hussain, 2013, personal communication).

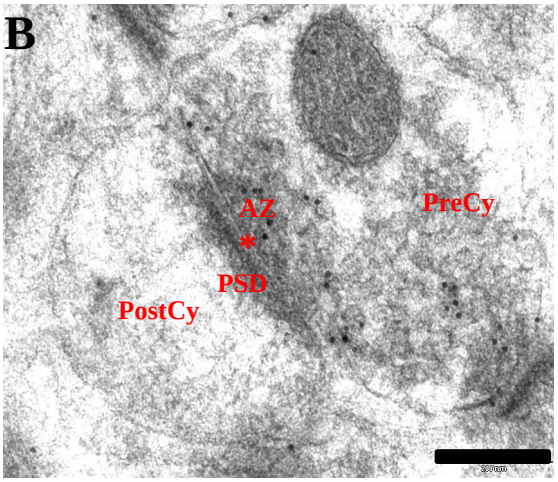
Figure 12

Syntaxin1 concentration is higher in the presynaptic cytoplasm of the LH and WT group compared to the NLH group. Syntaxin1 concentration is also significantly higher in the postsynaptic cytoplasm of the LH group compared to the NLH group.

A



B



C

Group	N	Min.	Max.	Mean	Std. Dev.
WT	180	3.82	272.97	65.72	38.85
LH	180	7.31	207.37	75.28	43.21
NLH	177	5.70	217.88	53.54	37.80

D

Group	N	Min.	Max.	Mean	Std. Dev.
WT	163	2.56	211.48	36.08	29.54
LH	167	4.43	327.99	44.61	36.79
NLH	156	3.01	153.00	31.45	24.55

E

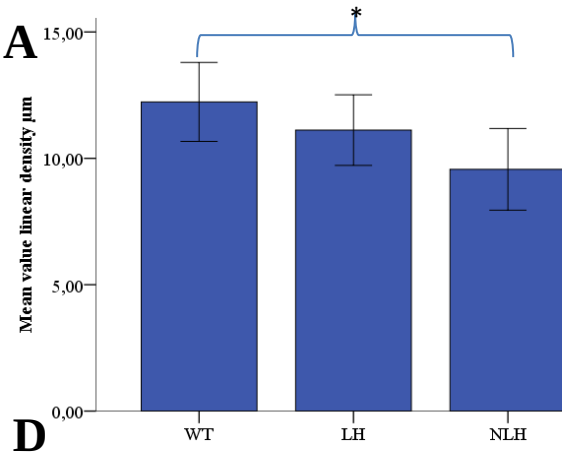
Region of interest	Finding	Two-tailed p-value
PreCy	LH > NLH	0.001
PreCy	NLH < WT	0.002
PostCy	LH > NLH	0.001

Density of syntaxin1 in the presynaptic cytoplasm is significantly greater in the LH compared to the NLH group at p = 0.001, and in the WT compared to the NLH at p = 0.002. Density of syntaxin1 is also greater in the postsynaptic cytoplasm in the LH group compared to the NLH group at p = 0.001. **A**: Semiquantative analysis of syntaxin1 gold particle density in the WT, LH and NLH rats. Bars represent mean value of linear density +/- 2 standard errors of the mean of the different groups. **B**: Electron micrograph from a section of a WT rat showing syntaxin1 immunogold labeling of an asymmetric synapse in the stratum radiatum. **C**: Descriptive statistics of density values in the presynaptic cytoplasm of each group. **D**: Descriptive statistics of density values in the postsynaptic cytoplasm of each group. **E**: Summary of main results. WT=wild-type, LH=learned helpless, NLH=non-learned helpless, AZ=active zone, PSD=postsynaptic density, PostCy=postsynaptic cytoplasm, PreCy=presynaptic cytoplasm. Scale bar = 200 nm. Size of gold particle = 10 nm. Asterisk=synaptic cleft.

Figure 13

Syntaxin1 concentration is significantly lower in the PSD of the NLH group compared to the WT group.

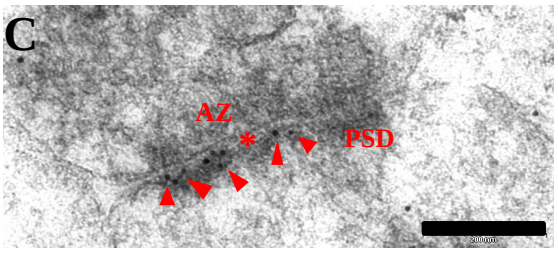
A



B

Group	N	Min.	Max.	Mean	Std. Dev.
WT	123	2.27	44.64	12.17	8.60
LH	119	2.55	47.25	10.73	7.57
NLH	93	2.89	45.13	9.49	7.03

C



D

Region of interest	Finding	Two-tailed p-value
PSD	NLH < WT	0.024

Linear density of synatxin1 in the PSD is significantly lower in the NLH group compared to the WT group at p = 0.024. **A**: Semiquantative analysis of syntaxin1 gold particle density in the WT, LH and NLH rats. Bars represent mean value of linear density +/- 2 standard errors of the mean of the different groups. **B**: Descriptive statistics of linear density values of each group. **C**: Electron micrograph from a section of a WT rat showing syntaxin1 immunogold labeling of an asymmetric synapse in the stratum radiatum. **D**: Summary of main finding. WT=wild-type, LH=learned helpless, NLH=non-learned helpless, AZ=active zone, PSD=postsynaptic density. Scale bar = 200 nm. Size of gold particle = 10 nm. Asterisk=synaptic cleft.

3.2 Synaptic concentration of the NMDAR subunit NR2B

I hypothesized that the synaptic concentrations of NR2B in Schaffer collateral synapses in CA1 are different between the learned helpless, non-learned helpless and wild-type group. As with the analysis of syntaxin1, I split the data file according to the region of interest and analyzed effects between the groups.

3.2.1 Postsynaptic cytoplasm

The assumption of normality was not met by the data and a suitable log (x+1) transformation did not lead to normality. Therefore, I opted for the non-parametric version of the one-way ANOVA, the Kruskal-Wallis test, to examine effects between the dependent variable *density* (μm^2) and the independent variable *group belonging* (WT, LH, NLH). As displayed in *figure 14,* a Kruskal-Wallis test revealed that the particle density (μm^2) in the three groups (WT, LH, NLH) differed significantly in the postsynaptic cytoplasm ($_x^2$ = 16.20, *df* = 2, two-tailed $p <$ 0.00). Determining which groups differ significantly, a Mann-Whitney *U*-test found that the density (μm^2) score of particles in the learned helpless group (M= 27.33, SD = 25.04) was significantly higher than in the wild-type group (M= 20.95, SD = 22.50) in the postsynaptic cytoplasm (U = 12445.00, N_1 = 174, N_2 = 174, two-tailed p = 0.012). A further Mann-Whitney *U*-test revealed that the density (μm^2) score of particles in the non-learned helpless group (M= 31.46, SD = 28.19) was significantly higher than in the wild-type group (M= 20.95, SD = 22.50) in the postsynaptic cytoplasm (U = 11635.5 N_1 = 175, N_2 = 174, two-tailed $p <$ 0.00). Therefore, the concentration of NR2B in the postsynaptic cytoplasm is significantly higher in the learned helpless and non-learned helpless group compared to the wild-type group. Though there was no statistically significant between the groups with respect to the concentration of NR2B in the presynaptic cytoplasm, the mean value of NR2B concentration in the presynaptic cytoplasm is greatest in the learned helpless group (M = 9.72, SD = 12.96) compared to the non-learned helpless (M = 8.56, SD = 11.7) and the wild-type group (M = 7.37, SD = 8.4).

3.2.2 PSD

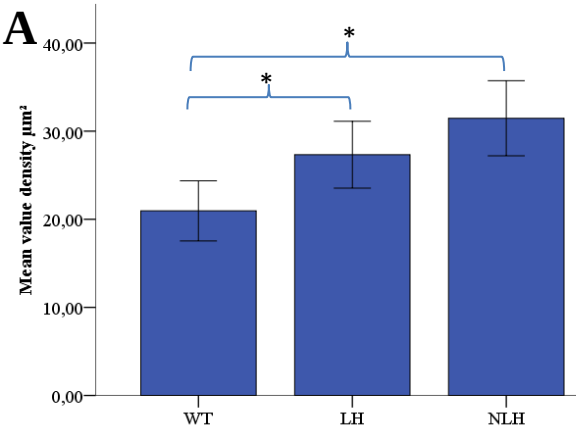
For the dependent variable *linear density* (µm) and independent variable *group belonging* (WT, LH, NLH) an overall significant effect was found for the PSD. As demonstrated in *figure 15,* a Kruskal-Wallis test revealed that the linear density (µm) of particles in the three

groups (WT, LH, NLH) differed significantly in the PSD ($_x^2$ = 19.88, *df* = 2, two-tailed $p <$ 0.00). A Mann-Whitney *U*-test found that the linear density (μm) of particles is significantly higher in the learned helpless (M= 11.32, SD = 11.26) than in the wild-type group (M = 7.43, SD = 10.71) in the PSD (U = 11739.00, N_1 = 175, N_2 = 179, two-tailed $p < 0.00$). A further Mann-Whitney *U*-test revealed that the linear density (μm) of particles is significantly higher in the non-learned helpless group (M= 10.40, SD = 10.88) than in the wild-type group (M = 7.43, SD = 10.71) in the PSD (U = 12358.00, N_1 = 175, N_2 = 177, two-tailed p = 0.003). Though there was no significant effect between the groups with respect to NR2B concentration in the active zone, the mean value of the NR2B concentration was greatest in the learned helpless group (M = 5.74, SD = 9.23) compared to the non-learned helpless group (M = 4.84, SD = 8.47) or the wild-type group (M = 5.08, SD = 8.31). Taken together, the concentration of NR2B is increased in the PSD in both the learned helpless and the non-learned helpless group compared to the wild-type group.

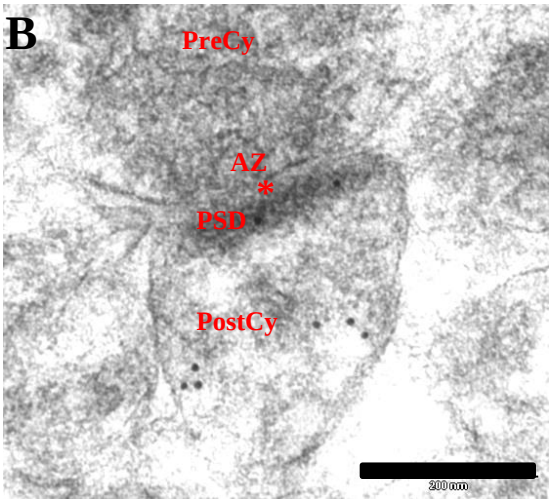
Figure 14

NR2B subunit concentration is greater in the postsynaptic cytoplasm in both the LH and NLH group compared to the WT group.

A



B



C

Group	N	Min.	Max.	Mean	Std. Dev.
WT	174	.00	92.62	20.95	22.50
LH	174	.00	141.23	27.33	25.04
NLH	175	.00	125.18	31.46	28.19

D

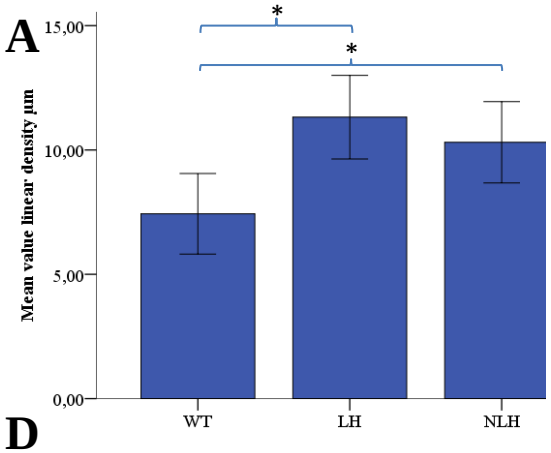
Region of interest	Finding	Two-tailed p-value
PostCy	LH > WT	0.012
PostCy	NLH > WT	0.00

Density of NR2B in the postsynaptic cytoplasm is significantly greater in the LH compared to the WT group (U = 12445.00, N_1 = 174, N_2 = 174, two-tailed p = 0.012), and in the NLH compared to the WT group (U = 11635.5 N_1 = 175, N_2 = 174, two-tailed p < 0.00). **A**: Semiquantative analysis of NR2B gold particle density in the WT, LH and NLH rats. Bars represent mean values of density +/- 2 standard errors of the mean value of the different groups. **B**: Electron micrograph from a section of a WT rat showing NR2B immunogold labeling of an asymmetric synapse in the stratum radiatum. **C**: Descriptive statistics of linear density values in the PSD of each group. **D**: Summary of main findings. WT=wild-type, LH=learned helpless, NLH=non-learned helpless, AZ=active zone, PSD=postsynaptic density, PostCy=postsynaptic cytoplasm, PreCy=presynaptic cytoplasm. Scale bar = 200 nm. Size of gold particle = 10 nm. Asterisk=synaptic cleft.

Figure 15

NR2B subunit concentration is greater in the PSD in both the LH and NLH group compared to the WT group

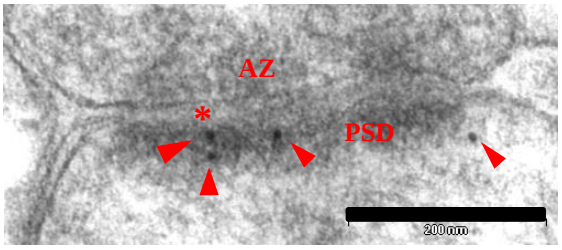
A



B

Group	N	Min.	Max.	Mean	Std. Dev.
WT	175	.00	44.05	7.43	10.71
LH	179	.00	49.98	11.32	11.26
NLH	179	.00	43.08	10.40	10.88

C



D

Region of interest	Finding	Two-tailed p-value
PSD	LH > WT	0.00
PSD	NLH > WT	0.003

Linear density of NR2B in the PSD is significantly greater in the LH compared to the WT group (U = 11739.00, N_1 = 175, N_2 = 179, two-tailed p < 0.00), and in the NLH compared to the WT group (U = 12358.00, N_1 = 175, N_2 = 177, two-tailed p = 0.003). **A**: Semiquantative analysis of NR2B gold particle density in the WT, LH and NLH rats. Bars represent mean value of linear density +/- 2 standard errors of the mean value of the different groups. **B**: Descriptive statistics of linear density values in the PSD of each group. **C**: Electron micrograph from a section of a WT rat showing NMDAR2B immunogold labeling of an asymmetric synapse in the stratum radiatum. **D**: Summary of main findings. WT=wild-type, LH=learned helpless, NLH=non-learned helpless, AZ=active zone, PSD=postsynaptic density. Scale bar = 200 nm. Size of gold particle = 10 nm. Asterisk=synaptic cleft.

3.3 Synaptic concentration of Arc

I claimed that the synaptic concentrations of Arc in Schaffer collateral synapses in CA1 are different between the learned helpless, non-learned helpless and wild-type group. In accordance with prior analyses I split the data values according to the region of interest and then analyzed effects between the different groups.

3.3.1 PSD

As shown in *figure 16,* there exists an overall statistically significant effect of the independent variable *group belonging* (WT, LH, NLH) on the dependent variable *linear density* (µm) for the PSD as revealed by the Kruskal-Wallis test. The linear density (µm) of particles in the PSD significantly differs between the three groups (WT, LH, NLH) ($_x^2$ = 6.03, *df* = 2, two-tailed p = 0.049). A Mann-Whitney *U*-test found that the linear density (µm) of particles is significantly higher in the learned helpless (M= 7.06, SD = 8.00) than in the wild-type group (M = 5.14, SD = 6.78) (U = 13037.00, N_1 = 177, N_2 = 172, two-tailed p = 0.048). The concentration of Arc is increased in the learned-helpless compared to the wild-type group in the PSD. The high presence of Arc in the PSD is in accordance with other investigators (Moga et al., 2004).

Mann Whitney *U*-tests (with a Bonferroni correction) comparing the differential location of Arc in the active zone and the PSD within each group revealed that Arc concentration in the active zone is greater than the concentration of Arc in the PSD in the wild-type (U = 12647.00, N_1 = 178, N_2 = 172, two-tailed p = 0.004), as well as in the learned-helpless group (U = 12699.00, N_1 = 178, N_2 = 177, two-tailed p = 0.002).

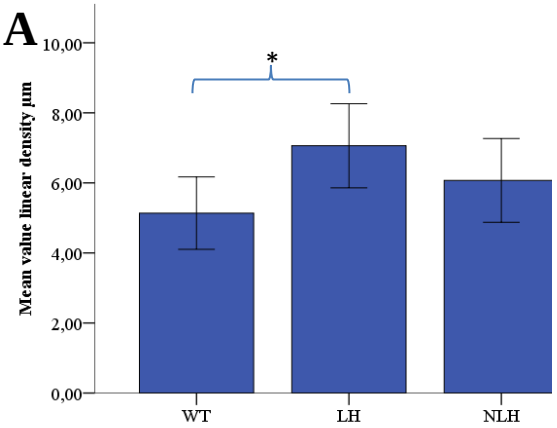
No significant changes of Arc concentration in the pre- or postsynaptic cytoplasm of nerve terminals were found between the groups.

File names of the electron micrographs are included in *appendix 5*.

Figure 16

Arc concentration is greater in the PSD of the LH group compared to the WT group.

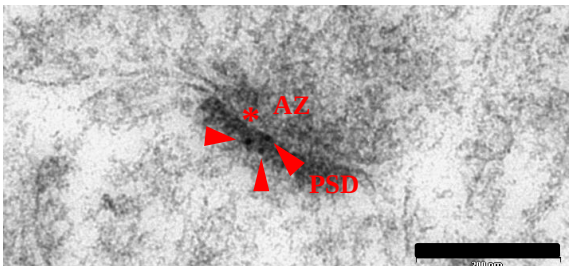
A



B

Group	N	Min.	Max.	Mean	Std. Dev.
WT	172	.00	39.67	5.14	6.78
LH	177	.00	48.40	7.06	8.00
NLH	178	.00	32.08	6.07	7.92

C



D

Region of interest	Finding	Two-tailed p-value
PSD	LH > WT	0.048

Linear density of Arc in the PSD is significantly higher in the LH than in the WT group (U = 13037.00, N_1 = 177, N_2 = 172, two-tailed p = 0.048). **A**: Semiquantative analysis of Arc gold particle density in the WT, LH and NLH rats. Bars represent mean value of linear density +/- 2 standard errors of the mean value of the different groups. **B**: Descriptive statistics of linear density values of each group. **C**: Electron micrograph from a section of a WT rat showing Arc immunogold labeling of an asymmetric synapse in the stratum radiatum. **D**: Summary of main finding. WT=wild-type, LH=learned helpless, NLH=non-learned helpless, AZ=active zone, PSD=postsynaptic density. Scale bar = 200 nm. Size of gold particle = 10 nm. Asterisk=synaptic cleft.

4. Discussion

4.1 Purpose and main findings of the work

MDD is associated with a volumetric loss of neural areas such as the PFC, the amygdala and the hippocampus. These neural changes may contribute to the disturbances in cognitive functioning and emotional stability as seen in the course of MDD. Various biological hypotheses of MDD focus on plastic changes in the brain as a causing, or accompanying, factor of this disease. Though these hypotheses comprehensively account plastic changes at the cellular level during MDD, the molecular correlates of these plastic changes require further experimental analyses.

The objective of this study was to investigate the involvement of synaptic proteins implicated in synaptic plasticity in the rat learned helpless model of depression. The proteins of interest were syntaxin1, the NMDA receptor subunit NR2B, and Arc. I tested my claim of a difference in the synaptic concentrations of these proteins in Schaffer collateral synapses in CA1 between the learned helpless, the non-learned helpless and the wild-type group.

By comparing the learned helpless group to the wild-type group, this work revealed that the concentration of NR2B is greater in the postsynaptic cytoplasm and in the PSD, as well as that the concentration of Arc in the PSD is greater in the learned helpless group compared to the wild-type group. The non-learned helpless group differed from the wild-type group with respect to a lower concentration of syntaxin1 in the presynaptic cytoplasm and in the PSD, as well as an increased concentration of NR2B in the postsynaptic cytoplasm and in the PSD. The concentration of syntaxin1 was greater in the pre- and postsynaptic cytoplasm of the learned helpless group compared to the non-learned helpless group.

4.2 Interpretation

The up- and down-regulation of syntaxin1, NR2B and Arc stems from an interplay between synaptic scaling (homeostatic plasticity) and an alteration of synaptic strength. As described previously, synaptic scaling allows neurons to increase or decrease the amount of AMPARs/NMDARs in order to keep ongoing synaptic activity in balance. Synaptic strength can be altered either through changes in the presynaptic machinery releasing the neurotransmitter, or by changing the concentration and functions of receptors at the

postsynaptic plasma membrane that sense the neurotransmitter signal. *Figure 17* displays a summary of the between-group differences in the concentration of proteins in the regions of interest.

4.2.1 Syntaxin1

I found a significant increase in syntaxin1 concentration in the presynaptic cytoplasm in the learned helpless group compared to the non-learned helpless group. Though the common assumption is that syntaxin1 is localized in the cell membrane of the active zone, an increased concentration of this protein presynaptically might indicate a potential enhancement of neurotransmitter release at later vesicle-membrane fusion stages (i.e a greater concentration of transport vesicles that deliver the neurotransmitter to the release site of the active zone). Because glutamate is the major excitatory neurotransmitter, one may speculate that an increase of syntaxin1 in the presynaptic cytoplasm prepares the presynaptic cell for an increased release of glutamate into the synaptic cleft. For the same token, an increased concentration of syntaxin1 in the presynaptic cell might indicate an increased turn-over of presynaptic vesicles. During the endocytosis of vesciles, the membrane might invaginate and detach from the plasma membrane with syntaxin1 being attached and transported back.

The notion that syntaxin might be particularly involved in the release of glutamate is supported by the finding that this SNARE protein is primarily expressed in excitatory synapses (Koh et al., 1993). In addition, one study has found that antidepressant treatment disruptes the participation of syntaxin1 in the SNARE complex presynaptically leading to a lowered release of glutamate into the synaptic cleft (Bonanno et al., 2005). In particular, the interaction between syntaxin1 and Thr^{286} phosphorylated αCaMkinaseII that usually leads to an enhanced neurotransmitter release was diminished, while the interaction between syntaxin1 and Munc-18, an interaction reducing neurotransmitter release, was enhanced.

Besides the possible restriction of syntaxin1 being present in excitatory synapses only, Koh et al. (1993) also found that syntaxin1 concentration is greater in the presynaptic cytoplasm and is almost absent in the active zone (Koh et al., 1993). This finding is in stark contrast with the general notion that syntaxin1 concentration is restricted to the active zone where it regulates vesicle fusion (Sudhof and Rizo, 2011).

A lack of differences between the different groups in the concentration of syntaxin1 in the active zone does not imply a lack of antigen detection of the antibody because syntaxin1 was - without any significant differences - expressed in the active zone. It could be that syntaxin1 might not be as easily detectable by antibodies due to the accumulation of various other proteins in the active zone. In addition, once at the active zone, syntaxin1 might already be engaged in the vesicle fusion process which might hinder the detection of the antigen by the antibody.

To give an account why there was only a significant difference in the concentration of syntaxin1 in the cytoplasm of nerve terminals and not in the plasma membranes is problematic. However, research indicates that different isoforms of syntaxin (syntaxin 1-4) are confined to different compartments of the pre- and postsynaptic cell in the retina (Sherry et al., 2006). These different isoforms of syntaxin are associated with functionally different synapses in amacrine cells (syntaxin1) or bipolar cells (syntaxin3). In particular, Sherry et al. (2006) found that the isoforms of syntaxin2 and syntaxin4 are located postsynaptically, thereby rejecting the assumption that syntaxin2 and syntaxin4 are involved in neurotransmitter release at the presynaptic plasma membrane.

I further found that the concentration of this protein is increased in the postsynaptic cytoplasm of the learned helpless group. The increased concentration of syntaxin1 in the postsynaptic cytoplasm of the learned helpless group suggests that syntaxin1 is involved in the trafficking of molecules to the postsynaptic plasma membrane or in the recycling of glutamate receptors. As it has been suggested by others (Suh et al., 2010), SNAP-23 a SNARE protein interacting with syntaxin1 is also found in the postsynaptic cytoplasm regulating the trafficking of NMDARs. A similar role of synaptic scaling could be attributed to synatxin1 where it regulates the trafficking and insertion of NMDARs into the postsynaptic membrane in the learned helpless group (as shown later, the NMDAR2B subunit is up-regulated in the PSD). In addition, a recent study has noted the importance of the isoform synatxin3 in postsynaptic regulation of AMPAR delivery to synapses during LTP, adding more evidence to syntaxin's a possible involvement in the postsynaptic cell (Jurado et al., 2013).

The findings of an increased concentration of syntaxin1 presynaptically and postsynaptically as described above should be regarded in connection with a higher concentration of NMDARs in the learned-helpless group as discussed next.

4.2.2 NMDAR2B subunit

The concentration level of the NMDAR2B subunit was greater in the PSD and postsynaptic cytoplasm of the learned helpless group compared to the wild-type group. The increased concentration level of NMDARs is in line with others (Karolewicz et al., 2009) and underscores the importance of NMDAR antagonists such as ketamine as potential antidepressants (Zarate et al., 2006). A possible explanation why the concentration level of NR2B is increased in the PSD and the postsynaptic cytoplasm requires that the results are regarded in two divergent, yet interconnected, scenarios.

First, it has been proposed that NMDARs are heavily involved in homeostatic plasticity by scaling the strength of incoming synaptic input up or down while preserving the relative weights between the inputs (Perez-Otano and Ehlers, 2005). Increasing the concentration of NMDARs, in this case the NR2B subunit could possibly increase the potential for synaptic plasticity or change. It has been suggested that MDD might be an evolutionary adaptive response to activate resources and make changes to the current painful situation (Nesse, 2000). NMDARs are particularly involved in synaptic changes, or learning and memory, as outlined above. Therefore, learned helpless rats may display enhanced learning to make changes to the current depressive state. Previous research has indicated that depressed patients are impaired in selective attention (Lemelin et al., 1996). Impaired selective attention might benefit depressed patients in broadening their attention to find and adapt to changes in life. That MDD might be an evolutionary adaptive response is furthermore supported by a study showing that depressed patients are faster in making motivational responses (Chase et al., 2010). Enhanced cognitive processing with respect to rewards might enable and motivate depressed patients to change current situations in life.

Second, it is known that activation of NMDARs during LTD in the hippocampus leads to the endocytosis of AMPARs through a calcium influx and CN activation (Beattie et al., 2000). An increasing rate of AMPAR endocytosis due to the increased concentration of NMDARs amplifies the induction of LTD. In previous work, our group found a tendency that AMPAR concentration is reduced in the brains of learned helpless rats (Daaland, 2012). The findings above support the notion that LTD is more pronounced during MDD and - because LTP is impaired when glucocorticoid levels are either very low or very high (as in MDD) - that LTP is impaired (Kim and Diamond, 2002). Furthermore, a greater activation of NMDARs is

accompanied by an increased influx of Ca^{2+} which, when excessive, leads to neurotoxicity and spine retraction (Christian et al., 2011).

As mentioned above, antidepressant treatment seems to disrupt the participation of syntaxin1 in the SNARE complex leading to a lowered release of glutamate into the synaptic cleft (Bonanno et al., 2005). The increased NMDAR concentration during MDD might lead to glutamate excitotoxicity through an uncontrolled increase of Ca^{2+} postsynaptically (Duman, 2009). Excitotoxicity is central to mood disorders, and several studies have supported the notion that an excess amount of extracellular glutamate is related to the pathology of MDD. For instance, it has been shown that stress rapidly leads to an increase of glutamate in the hippocampus disturbing LTP (Cazakoff and Howland, 2010). In addition, research has shown that extracellular glutamate concentration was increased in a learned helpless paradigm (Almeida et al., 2010). The high level of glutamate in the extracellular space might be attributed to an impaired uptake of glutamate by glial cells (Zink et al., 2010).

It has to be taken into consideration that previous work which found a down-regulation of the NMDARs did not distinguish between the different subunits of the receptor (Daaland, 2012). The discrepancy between the present work and the latter study may be due the combinatorial concentration measurement of NR2A/NR2B subunits.

4.2.3 Arc

The concentration of the protein Arc was highly increased in the PSD of the learned helpless group compared to the wild-type group. It has been shown that Arc transcription depends on a high NMDAR/AMPAR ratio (Rao et al., 2006) and that Arc is involved in the endocytosis of AMPARs (Chowdhury et al., 2006).

It is likely that an increased concentration level of NMDARs, as shown in this work, lead to the increase in the concentration of Arc in the PSD. It has been shown that Arc is predominantly localized in postsynaptic sites of recently activated synapses (Moga et al., 2004). This makes Arc an ideal candidate as a neural marker to explore the history of neural activity in the brain because increased concentration of Arc mRNA is immediately observed upon neural activation (Guzowski et al., 1999). It could be that recently activated synapses in the learned helpless group, as revealed by the immunoreactivity of Arc, underwent

intracellular signaling mechanisms due to glutamate excitotoxicity (increased NR2B concentration).

It has also been shown that overexpression of Arc blocks the up-regulation of AMPARs, while Arc KO neurons exhibit an increase in AMPARs, thereby permitting the homeostatic scaling of synaptic strength (Shepherd et al., 2006). A recent study supports the notion that Arc KO neurons display a deficiency in the synaptic scaling mechanism (Beique et al., 2011). In Arc KO mice, a prolonged reduction of glutamate release did not lead to a homeostatic up-regulation of AMPARs.

Surprisingly, the present work showed that Arc is also found in the active zone which is in contrast to the literature claiming that Arc is absent from the presynaptic cell (Moga et al., 2004, Shepherd and Bear, 2011). Though there was no significant difference between the groups, immunoreactivity for Arc was even greater in the active zone than in the PSD within the groups. Though there were no significant effects within or between the groups, Arc concentration was also present in the presynaptic cytoplasm. Because Arc mRNA is targeted to recently activated synaptic sites where it is translated and enhancing the synthesis of proteins required for synaptic plasticity (Tzingounis and Nicoll, 2006), one may speculate that the function of Arc in the active zone is to integrate the expression of genes that direct the production of presynaptic proteins. Because one way of increasing synaptic strength is in modulating the neurotransmitter releasing machinery, an increased concentration of Arc in the active zone might indicate that the concentration of other SNARE proteins besides syntaxin is increased.

4.2.4 Resilient animal type

I found additional effects particularly for the non-learned helpless group when comparing it to the learned helpless or wild-type group. Because non-learned helpless rats represent a resilient animal type towards stress, the results of this animal group are appealing with respect to the increasingly valuable resilience-related research. Resilience-related research informs us why stressful life events are controllable to some persons while the same events are uncontrollable to others.

In this work, I found a significant decrease of syntaxin1 concentration in the presynaptic cytoplasm and in the PSD of the non-learned helpless group compared to the wild-type group.

Furthermore, the NR2B concentration was significantly higher in the PSD and in the postsynaptic cytoplasm in the non-learned helpless group in comparison to the wild-type group.

Because syntaxin is instrumental in exo-endocytotic activity, I assume that a lower concentration in the presynaptic cell is associated with a lower release of glutamate. Given the lower concentration of syntaxin1 in the non-learned helpless group, the results likewise suggest that vesicle turn-over is decreased, possibly transporting less syntaxin away from the active zone. A lower level of synaptic vesicle turn-over might therefore determine a resilient animal type. In line with stress-resiliency, a previous study has shown that disturbing the participation of syntaxin in the SNARE complex leads to an antidepressant effects by lowering the release of glutamate into the synaptic cleft (Bonanno et al., 2005). The involvement of syntaxin in the endocytosis of AMPARs in the postsynaptic cell has been reported previously.

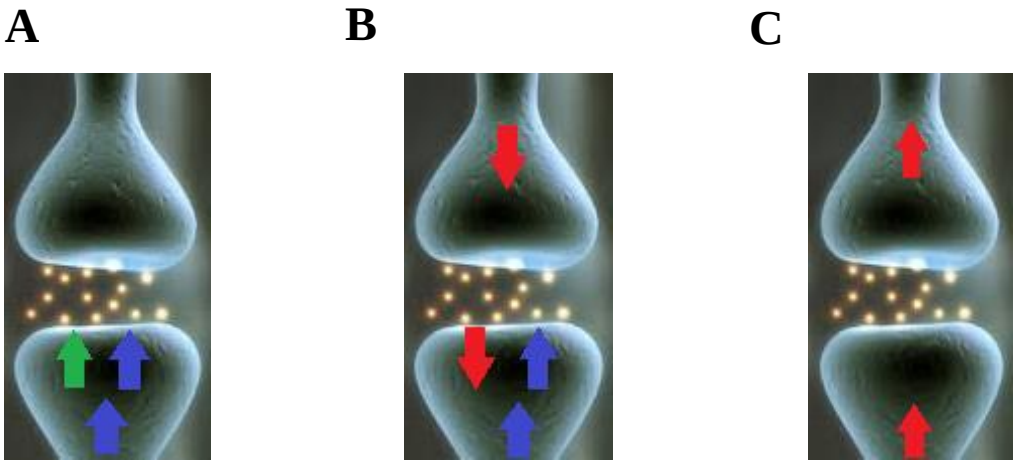
The distinctions between the learned helpless and the non-learned helpless group with respect to the wild-type group are less clear for the concentration level of NR2B. The results showed that the concentration levels of NR2B in the PSD as well as in the postsynaptic cytoplasm of the non-learned helpless group increased in accordance with the learned-helpless group. The concentration of NR2B in the latter two groups were both significantly higher compared to the concentration in the wild-type group, but were not significantly different from each other. Comparing the learned helpless and the non-learned helpless group with each other revealed a trend in the differential concentrations. The concentration level of NR2B in the PSD of the learned helpless group increased slightly more than the level in the non-learned helpless group, while the concentration of NR2B subunit in the postsynaptic cytoplasm of the non-learned helpless group increased slightly more than the level in the learned helpless group. However, this differential effect is not statistically significant and is only a tendency.

Nevertheless, the relationship between the learned helpless and the non-learned helpless group follows a linear function where a certain threshold of NMDAR concentration determines whether a stress response is maladaptive (learned helpless) or adaptive (non-learned helpless). The small, though not significant difference, between the non-learned helpless and learned helpless group might resemble the threshold where an increasing glutamate excitation (increased number of NMDARs) develops into a maladaptive adaptation to stress causing the animal to be vulnerable to learned helpless behavior.

However, these speculations need to be further verified at the functional and molecular level by resilience-related research.

Figure 17

Between-group differences



Protein concentration in the different groups. **A**: LH (compared to WT), **B**: NLH (compared to WT), **C**: LH (compared to NLH). Arrows indicate up- or downregulation of the proteins. Green=Arc, blue=NR2B, red=synatxin1. Background picture retrieved from http://cargocollective.com/synapses#what-is-a-synapse

4.3 Limitations

4.3.1 Methodology

It should be taken into consideration that the labeling efficacy in immunocytochemical procedures is influenced by the quality of the antibody, the fixation procedure, the embedding medium, and the incubation parameters. These factors have an impact in any experiment making use of immunolabeling, and very likely influenced the results of my work as well.

When evaluating the results obtained in this work, it should further be taken into consideration that whether the lack of escape behavior as expressed by animals in the learned helpless model can unhesitatingly be applied to MDD in humans is under debate. There seems to be major overlap in neural activation between the LH model and MDD in humans (Pryce et al., 2011). However, the important question about what neural structures and functions have been maintained between species and what neural structures and functions underwent species specific adaptations is unconditionally ignored in the evaluation of animal models.

Furthermore, with respect to MDD, clinical diagnosis is largely based on communication between patient and health staff. Obviously, animals lack the ability of self-reflection and have no means to communicate effectively with researchers. Therefore, not all clinical symptoms in animals can be modeled due to the reliance on verbal report in psychiatric diagnostics.

The learned helpless model has strong validity criteria (Vollmayr and Henn, 2003). At the same time, these criteria resemble one of the greatest weaknesses of the learned helpless model. The *face validity* criterion creates a relationship between the learned helpless model and MDD on the basis of known signs and symptoms of MDD. A shortcoming of this approach is that, as the DSM-V demonstrates, some symptoms are weighted more than others differentiating between core and peripheral signs of MDD. According to the DSM-V, the core symptoms are anhedonia and depressed mood. While anhedonia, which is also a major symptom in schizophrenia, can be modeled in animal models, depressed mood (such as guilt or suicidality) cannot. The symptom of anhedonia therefore captures a great role in determining a depressed state in an animal neglecting the criterion of depressed mood. Peripheral symptoms of MDD such as psychomotor changes, fatigue or loss of energy, are the preferred and prominent symptoms in animal models when judging whether an animal is depressed i.e. learned helpless (Willner and Mitchell, 2002).

Construct validity requires that neurobiological and psychosocial concepts of MDD are in alignment with the exhibited behavior of the modeled animal. This approach is clearly hampered by the lack of a comprehensive etiological theory on the development of MDD. It is far from being known how psychosocial factors (undesirable life events, chronic mild stress, adverse childhood experiences, and personality traits) are involved in the physiological processes underlying mood. In addition, there does not exist one common etiological factor that precedes depression, rather it is a diverse set of causes (Willner and Mitchell, 2002).

An additional shortcoming is the discriminative effect of stress on the development of MDD. If very severe stress is used to induce symptoms of MDD in animals, it is possible that the model approximates post-traumatic stress disorder (PTSD) rather than MDD. Furthermore, the learned helpless model links the inability to terminate the foot shock by pressing the level with a depressed helpless state. However, the inability to escape or terminate the shock might also be related to behavioral impairments such as decreased locomotor activity or analgesia (Willner and Mitchell, 2002).

Despite the use of animal models in research of MDD, the above described limitations need to be taken into consideration when evaluating the results. An animal model such as the learned helpless model is an approximation of MDD.

4.3.2 Subdivisions of the hippocampus

The results obtained in this work could have been influenced by the selection of neural tissue from the CA1. The tissues used in this work have been dissected from the dorsal hippocampus. Focusing only on the ventral division of the hippocampus might have led to different results of the protein concentrations. The ventral hippocampus seems to be the subdivision which ultimately needs to be explored in research related to MDD as supported by the findings outlined below.

Cognitive functioning and emotional stability are major parts of the pathology of MDD. It seems that these two functions of the human mind are interrelated, and also that they are regulated by activity in one neural area, the hippocampus (Pessoa, 2008). Despite the strong relationship between cognition and emotion, the hippocampus is commonly regarded as being purely responsible for cognitive functioning such as memory, or being solely a regulator of emotion.

The hippocampus is involved in the formation of episodic memories and spatial navigation. However, in 1937, the hippocampus was already described by Papez as being part of an emotion processing system, known today as the limbic system (Papez, 1937). Given the different functional roles of the hippocampus it suggests itself that different regions of this structure may be important for different (cognitive and emotional) functions.

Evidence for a fragmentation of the hippocampus was reviewed by Moser and Moser (1998). They outlined that the hippocampus contains different subdivisions because (i) the ventral and dorsal hippocampus have different input structures, (ii) spatial memory only appears to depend on the dorsal hippocampus, and (iii) stress responses and emotional behavior are only altered by lesions to the ventral hippocampus. In addition, the dorsal hippocampus sends projections mainly to areas responsible for visuospatial processing, memory processing and spatial exploration. In contrast, the ventral hippocampus sends projections to nuclei of the amygdala, the PFC, and medial zones of the hypothalamus regulating endocrine functions and motivated behaviors.

Others have found that also the induction of LTP differs between the dorsal and the ventral hippocampus (Segal et al., 2010) and hypothesized that the induction of LTP in the dorsal or the ventral hippocampus might regulate the stress responses. In normal conditions, the ability to evoke LTP is greater in the dorsal hippocampus. During acute stress; however, LTP is facilitated in the ventral hippocampus and inhibited in the dorsal region. Opposite effects might allow the ventral part of the hippocampus to enhance the connectivity with the amygdala and the PFC.

4.3.3 Glial cells

Given the current knowledge of MDD, it could be that an excess amount of extracellular glutamate might mirror the highly increased amount of NMDARs in the PSD and postsynaptic cytoplasm of the learned helpless group as shown in this work. An increased amount of glutamate receptors allows more Ca^{2+} to enter the postsynaptic cell leading to excitotoxicity and eventual cell death or retraction of spines. Studies suggest that the amount of astrocytes is reduced in MDD contributing to the pathological alterations in glutamate neurotransmission (Cotter et al., 2002, Ongur et al., 1998). A study has shown that glutamate uptake in the hippocampus is disturbed in learned helpless rats (Almeida et al., 2010) which is in line with a study showing that learned helpless rats have lower levels of the glutamate transporters GLT-1 in the hippocampus (Zink et al., 2010). A critical role in the removal of glutamate is performed by astrocytes containing the glial glutamate transporter (GLT-1) which eventually reduces the risk of excitotoxicity (Rothstein et al., 1996). It has also been shown that in patients suffering from MDD the level of glutamate is increased and is possibly related to the decreased amount of GFAP found in postmortem brains (Si et al., 2004).

In sum, the general finding of reduced hippocampal volume might not solely be related to synapse or neuron loss. Though I looked at synapses in the hippocampus, investigating proteins such as GFAP might be promising in order to establish a link between the loss of glial cells and volumetric reductions of the hippocampus. Above all, glial cells might be heavily involved in synaptic plasticity by inducing new synapses in the CNS and maintaining them over time as indicated above (Pfrieger and Barres, 1997, Nedergaard et al., 2003).

4.3.4 Group size and outliers

The study revealed significant effects between the learned helpless and wild-type group and also between the non-learned helpless and the wild-type group. However, it should be noted that the groups differed in the amount of animals they contained (the learned helpless and non-learned helpless group contained four animals each while the wild-type group contained two animals).

Different group sizes affect the criterion of homogeneity of variance of the ANOVA. The ANOVA computes effects by comparing the variance between the groups (*between group variance*) with the variance within the groups (*within group variance/error variance*). A greater variance between the groups compared to the variances within the groups leads to statistically significant results. However, the greater the *within variance,* or *error variance,* the more likely it is to get statistically significant results by chance (Howitt and Cramer, 2006). Since the wild-type group only contained two animals this group is more likely to display a greater within variance than the learned helpless or the non-learned helpless group that contained twice as many animals as the wild-type group.

Because significant results could be obtained by chance by having unequal group sizes, I regarded the group size not as the number of animals but as the number of studied synapses. It could be argued that the criterion of independence might be violated. However, each synapse contributed to one group only once and the concentration of proteins in one synapse had no effect on the concentration of proteins in other synapses, i.e. they were independent. In order to conform both groups with respect to size, I increased the number of pictures taken of each animal (WT $N = 180$, LH $N = 180$, NLH $N = 180$). If differences in the variances occur nevertheless, the ANOVA is robust to differences in error and within variances when group sizes are equal (Howitt and Cramer, 2006). I applied the Kruskal Wallis test when the variances of the groups differed significantly and violated the homogeneity of variances criterion.

As stated in section 3.9, I removed outliers which were further than three interquartile ranges (IQR) away from the end of a boxplot. I assumed these observations must have been caused by pure chance. Though there is no objective way to remove outliers and no real evidence that these are actual true values I kept outliers which were 1.5 IQRs away from a boxplot in the analysis. Given the very large values of outliers being further than three IQRs away from the

end of the boxplot, I considered them to be occurring by chance and not resembling true detection of antigens by the antibodies.

4.4 Implications and suggestions for future work

The results demonstrate at the molecular level that synaptic proteins such as syntaxin1, the NMDAR2B subunit or Arc are involved in changes of synaptic plasticity in the learned helpless model of depression. An altered concentration of synaptic proteins lies at the heart of the neuroplasticity hypothesis of MDD which implicates that malfunctioning plasticity mechanisms lead to a volumetric loss of the hippocampus in MDD.

A vast amount of other synaptic proteins continue to require scientific investigation in unraveling their role in the pathology of MDD. A possible protein to explore is the AMPAR. A down-regulation of AMPARs would be in line with the results of this work and would further support the notion of increasing Ca^{2+} influx into the postsynaptic cell enhancing LTD, excitotoxicity and eventual cell death, i.e. volumetric decreases of the hippocampus. In fact, a moderate, but not significant reduction in synaptic AMPAR concentration was noted previously in our lab (Daaland, 2012).

It is plausible that the reduction of neural tissue in the hippocampus during MDD might be mediated or even attributed to alterations in glial cells. This would support the idea of disturbed astrocytic clearance mechanisms of glutamate from the synaptic cleft due to the reduced concentration of glutamate transporters that are located in glial cells (Zink et al., 2010). It needs to be further examined whether the neural areas displaying a volume loss are associated with a loss in glial cells.

5. Conclusion

I have examined the relative concentration level of three important synaptic proteins. I found an increased concentration level of syntaxin1 in the pre- and postsynaptic cytoplasm, as well as an increased NMDAR2B subunit concentration in the postsynaptic cytoplasm and the PSD, and an increased concentration of Arc in the PSD of learned helpless rats. The non-learned helpless rats showed a significant reduction in the concentration level of syntaxin1 in the pre- and postsynaptic cytoplasm, as well as an increased concentration of the NR2B subunit in the postsynaptic cytoplasm and the PSD. The results of this work demonstrate that mechanisms of synaptic plasticity are altered in the learned helpless model of depression. Disturbed forms of synaptic plasticity might be related to the morphological and behavioral changes in the learned helpless model. Hence, these latter changes may be connected to the expressed cognitive and emotional symptoms accompanied by a volumetric decrease of the hippocampus as seen during MDD.

6. Appendix

Appendix 1

Information about the experimental rats

Animal	Group	Stock no.	DOB	LPs	Time (min)
SL3	LH	6007	11.02.2010	1	20.7
Sl4	LH	6008	11.02.2010	0	21
SN11	NLH	6010	12.06.2010	15	7.71
SN12	NLH	6011	12.06.2010	14	8.7
WT_A	WT	5773			
WT_B	WT	5774			
CLH_A	LH	5769			
CLH_B	LH	5770			
CNLH_A	NLH	5771			
CNLH_B	NLH	5772			

DOB = date of birth, LP = lever press, Time = time to finish testing session, CLH = congenital learned helpless, CNLH = congenital non-learned helpless, WT = wild-type.

Appendix 2

Chemicals, supplier, catalogue number

Product	Supplier	Catalogue number
Trizma base	Sigma	T1503
Sodium chloride	AnalaR Normapur	27810.296
Triton X-100	Sigma	T8787
Albumin, human serum	Sigma	A1653
Polyethylene glycol	Sigma	P2263
Glycine	Sigma	G7126
HCI	AnalaR Normapur	20252.290

Appendix 3

Composition of solutions

TBST (200ml)	Tris solution	TBST with 50mM glycine (5 ml)	TBST with 2% HSA (2 ml)	TBST with 2% HSA and PEG (1 ml)	PEG solution (1 ml)
20ml 0.05M Tris-HCI pH7.4	1,211g Tris (Trizma Base) in 170 ml dH20	18.75 mg glycine	40 mg HSA	100 µl PEG solution	5 mg PEG
+	+	+	+	+	+
180ml dH20 with XXg NaCl (0.3% = 0.54g, 0.4% = 0.79g, 0.5% = 0.9g, 0.6% = 1.08g, 0.9% = 1.62g)	Adjust HCI to reach p.H7.4 and add up dH20 until 200 ml	5 ml TBST	2 ml TBST	900 µl TBST with 2% HSA	1 ml TBST
+					
0.02g (0.01%) or 0.2g (0.1%) Triton X-100					

Appendix 4

Protocol for post-embedding immunochemistry

1)	Place grids into a grid support plate and put on 50mM glycine in TBST (Tris buffered saline with Triton X-100)	10min.
4)	Preincubate in TBST containing 2%HSA (human serum albumin)	10min.
5)	Incubate in primary antibody diluted in TBST containing 2% HSA	over night
6)	Rinse in TBST	3 X short 1 X 10min. 3 X short 1 X 10min.
7)	Preincubate in TBST containing 2%HSA	10min.
8)	Incubate in secondary antibody (immunoglobulins coupled to colloidal particles, 15nm). Diluted as recommended from the company in TBST with 2%HSA and 0.05% PEG (polyethylenglycol)	90min.
9)	Rinse in UF-water Dry sections	6 X short
10)	Incubate in 5% uranylacetate in 40% ethanol	90sec.
11)	Rinse in UF-water Dry sections	3 X short
12)	Incubate in lead nitrate	90sec.
13)	Rinse in UF-water	3 X short

Let sections dry completely before examination in the microscope.

Appendix 5

File names of electron micrographs

Figure 12B: Syntaxin_cy_WT_5773-2_H1_23.tif
Figure 13C: Syntaxin_PSD_WT_5773-2_H2_28.tif
Figure 14B: NMDAR_cy_WT_5773_J1_Tv15.tif
Figure 15B: NMDAR_PSD_WT_5773_E3_Tv41.tif
Figure 16C: ARC_PSD_WT_5773-2_J1_26_Tv80.tif

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