Molecular and cellular neuroplasticity in animal models of depression

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Avoid boring people

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Chapter 1

Genaral introduction

1. Major depressive disorder

1.1 Clinical diagnosis of major depressive disorder

Major depressive disorder (MDD) is among the most common psychiatric disorders with a lifetime prevalence estimated at 16.2% in the United States[1] (see www.hcp.med.harvard.edu/ncs/ for up to date and comprehensive statistics) and 17.5% in The Netherlands[2], with females at higher risk than males. It is estimated that in the year 2020 MDD will be the second leading cause of disability, only to be surpassed by ischaemic heart disease[3]. The consequences of MDD are of compound nature and characterized by the fact that MDD has a high tendency towards relapse, recurrence and chronicity[4].

Diagnosis of MDD is based on symptomatic criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). The diagnostic criteria for MDD require persistence of either depressed mood or loss of interest and pleasure (anhedonia), in association with at least four out of the following symptoms: inattention, fatigue, self-depreciating or suicidal thoughts, and disturbances of psychomotor activity, sleep, appetite and weight. Symptoms need to be present consistently for at least two weeks. The variety in clinical symptoms of MDD underscores the complexity of the disease and, therefore, MDD is regarded as a heterogeneous disorder comprising of many different syndromes rather then a single disease. In particular, the clinical course of MDD is pleiomorphic and varies from subthreshold syndromes, single episodes (short or long), multiple recurrent episodes with or without inter-episodic recovery, residual symptoms after an episode, to chronicity[5].

1.2 The pathogenesis of depression

An open question in MDD is which factors underlie a person's vulnerability (or resilience) to the disease. Various putative risk factors have been suggested arising from epidemiological studies but difficulties in differentiating between association and causation have left many inconsistencies. However to date, it is generally accepted that both genetic and non-genetic factors, and the interaction between those, comprise disease liability[6,7].

Genetic epidemiological studies have shown depression to be a familial disorder with increased risk odd ratios (ratio of risks of first-degree relatives of MDD probands vs. the general population) of ~2.8 [7]. Familial studies can, however, not distinguish between genetic and shared environmental influences. Therefore, twin studies using mono- and dizygotic twins have been performed. These confirmed that genetic influences for the most part contribute to this familial aggregation, and heritability of liability is estimated to be ~37%[7]. In

terms of genetic contribution in psychiatric disorders this makes MDD at the lower end of the scale[8].

Of the environmental factors, the most consistent and dominant one is exposure to stressful live events. Twin studies have shown a causal relation between MDD and events related to 'bad luck' or stressful experiences related to a persons own behavior (65 – 75%), with odd ratios – the ratio of the risk of disease to individuals experiencing an event compared with persons without the stressful event – of 2.33 and 5.64, respectively[6]. Furthermore, the occurrence of stressors are 2.5 times more likely in depressed patients compared with controls, and in community samples, 80% of the depressed cases have experienced major life events prior to the onset[9]. In particular chronic forms of stress, often psychosocial in nature, may predict precipitation of depression[10].

1.3 Brain structures involved in MDD

Despite the prevalence of depression and its considerable impact, knowledge about the pathophysiology of MDD is limited. This is caused by the fact that MDD is heterogeneous in terms of disease and underlying causes. Additionally, the impossibility to take a brain biopsy from depressed individuals hampers investigation of the affected brain tissue.

Since currently available antidepressants adjust monoaminergic signaling (Box 1), early studies focused largly on monomaminergic nuclei such as the dorsal raphe nucleus and the locus coeruleus. From these nuclei, serotonergic and noradrenergic neurons innervate most other brain regions, respectively.

However, the development of structural en functional neuroimaging technologies has permitted *in vivo* characterization of the anatomical correlates of mood disorders. Among these are brain regions and circuits that are known to regulate emotion, reward and executive function. Dysfunction of these highly interconnected 'limbic' regions has been implicated in depression and antidepressant action. In particular, these regions include the amygdala (anxiety and emotional memory), hippocampus (cognition), mediodorsal and midline thalamic nuclei (emotional expression), hypothalamus (vegetative symptoms and hormonal regulation), subgenual anterior cingulate cortex (negative mood states), and the ventral striatum (anhedonia)[11]. However, published findings are not consistent and are often complicated by comorbid diagnoses and medication history, and there has been little success in demonstrating any clear cause–effect relationships of pathological changes and MDD.

Among the most consistent findings are a reduced grey-matter volume in the hippocampus[12,13] and prefrontal cortex[14]. These pathological changes have been linked to

Box 1. Most commonly prescribed antidepressants

Drug	Brand	Class	Mechanism of action
Sertraline	Zoloft	SSRI	Inhibits serotonin transporter, and partly dopamine reuptake
Escitalopram	Lexapro	SSRI	Inhibits serotonin transporter
Fluoxetine	Prozac	SSRI	Inhibits serotonin transporter
Bupropion	Wellbutrin	NDRI	Inhibits norepinephrine and dopamine transporters
Imipramine	Tofranil	TCA	Inhibits serotonin, norepinephrine, and dopamine transporters. Is an antagonist of acetylcholine and histamine receptors. And is a dopamine receptor agonist
Venlafaxine	Efexor	SNRI	Inhibits serotonin and norepinephrine transporters
Citalopram	Cipramil	SSRI	Inhibits serotonin transporter
Trazodone	Trazolan	SARI	Inhibits serotonin transporter and antagonizes several serotonin and noradrenalin receptors
Amitriptyline	Tryptizol	TCA	Inhibits serotonin, and norepinephrine, transporters. Is an antagonist of serotonin and adrenergic receptors

SSRI: specific seretonin reuptake inhibitor; NDRI: norepinephrine-dopamine reuptake inhibitor; TCA: tricyclic antidepressant; SNRI: Serotonin–norepinephrine reuptake inhibitor; SARI: Serotonin antagonist and reuptake inhibitors.

the cognitive aspects of depression, potentially underlying a 'diminished ability to think or concentrate'. Cognitive difficulties in major depression fall into at least two domains. First, impairment of concentration and attention is likely to relate to the well-documented abnormalities of dorsolateral prefrontal cortex (DLPFC)-function in MDD subjects[15]. Second, MDD patients also exhibit prominent deficits in explicit memory[16], a cognitive capacity well-known to depend on the function of the hippocampus and the medial temporal lobe[17]. Apart from structural changes, hippocampal atrophy has been repeatedly documented in MDD. Whereas the total number of neurons and glia has not been found altered, neurons are reduced in size and the volume of the neuropil is reduced[18]. Disruption of hippocampal function, including the capacity for neuroplasticity, might contribute to several cognitive aspects of severe forms of MDD.

In addition to its role in declarative memory, the hippocampus is a key regulator of prefrontal cortical function; the hippocampus and DLPFC act concertedly to regulate explicit memory. Disruption of hippocampal function in MDD might therefore contribute to the observed deficits in concentration, described above. The hippocampus is also a critical activity regulator of both nucleus accumbens and ventral tegmental area (VTA). It has been hypothesized that an indirect excitatory projection from hippocampus to VTA is important for coordinating the firing of VTA cells in response to novelty[19]. Impairment of this hippocampal function might lead to reduced dopaminergic tone and contribute to anhedonia[20]. Finally, the hippocampus provides an important source of negative modulation of the hypothalamus-

pituitary–adrenal (HPA) stress hormone axis through its projections to the hypothalamus; hippocampal dysfunction therefore may contribute to dysregulation of the stress response[21] that is seen in major depression.

1.4 Treatment of MDD

There are several effective treatments for depression. The large majority (80%) of people with MDD show improvement during treatment with antidepressants or with electroconvulsive therapy (ECT). In particular ECT is still being considered the most effective[22,23] treatment for patients with melancholic or psychotic depression. In addition, several forms of psychotherapy – in particular, cognitive and interpersonal psychotherapy – can be effective for patients with mild to moderate symptoms.

The first antidepressants were discovered by chance almost 50 years ago, when iproniazid, a drug registered for the treatment of tuberculosis, was found to elevate mood in MDD patients[24]. Simultaneously and independently, imipramine, an experimental antihistamine with a tricyclic structure, was found to have antidepressant effects[24]. Soon after this, drugs with antidepressant activity were shown to increase the extracellular concentrations of two important monoamine neurotransmitters in the brain, serotonin (5-hydroxytryptamine or 5-HT) and noradrenaline, by inhibiting their catabolism or reuptake into nerve endings. These findings were the basis for the monoamine hypothesis of depression, which proposes that mood disorders are caused by a deficiency in serotonin or noradrenaline at functionally important receptor sites in the brain[24,25]. Over the last few decades, the view that depression is produced by a chemical imbalance in the brain has become widely accepted among scientists, clinicians and the public, despite the lack of evidence for a direct role of 5-HT in this.

It soon became evident that the monoamine hypothesis in its original form did not explain all of the antidepressant effects[26]. In particular, available antidepressants immediately increase monoaminergic availability, while it takes up to several weeks for the clinical antidepressant response to occur[23]. Therefore, the focus of research was re-directed towards the receptors and intracellular signal transduction molecules that are regulated by antidepressant treatment[27], thereby generating new theories of the pathophysiology of MDD, the action of antidepressant medications, and identifying potential targets for novel antidepressant therapies[28]. A striking observation, as these downstream molecular events have been elucidated, is the degree of overlap between the molecular and cellular changes induced by antidepressant treatment and the molecular mechanisms of neuroplasticity, especially synaptic plasticity[29].

In line with this, accumulating evidence suggests that the glutamatergic system and its plasticity play an important role in the neurobiology and treatment of depression. For example, the N-methyl-D-aspartate receptor (NMDAR) antagonist ketamine has consistently shown antidepressant effects within a few hours of its administration[30,31]. Also, it is hypothesized that increased alpha-amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid receptor (AMPAR) throughput may represent a convergent mechanism for the rapid antidepressant actions of ketamine[30,32]. This raises the possibility that AMPA receptor potentiators might be useful in the treatment of MDD[30].

2. Stress, depression and neuroplasticity

As discussed above, there is a clear relationship between psychosocial stressors and MDD. Also, in animal models of depression, stress induces depressive symptom-like behaviors[33,34], and can lead to atrophy of the hippocampus similar to that seen in depression[35]. Increasing evidence shows that exogenously applied chronic stress has detrimental effects on neuroplasticity[18,36,37].

As outlined below, in these animal models an overlap between antidepressant-induced changes and mechanisms of neuroplasticity is observed. For example, antidepressant action in the hippocampus is found to be dependent on cAMP response element-binding (CREB) protein[38] that has a well-established role in synaptic plasticity and learning and memory.

These findings have resulted in the hypothesis that chronic stress, which can precipitate or exacerbate depression, disrupts neuroplasticity, whereas antidepressant treatment produces opposing effects and can enhance neuroplasticity (Fig 1). Experimental evidence for this hypothesis is discussed below, and originated for a large part from animal models on stress-related research (Box 2).

2.1 Stress and neuroplasticity

Memory, plasticity and cell survival

In animal models, transient mild stress has been shown to increase hippocampus-dependent memory performance[39]. However, more severe or chronic stressors induce hippocampus-dependent spatial memory impairments[40,41]. Similar results were obtained by treatment with glucocorticoid stress hormones[40]. Specific impairments of hippocampus-

dependent explicit memory were also observed after treatment of human subjects with glucocorticoids[42].

Hippocampus-dependent memory formation is known to be dependent on long-term potentiation (LTP) and long-term depression (LTD), the basic cellular mechanisms of synaptic plasticity within the hippocampus[29]. Interestingly, both stress and increased glucocorticoid levels inhibit LTP in the rodent hippocampus[43]. Conversely, paradigms inducing stress in rodents enhance hippocampal LTD[43].

Sustained levels of stress or glucocorticoids could damage hippocampal cells. Stress leads to atrophy and retraction of the apical dendrites of hippocampal pyramidal cells[44]. In addition, different forms of acute and chronic stress have been shown to reduce adult hippocampal neurogenesis in rodents[18]. It has been established that new neurons are generated in the dentate gyrus region of the hippocampal formation of the adult mammalian brain[45]. Neurogenesis appears to be required for the behavioral response to antidepressants in rodents[46] and impaired neurogenesis has been hypothesized to represent a core pathophysiological feature of MDD[18,47]. Thus, neurogenesis might mediate the effects of stress on mechanisms of neuroplasticity and may contribute to the development of MDD.

Similar effects of stress have been obtained in the PFC, such as reduced synaptic plasticity in projections coming from the amygdala[48], regression of the apical dendrites of pyramidal cells[49], and a reduction in the number of glial cells[50]. In contrast, in the amygdala, stress enhanced synaptic plasticity and the function of amygdala neurons[51]. Stress could also enhance amygdala-dependent learning[52], and the size and activity of the amygdala were increased in depressed patients[53]. This contrast in stress-related response of different brain areas makes clear that the well-documented effects of stress on hippocampal morphology and function are not the mere manifestations of a universal effect of stress hormones, or other aspects of stress, on neuronal integrity. Rather, the effects of stress on brain morphology and function are region- and circuit-dependent.

Stress and molecular plasticity

Accumulating evidence suggests a role for glutamate in response to stress that might subsequently yield depression[54]. Glucocorticoid excess increases glutamate release in the CA1 region of the hippocampus[55] and chronic behavioral stress increases extracellular levels of glutamate in the CA3 region[56]. This excess glutamate likely contributes to cell damage in these regions and possibly even cell death[35,57]. Chronic stress increases glutamate levels, which activates extrasynaptic NMDA receptors[58]. Extrasynaptic NMDA receptors have been found to inhibit LTP[58].

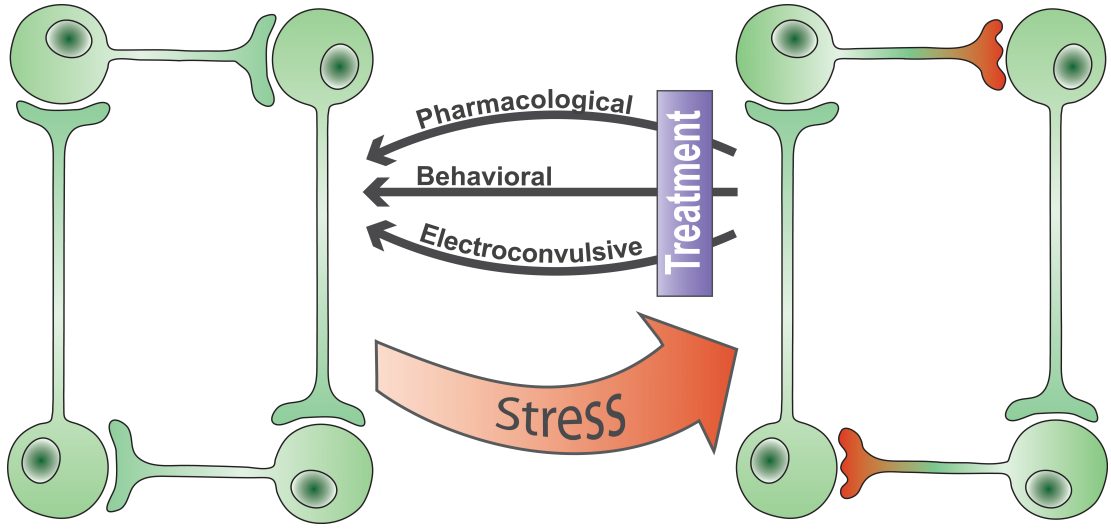

Figure 1. The neuroplasticity hypothesis for depression. In healthy humans or animals (left), stress can disrupt neuroplasticity in specific neuronal networks, i.e. the hippocampus or PFC (right). Antidepressant drugs, electroconvulsive treatment, of behavioral therapy can all enhance neuroplasticity, thereby bringing plastic processes back to normal levels and relieving (cognitive) depressive symptoms.

Stress can also alter downstream molecular signaling at the synapse in several ways. For example, both acute and chronic stress alter the activity of mitogen-activated protein kinase (MAPK)[59] and calcium-calmodulin-dependent kinase II (CaMKII)[60], two proteins that become activated by sufficient synaptic activity and therefore are involved in both early and late phase LTP. In addition, stress leads to reductions in hippocampal brain-derived neurotrophic factor (BDNF) mRNA levels, suggesting an impairment of some of the mechanisms of neuroplasticity. BDNF is known to be induced in activity-dependent LTP and it has a critical role in stabilizing synaptic change[61].

2.2 Antidepressants and neuroplasticity

Memory, plasticity and cell survival

As stress reduces neuroplasticity, and MDD is associated with a depressed synaptic state, it is likely that antidepressant treatment has the opposite effect. Indeed, accumulating evidence shows that antidepressants influence plasticity in a contrasting, but not exactly opposite way. In this respect, studies in healthy individuals / non-stressed animals are sparse and sometimes conflicting, but antidepressants consistently restore decreased plasticity.

Some lines of research show that, in healthy humans, antidepressant treatment increases memory and acts on other cognitive domains[62,63], although evidence is sparse.

Also in naïve animals, there is not a lot known of the effects of antidepressants. Some, although not all, antidepressants increase performance in the Morris water maze, a spatial learning and memory model[64].

At the synaptic level, antidepressant treatment predominantly increases plasticity. In naïve animals, several studies show increased LTP in dentate gyrus and CA1 synapses, although results have not been consistent and were dependent on the type of antidepressant[65]. More importantly, antidepressant treatment has consistently been found to rescue stress-induced reductions in LTP and increases in LTD[66,67]. Furthermore, chronic antidepressant treatment blocks the stress-induced changes in dendritic morphology and neurogenesis[68], and some classes of antidepressants also increase neurogenesis in naïve animals[68].

Molecular changes induced by antidepressants

Several lines of evidence suggest that antidepressants can directly modulate glutamate neurotransmission. For example, both riluzole and lamotrigine, which have antidepressant properties, increase the surface expression of alpha-amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid receptor (AMPAR) subunits GluR1 and GluR2, and riluzole reversibly attenuates AMPAR-mediated synaptic currents in cultured cells[69]. More typical antidepressants, such as fluoxetine, can regulate the phosphorylation state and thereby the function of AMPARs[70] in frontal brain areas. These effects of antidepressants, targeting other molecules and pathways, have led to the proposal that direct modulators of AMPAR function, such as AMPAkines, can act as antidepressants.

Furthermore, in rodents, chronic antidepressant administration increases cAMP levels[71], and activates cAMP-dependent protein kinase (PKA)[72] and CREB[73]. Since this cAMP-PKA-CREB pathway is important in the maintenance of LTP, it is hypothesized that activation of this pathway accounts for the antidepressant induced increase in LTP. However, this suggested direct link needs validation in future studies. Another line of evidence supporting the hypothesis that antidepressant treatment leads to altered neuroplasticity, is the regulation of neurotrophic factors by antidepressants. Chronic antidepressant administration increases the expression of BDNF in the hippocampus and PFC[74]. Roles for BDNF in depression and its antidepressant action are supported by studies showing that levels of BDNF were decreased in the brains of MDD patients[75] and were increased in patients receiving antidepressant treatment at the time of death[76]. Also, BDNF infusions produced an antidepressant response in animal models[77].

Non-pharmacological treatment

Apart from pharmacological treatment, also ECT and behavioral therapy have profound effects on neuroplasticity. Both these forms of treatment have been found to increase plasticity in frontal brain areas, i.e., reverse the stress-induced decrease in neuroplasticity in animal models of depression. Both treatments increase hippocampal LTP[78] and prevent stress-induced alterations in cellular morphology and neurogenesis[20].

2.3 Neuroplastcity and the relevance for depression

The effects of stress and antidepressants suggest that decreased neuroplasticity is a core pathophysiological feature of MDD. However, these data are best applicable to the hippocampus and PFC, whereas in other brain areas, such as the amygdala and the nucleus accumbens, increased plasticity after stress is related to depressive-like phenotypes in animal models[34,51]. For example, expression of CREB in the nucleus accumbens increased behavioral despair and helplessness in the forced swim and learned helplessness paradigms, and CREB inhibition has an antidepressant effect[79]. Therefore it is likely that decreased plasticity contributes to the installment of a depressive state only in certain brain areas.

Most of the behaviors used to assay antidepressant effects in rodents, e.g., learned helplessness, the forced swim test, and the tail suspension test (see below and Box 2), are models of behavioral despair and coping. Animals are placed in an adverse environment from which it is difficult or impossible to escape, and, after a period of struggle, they enter a behavioral state of passivity. It might be that enhancement of neuroplasticity, and the concomitant increased capacity to adapt and learn, lead to an enhanced potential repertoire of behaviors or capacities to explore new escape options in adverse circumstances, and thus reduces the tendency to enter a state of behavioral despair. This interpretation predicts that enhanced neuroplasticity is indeed of causal importance for reduced depression-like behaviors after antidepressant treatment. Additionally, considering the undisputed relation between neuroplasticity and learning and memory, it is tempting to speculate that cognitive impairments seen in depressed patients are associated to these neuroplastic changes. Future studies using animal models of depression should provide evidence for a causal relationship between depression-related cognitive impairments and reduced neuroplasticity, and in particular, what molecular changes might account for these impairments.

Box 2. Animal models used in depression research

Model	Main features of symptoms and antidepressant effects
Forced swim test	Lack of struggling when placed in a chamber of water, thought to represent a state of despair. Antidepressants acutely increase the time an animal struggles.
Tail suspension test	Lack of struggling when suspended by its tail, thought to represent a state of despair. Antidepressants acutely increase the time an animal struggles.
Learned helplessness	Animals exposed to inescapable foot shock take a longer time to escape, or fail to escape entirely, when subsequently exposed to escapable foot shock. Antidepressants acutely decrease escape latency and failures.
Chronic mild stress	Animals exposed repeatedly to several unpredictable stresses (cold, disruption of light-dark cycle, foot shock, restraint, etc.) show reduced sucrose preference and sexual behavior. Chronic antidepressant treatment reverses these symptoms.
Social stress	Animals exposed to various types of social stress (proximity to dominant males, odors of natural predators, defeat by a dominant conspecific) show behavioral abnormalities. Chronic antidepressant treatment reverses these symptoms.
Early life stress	Animals separated from their mothers at a young age show some persisting behavioral and HPA axis abnormalities as adults, some of which can be reversed by antidepressant treatments.
Olfacotry bulbectomy	Chemical or surgical lesions of the olfactory bulb cause behavioral abnormalities, some of which can be reversed by antidepressant treatments.
Anxiety-based tests	The degree to which animals explore a particular environment (open space, brightly lit area, elevated area) is increased by anxiolytic drugs (e.g., benzodiazepines).
Reward-based tests	Animals show highly reproducible responses to drugs of abuse (or to natural rewards such as food or sex) in classical conditioning and operant conditioning assays.
Cognition-based tests	The ability of animals to attend, learn, and recall is measured in a variety of circumstances. This possibly models cognitive impairments.

3. Rodent models of depression

Animal models of depression are evaluated for their validity based on four criteria: (1) similarity in the symptom profile presented, such as decreased interest in pleasure (face validity), (2) amelioration or attenuation by treatments effective in treating the human condition i.e. antidepressants and behavioral therapy (predictive validity), (3) provocation by events thought to be important in eliciting the human disorder, such as stressful life events (etiological validity), and (4) involvement of similar neurochemical processes, like decrease hippocampal BDNF expression (construct validity). This is a challenge. Many of the core symptoms of depression (e.g., depressed mood, feelings of worthlessness and suicide) cannot be easily measured in laboratory animals. As a result, most available animal models of depression rely on one of two principles: 1) effects of known antidepressants and 2) responses to stress, thereby modeling depressive symptoms (Box 2).

3.1 The forced swim test

Some of these tests, in particular the forced swim test, have been very effective at predicting the antidepressant efficacy of pharmacons (predictive validity)[80]. In this test, rodents immersed in a vessel of water develop an immobile posture after initial struggling. This immobility is considered as behavioral despair. Most antidepressants, administered acutely before the test, reverse immobility and promote struggling[80]. Obviously, models that use an acute stressor (for example, forced swimming) are better thought of as read-out of coping style, and cannot recapitulate a long-lived multidimensional syndrome such as depression. Therefore, this test should be considered as a fast and cost-efficient screening paradigm for potential antidepressants, rather than an animal model reflecting multiple aspects of the disorder. In this thesis this test was used to screen for the possible long-lasting antidepressant effect of the novel antidepressant ketamine.

3.2 The social defeat paradigm

Animal models that try to incorporate the multidimensional aspects of depression in order to study its neurobiological underpinnings are limited to stress models such as social defeat or chronic mild stress. These are more technically challenging paradigms, however, they show unique sensitivity to chronic and not acute antidepressant administration, which is comparable to the therapeutic delay of 3–6 weeks that is required for all available antidepressant drugs to adequately treat depression in humans[26]. In the social defeat model the experimental male rodent is placed in the territory of a large dominant male, after which a fight takes place and the intruder gets (socially) defeated. Apart from having etiological validity, because of the social stressor used as trigger, this model also has face validity, in which certain behavioral changes brought about by stress superficially resemble depressive symptoms. For instance, the defeated animal shows decreased sucrose intake or a reduced anticipation towards sucrose after chronic stress, which is thought to model anhedonia[33,81]. However, most studies of these models mimic the effects of acute stress, i.e. effects are studied acutely after the last stress-encounter, whereas we know from human studies that depressive symptoms are still apparent long after cessation of the stressor[82]. Moreover, these paradigms do not model the effects of more passive forms of stress, such as social isolation[83]. This is unfortunate since the lack of social support is known to be important especially for maintenance of depressive symptoms[33]. Therefore, to be able to study the long-term consequences of social defeat stress on depressive-like behavior, I adopted a social defeat paradigm[33] in which experimental rats get socially defeated after which they are kept in social isolation for an additional 10-12 weeks. This paradigm might match human

depression in which active stress is often involved in the onset of depression, while passive stress, e.g., in the form of impaired social interaction, has strong precipitating effects on the development of the disease[84].

4. Aim and outline of the thesis

MDD is a devastating brain disease that negatively impacts on the lives of many people worldwide. The aim of this thesis is to identify novel molecular targets that can be used to address the cognitive dysfunction that comes with MDD. For this I made use of two animal models, 1) temporal effects of ketamine in the mouse forced swim test, 2) social defeat (SD) stress in rats, which has best validity in terms of modeling aspects of MDD. In particular, I investigated the molecular and cellular neurogenic and plasticity changes that might affect hippocampal function, short and long-term after SD stress. I investigated whether typical antidepressants have a positive effect on the changes that are brought about by SD stress. Also I investigated the effect of behavioral therapy in this model. In this thesis there are several specific questions concerning stress-induced hippocampal plasticity that are addressed in the four experimental chapters.

First of all the antidepressant action of ketamine was investigated. This NMDA receptor antagonist produces rapid (1 hour), robust and sustained (up to two weeks) antidepressant actions in treatment-resistant MDD patients[31] and in preclinical models of depression following a single systemic infusion[32]. The rapid antidepressant effect of ketamine is of great interest since all other available antidepressants take weeks to exert their antidepressant effects. However, the mechanism through which ketamine exerts its effects is largely unknown. In **chapter 2** I investigated the synaptic mechanism through which ketamine produces its antidepressant effect.

Then I went on to get better insight into the neurobiology of MDD by using an animal model with profound validity for the human disorder. First, I investigated cognitive functioning and molecular changes at the level of the synapse that might occur directly after severe SD stress exposure when compared to the long-term effect of SD. These molecular alterations are involved at the onset of neuroplastic changes and the installment of a depressive-like phenotype, and are addressed in **chapter 3** of this thesis.

A second issue concerns the longitudinal effects of stress on neuroplasticity. As we know from human studies (see[82] for review), depressive symptoms are often still apparent long after the end of a stressful life event and MDD is considered a chronic disease.

Therefore, it is of interest to determine whether the long-term effects of SD stress on depressive-like (cognitive) symptoms are related to altered hippocampal plasticity. In **chapter 4** I questioned whether the depressive symptoms that are seen long after the cessation of social defeat stress exposure and are maintained by social isolation, are associated with reduced hippocampal memory performance. Ongoing, I investigated which type of cellular and molecular plasticity occurred at hippocampal synapses that could be involved in hippocampus-dependent memory impairment long after SD stress, and whether a form of behavioral therapy or chronic antidepressant treatment could restore these changes. In addition, I investigated whether long-term effects of SD stress were also associated with changes in the neurogenic process in the hippocampal dentate gyrus, which is discussed in **chapter 5.** Here, I also investigated the antidepressant action on SD-affected neurogenesis.

Finally, **chapter 6** of this thesis brings together my data, and here the most important conclusions are discussed in the context of MDD.

Chapter 2

Hippocampal AMPARs mediate the enduring antidepressant effects of a single treatment with ketamine

Biological psychiatry, under review

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Abstract

The NMDA receptor antagonist ketamine produces rapid, robust and prolonged antidepressant actions in treatment-resistant patients and preclinical models of depression following a single systemic infusion. Elucidating the neural sites and mechanisms underlying its effects are crucial for further development of novel treatments. Here, we examined the contribution of synaptic hippocampal glutamate receptors in depressive-like behavior as measured using an optimized set-up and automated protocol for the forced swim test (FST) in mice, a test of behavioral despair. Increased behavioral despair correlated with higher synaptic membrane expression of hippocampal NMDA receptor (NMDAR) subunit GluN2A. Subsequently, we analyzed the short- and long-term behavioral effects, and long-term molecular effects of a single dorso-hippocampal infusion of ketamine. Ketamine caused both rapid and enduring antidepressant effects without impairing memory retention. These long-term antidepressant effects were paralleled by increased turnover of AMPA receptors (AMPARs) as shown by increased Ser-845 phosphorylation of GluA1. The relative contribution of hippocampal AMPAR over NMDAR appeared to be crucial for the antidepressant-like effect, as molecular blockade of regulated AMPAR endocytosis by a TAT-$Glu2_{3Y}$ peptide in the CA1 region of the dorsal hippocampus partially mimicked the effects of ketamine. The well-known long-term antidepressant effect of a single treatment with ketamine is potentially mediated by increased hippocampal AMPAR function.

Introduction

Major depressive disorder (MDD), also referred to as major depression, is a common, chronic, recurrent mental illness that affects millions of individuals worldwide. Since current available antidepressants target the monoaminergic systems (serotonin, norepinephrine, and dopamine), these transmitter systems have received most attention in depression-related research. However, these antidepressants take weeks to achieve their therapeutic effect and leave patients particularly vulnerable to the devastating symptoms and the risk of self-harm. Therefore, treatment strategies that exert a rapid and prolonged antidepressant effect within days or even hours could substantially benefit public health. Previously, clinical trials have shown the immediate improvement of depressive symptoms in treatment-resistant patients by the use of a sub-anesthetic single dose of ketamine[31,85]. This antidepressant effect is characterized by a very rapid onset of action (within hours) and a robust and prolonged effect, lasting one to two weeks. Recently, the rapid activation of the mTOR pathway, leading to increased synaptic signaling proteins and increased number and function of new spines, was shown as mechanism for the acute antidepressant effects of ketamine[86]. As yet, molecular insight into the more lasting effects of ketamine, as well as the main anatomical site of action in the brain is not available.

Ketamine is a non-competitive *N*-methyl-D-aspartic acid (NMDA) receptor antagonist[87] and has been used clinically as a dissociative anesthetic in both pediatric and adult patients[88]. Its primary mechanism of action is blocking the NMDA receptor (NMDAR) at the phencyclidine site in the ion channel. In addition, ketamine induces rapid increases in presynaptic release of glutamate, a process hypothesized to be mediated by NMDA autoreceptors, and/or by activated GABAergic neurons[89]. Apart from ketamine, other NMDAR antagonists, such as MK-801 and CGP 37849, have antidepressant-like effects in several preclinical paradigms[90,91]. A better understanding of the antidepressant effect of ketamine might aid future development of rapidly acting and effective pharmacological therapies.

Apart from the antidepressant effect of ketamine, other clinical and preclinical lines of research points to the involvement of the glutamatergic system in the pathophysiology of depression. Several reports showed increased glutamate levels in blood and cerebrospinal fluid in patients with MDD[92]. In rodent models of depression, synaptic plasticity was affected in glutamatergic neurons in both the CA1 and the dentate gyrus of the hippocampus[93]. Growing evidence supports the idea that antidepressants, via a cascade of time-dependent signaling, ultimately converge to regulate α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and NMDA receptor-mediated synaptic plasticity (reviewed in[94]). Consequently,

several studies showed that positive allosteric modulators of AMPA receptors (AMPARs), referred to as AMPA potentiators, display antidepressant effects in both clinical[31] and preclinical[95] studies.

Recently, a large body of evidence from preclinical studies indicates that the acute effect of ketamine is mediated through a functional interplay between AMPARs and NMDARs[32,96]. The increased presynaptic release of glutamate preferentially acts on AMPARs, due to the ketamine inhibition of NMDARs. This direct effect of ketamine on glutamatergic signaling explains the rapid onset of action when compared to other antidepressants. Given these acute effects, the prolonged antidepressant action of up to at least a week[31,86,97] remains unexplained.

Here, we investigated how ketamine prolongs its antidepressant effects much beyond its short half-life (~13 min in serum of mice;[98]) and duration of NMDAR blockade. Secondly, we investigated through which neuronal circuits ketamine exerts its antidepressant effects. We show, by using an automated forced swim test (FST) analysis of depressive-like behavior in mice, that the long-term effects of ketamine are likely to be regulated by persistently increased AMPAR function in the dorsal part of the CA1 region in the hippocampus.

Methods & Materials

Animals

All experiments were carried out in accordance to the Animal User Care Committee (VU University). Adult male (>8 weeks) C57BL/6J and DBA/2J mice (20-25 g, Charles River) were singly housed on a 12/12 h light/dark cycle with ad libitum access to food and water, light on at 7 AM. All mice were habituated to the facility for at least 1 week prior to testing, and were allowed to recover for at least 1 week after surgery.

Pharmacalogical Interventions

Acute systemic injection – Imipramine (Sigma Aldrich, Germany; 8 or 16 mg/kg) was injected intraperitoneally 30 min before testing.

Chronic treatment – An osmotic minipump (Model 1004; Alzet, Cupertino, CA) containing 60 mg/ml imipramine was placed subcutaneously during isoflurane anesthesia, resulting in 6-8 mg/kg/day (for mice weighing 26-20 gram, respectively) administration of imipramine (28 days).

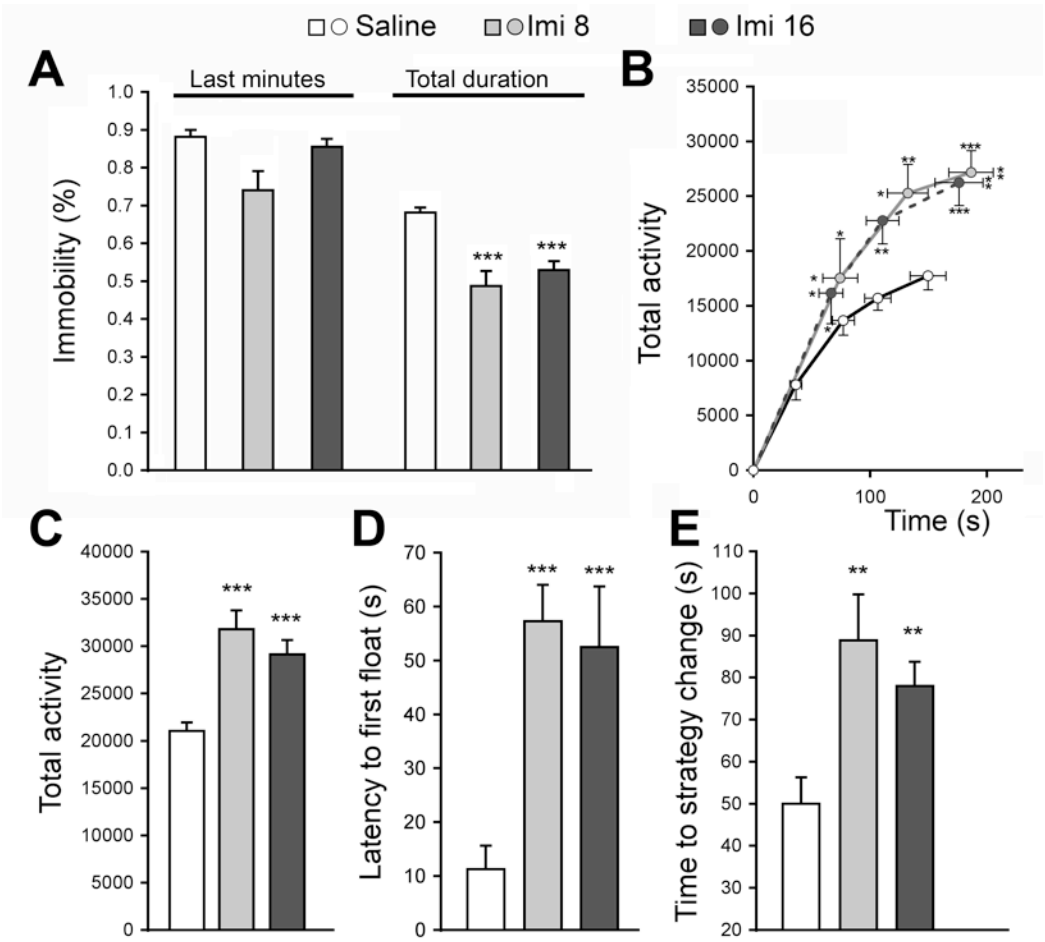

Figure 1. Automated analysis of FST performance detects low-dose imipramine effects. Imipramine (Imi; 30 min prior to testing, i.p., 8 and 16 mg/kg) had no significant effect on immobility, as often measured during the last 4 min of the FST (A, left). However, measuring immobility during the total 6-min test time (A, right), as well as novel parameters resulting from our automated analysis like strategy (i.e. activity over time; B), total activity C), latency to first float (D) and time to change swim strategy (E) showed robust effects due to imipramine. Data derived from digital images are explained in detail in Fig. S1. For detailed ANOVA results, see Table S1. *** P<0.001, ** P<0.01, * P<0.05 vs. saline.

Hippocampal injection – Mice were implanted with double guide cannulae (Plastics One, Roanoke, VA) targeting the CA1 region of the dorsal hippocampus as described previously[99,100], using mouse brain atlas coordinates[101]. Ketamine hydrochloride (Alfasan, Woerden, The Netherlands) at a dose of 0.25 µg (0.125 µg/side) and 2.5 µg (1.25 µg/side) was infused in a volume of 0.25 µl/hemisphere during 2 min. To block regulated endocytosis of AMPARs in the CA1 region of the dorsal hippocampus, a synthetic peptide derived from the GluA2 carboxyl terminal fused to the cell membrane transduction domain of the HIV-TAT protein ($GluA2_{3Y}$: $_{869}$YKEGYNVYG$_{877}$[102]) or control peptide ($GluA2_{3A}$: AKEGANVAG) were injected at (15 pmol/side; 60 µM) similar to ketamine delivery. Injection sites were verified (0.25 µl methylene blue), and mice that did not receive symmetrical and bilateral injections in

the CA1 region of the dorsal hippocampus were excluded. Days of testing after insertion of the pump or hippocampal injection are indicated (Tables S1 and S3).

Tissue Preparation and Immunoblotting.

The hippocampus (strain differences) or its dorsomedial part (ketamine effect) were dissected from fresh brains and immediately frozen (–80 °C). Synaptic membranes were isolated (pool of 2 or 3 mice per sample) on a discontinuous sucrose gradient, as described previously[103]. SDS-PAGE (8%) was run using 5 µg/sample (Laemli lysis buffer). Proteins were blotted onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA). After blocking and incubation of the first antibody, against GluA1 (1:1,000; Genscript, Piscataway, NJ), GluA1-Ser845 (1:1,000; Phosphosolutions, Aurora, CO) GluA2 (1:1,000; Neuromab, Davis, CA), GluN1 (1:1,000; Millipore, Billerica, MA), GluN2A (1:1,500; Abcam, Cambridge, UK) and GluN2B (1:1,000; Neuromab, Davis, CA) the blot was washed and incubated for 1 h at RT with AP-conjugated secondary antibody (1:10,000; GE Healthcare, Diegem, Belgium) allowing detection using the ECF immunoblotting detection system (GE Healthcare, Diegem, Belgium). The lower half of the same gel was cut and stained for coomassie, as shown previously to be a good control for general input[103]. Quantification of immunoblots was performed using Quantity One® 1-D analysis software (Bio-Rad, Veenendaal, The Netherlands).

Forced Swim Test

Mice were placed in a rectangular transparent Perspex tank (22 x 14 x 35.5 cm, length x width x height), filled up to a height of 30 cm with water (25 °C)(Fig. S1). Traditionally, a round tank is used in many FST setups. However, round tanks distort the image of the mouse in the tank particularly on the sides thereby confounding the precision of quantitative motion-detection analysis. A single swim session of 6 min was conducted, as the swim strategy is modified in a second swim session (Fig. S1D). After the session, mice were placed on a clean dry tissue under a warm light bulb (max. 5 min) in their home cage. A high-resolution digital camera (Sunkwang B140XP/SO; RF Concepts, Dundonald, UK), in combination with Virtualdub software (v1.9; www.virtualdub.org) was used to record the swim session (AVI files) at a rate of 25 frames per sec.

The digital movie was used offline for automatic analysis, using custom-developed software for motion detection (R.F. Jansen and O. Stiedl; see http://www.falw.vu/~ngc/FST.html) (Fig. S1A). This program yields the activity diagram (Fig. S1B). As the cumulative plot of activity, i.e., the strategy plot, yields non-stationary data due to changes in swim strategy of the

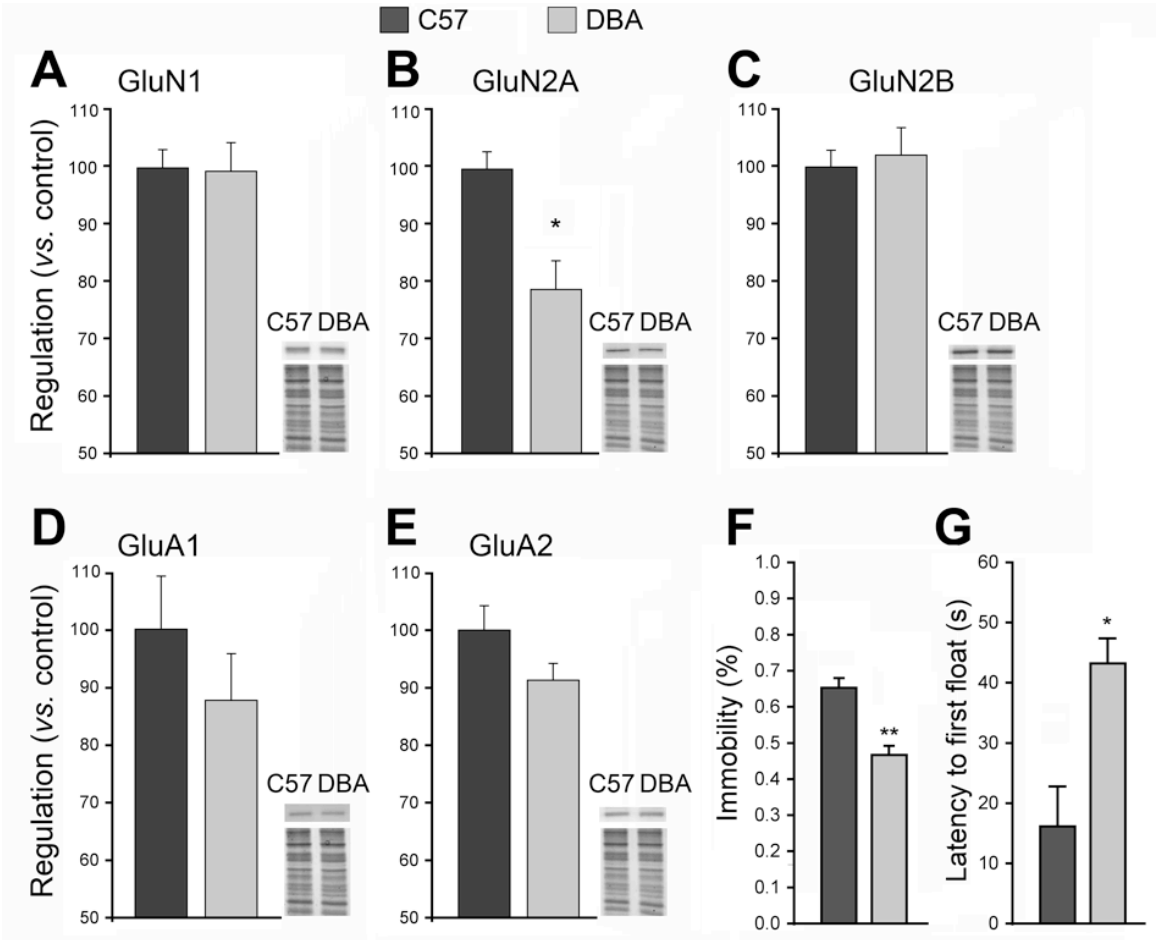

Figure 2. Mouse strain differences in expression of synaptic hippocampal glutamate receptors and behavioral despair. Hippocampal synaptic membrane fractions from C57BL/6J (C57) and DBA/2J (DBA) mice (n=4 samples) were used for immunoblotting with glutamate receptor subunit antibodies; GluN1 (A), GluN2A (B), GluN2B (C), GluA1 (D), GluA2 (E). Bar graphs (left) show quantification of samples. (Right) A typical example of the immuno-detected protein is shown for each strain, together with the coomassie-stained lower part of the gel. * P=0.013. (F,G) A strain-specific effect (C57BL/6J mice (n=11), DBA/2J mice (n=10)) was apparent for immobility (F) and latency to first float (G), as well as other parameters (see Fig. S4), ** P<0.01 and * P<0.05 vs. C57BL/6J.

animal over time, cumulative movement data were fitted using a Douglas-Peucker polyline algorithm[104] with an 8-pixel tolerance (Fig. S1C). The program for automatic analysis, using a custom-developed motion detection algorithm, is made available upon contact (R.F. Jansen and O. Stiedl, oliver.stiedl@cncr.vu.nl).

Immobility – Time (%) spent inactive with very low activity (change between frames < X pixels) below the threshold set per time bin (0.6 s per bin).

Latency to immobility – Time (s) before the first bout of inactivity lasting > 1 s.

Strategy plot – The coordinates of the average cumulative activity (y) by time (x) of each fitted line segment was calculated and plotted. This parameter gives detailed information about the average swim strategy over time. Because the swim strategies were mostly

captured by at least 3 line segments for every mouse, only those segments were used for analysis.

Total activity – The sum of the activity during the 6-min swim session calculated by the motion-detection analysis.

Time to change strategy – Total time before activity was less than before. The latency to reach the fitted line segment of which the slope was lower than before, i.e. when the second derivative was at a minimum, was taken.

Statistical Analyses

Behavioral data was subjected to univariate ANOVA/Kruskal-Wallis with dose or strain as between-subject variables. For statistically significant main effects, post-hoc comparisons were conducted using Student-Newman-Keuls/Mann-Whitney-U tests. Immunoblot and behavioral results using single drug injections were analyzed using student's t-test with either strain or treatment as factors. The level of probability for statistically significant effects was set at $P<0.05$. All data are displayed as mean values ± SEM (except for EPM parameters that are displayed as box plots with mean ± maximum values).

Results

Novel FST parameters for detection of antidepressant action

Predictive validity of the behavioral despair test is important in preclinical settings to assess effectiveness of novel pharmacological agents with antidepressant effects. Here, we first investigated whether the two most commonly used parameters, namely immobility[105], and latency to first float[106], are optimal to segregate between different classes of antidepressants used at clinically relevant doses.

Traditionally, the last 4 min of a 6-min FST are used to measure immobility[80,105]. Relative low doses of imipramine (8 and 16 mg/kg) that were previously reported to be ineffective in C57BL/6J mice[106,107] did indeed only reveal a trend ($P=0.064$) for immobility when the last 4 min were used for analysis (Fig. 1A). However, based on total test-time, a highly significant effect on immobility was obtained ($P=0.001$, Fig. 1A, Table S1). In addition, our optimized FST set-up and its analysis procedure increased sensitivity of detection as these relative low doses had a significant effect on latency to first float ($P<0.001$), that went previously undetected[106].

Movement detection of digital images allows more relevant information to be extracted, such as total amount of activity displayed, as well as changes in patterns of activity on a high-resolution time scale (Fig. 1B, Fig. S1). Mice have a tendency to start swimming

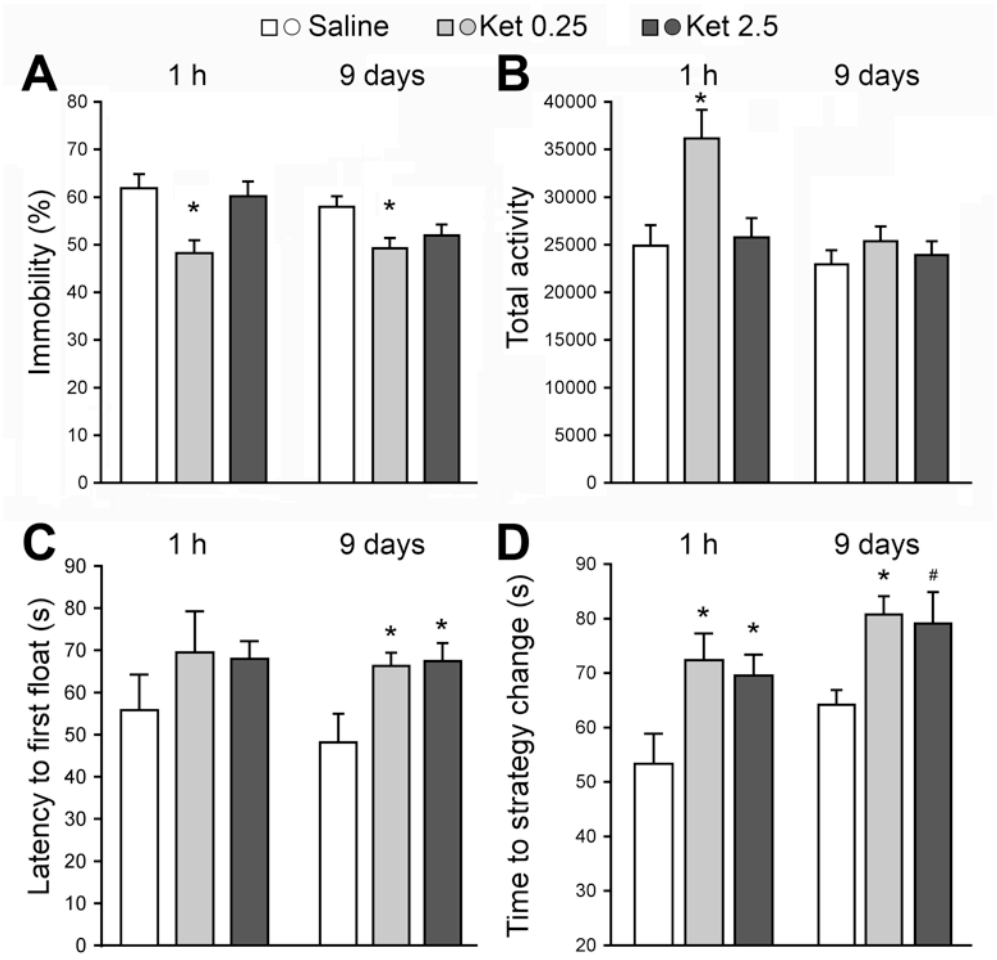

Figure 3. Acute and long-term effect of hippocampal ketamine on behavioral despair in C57BL/6J mice. Bilateral dorso-hippocampal injection of saline (n=6) or ketamine (0.25 µg, n=6-8; 2.5 µg, n=8), were given 1 h or 9 days before the test. Ketamine treatment caused differences from saline controls with respect to immobility (A), strategy (see Fig. S5), and time to change swim strategy (D). Total activity (B) and latency to first float (C) were specifically affected on the short-term and the long-term, respectively. For detailed ANOVA results see Table S1. ** P<0.01, * P<0.05, # P<0.2 vs. saline.

actively and vigorously for a certain period before they change strategy resulting in a combination of swimming and floating (Fig. S1). Here we show for the first time that specifically total activity, strategy and hence latency to change swim strategy are very sensitive to antidepressant treatment (Fig. 1B,C,E; Table S1). In addition, chronic imipramine treatment at a relative low dose (6-8 mg/kg/day, s.c.) for 3 weeks revealed a significant effect (P<0.05) on these novel parameters (Fig. S2B,C,E; Tables S1, S2), and no significant persistent effect was measured using the classical parameter of immobility (Fig. S2A). Notably, at this low dose, the previously reported effects of imipramine on locomotion are not detectable (Fig. S3; Table S3). Thus, we provide evidence that our improved protocol and automated analysis is very sensitive in measuring antidepressant-like effects, and could thereby improve the predictive validity of the FST.

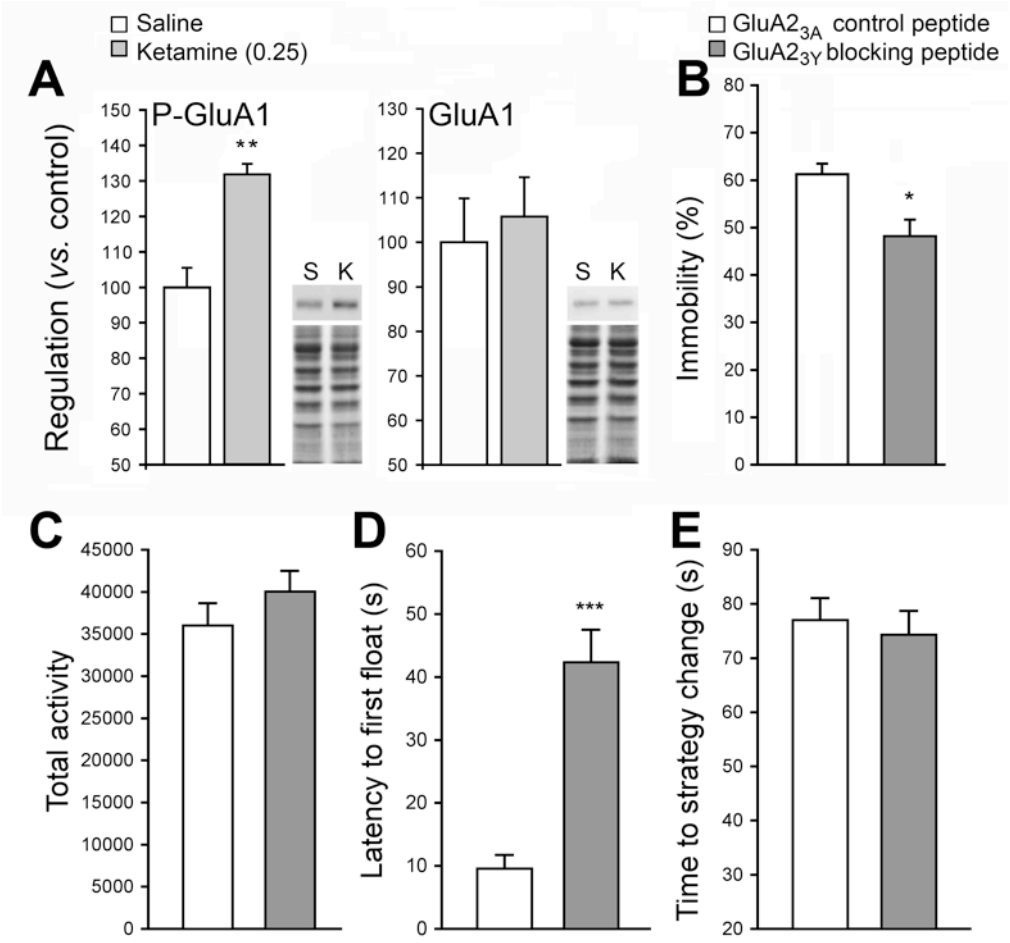

Figure 4. Synaptic hippocampal glutamate receptors are involved in the long-lasting antidepressant effect of ketamine. (A) Dorso-medial hippocampal synaptosome fractions of mice with dorso-hippocampal injections of saline or ketamine (0.25 µg, n=6) were used for immunoblotting with glutamate receptor subunit antibodies; P-GluA1 and total GluA1 (See Fig. S8). Typical examples of the immuno-detected protein levels are shown for each treatment (saline, S and ketamine, K) mice, together with the coomassie-stained lower part of the gel to the right of each bar graph. ** P=0.007. (B-E) Bilateral dorso-hippocampal injections of the GluA2$_{3Y}$ blocking peptide (60 µM, n=13) or GluA2$_{3A}$ control peptide (n=12) were given 1 h before the test. Treatment with this blocking peptide showed differences from control with respect to immobility (B), as well as novel parameters resulting from our automated analysis like latency to first float (D), but not total activity (C), and time to change swim strategy (E). *** P<0.001, * P<0.05 vs. control peptide.

Strain differences in behavioral despair and hippocampal NMDA receptor subunit expression

Depressive-like behavior in the FST is likely influenced by glutamatergic transmission[90]. Therefore we tested whether two inbred strains of mice (C57BL/6J and DBA/2J) that show well-known differences in behavioral despair[108,109] also show differences in synaptic hippocampal glutamate receptor expression. We performed immunoblotting for NMDAR and AMPAR subunits on hippocampal synaptic membranes (Fig. 2). A significantly reduced expression level of GluN2A was found in DBA/2J mice (79.3%±0.3, P=0.013), with no change in levels of AMPAR or other NMDAR subunits (Table S4).

We replicated the previously reported increased immobility[108] in C57BL/6J compared with DBA/2J mice (P<0.005; Fig. 2F,G; Table S2). In addition, C57BL/6J mice showed lower values for strategy (P<0.05), decreased total activity (P=0.007) and latency to the first float (P=0.021)(Fig. S4B-D). Thus, increased GluN2A levels correlated with a higher level of behavioral despair.

Rescue of depressive-like phenotype by inhibiting hippocampal NMDA receptors

According to current views[32,96], changing the balance between AMPAR and NMDAR involvement might contribute to antidepressant efficacy. Accordingly, AMPAR potentiators[110] and NMDAR antagonist have antidepressant effects[91,111,112]. Since C57BL/6J mice show increased behavioral despair and increased hippocampal synaptic NMDAR subunit expression, we tested the hypothesis that inhibiting hippocampal NMDARs in mice with endogenously elevated NMDAR subunits levels has antidepressant-like effects.

First, we showed that dorso-hippocampal injection of ketamine, a non-specific NMDAR antagonist, indeed had a clear acute antidepressant-like effect (immobility_last minutes: $F_{(2,17)}$=4.041, P=0.037; immobility_total time: $F_{(2,17)}$=5.542, P=0.014) (Fig. 3A, Table S1). In addition, the novel parameters of strategy (P<0.05; Fig. S5), total activity ($F_{(2,17)}$=5.897, P=0.011), and time to change strategy ($F_{(2,17)}$=4.957, P=0.020) showed that hippocampal ketamine had a significant antidepressant effect, with the highest effect at 0.25 µg (Fig. 3).

Second, when behavioral despair was measured 9 days after local injection of ketamine (Fig. 3), an antidepressant-like effect was still observed for immobility, but only using total test-time ($F_{(2,21)}$=3.523, P=0.048; Table S1). In addition, parameters time to change swim strategy ($F_{(2,21)}$=3.977, P=0.034; Table S1) and latency to first float ($F_{(2,21)}$=4.633, P=0.022) showed prolonged effects from ketamine that were more pronounced than the acute effects. In all parameters, the 0.25 µg dose was more effective than the 2.5 µg dose.

In an independent group of mice we observed that a hippocampal ketamine injection (0.25 µg) reduced anxiety in the novel exploration test (P=0.025) and elevated plus maze (P=0.044; Fig. S6, Table S3), with no effect on locomotion and anxiety in the open field test[113], nor in contextual fear learning (Fig. S7).

Antidepressant effects by increased AMPA functionality

As the long-term behavioral effect cannot be attributed to the antagonistic properties of ketamine on NMDARs, but rather reflects a down-stream molecular consequence of ketamine action reaching further than the recently described activation of the mTOR pathway[86], we determined the effect of ketamine on synaptic hippocampal glutamate

receptor expression nine days after injection in a separate group of mice not used in the FST experiment. A single ketamine injection increased the levels of P-Ser-845-GluA1 in the hippocampus (P=0.007; Fig. 4A), with no difference in expression of GluA and GluN subunits (Fig. S8, Table S4). Phosphorylation at this site increases the number of membrane-localized AMPARs[96,114]. Because we did not observe an increase in total level of GluA1, the long-term effect of ketamine might be attributable to stimulated receptor turnover in hippocampal synapses. This increased cycling could serve to increase functional non-desensitized AMPARs in the synaptic membrane.

If increased AMPAR functionality would play a role in the lasting antidepressant effect of ketamine, then increasing the total number of AMPARs might mimic the antidepressant effects. Therefore, we blocked the regulated endocytosis of AMPARs in order to create a phenocopy of the lasting antidepressant-like effect. For this, we used a molecular tool to impair retrieval of AMPARs from hippocampal synaptic membranes. We injected the GluA2 regulated endocytosis blocking peptide[102,103] into the dorsal hippocampus and tested the effect on behavioral despair. This peptide had no acute effects on basal locomotion (Fig. S9), and therefore appears to be a highly specific tool for behavioral intervention. Both immobility (P=0.021; total test time) and latency to the first float (P=0.0004) were decreased significantly (Fig. 4B,D). No effect was observed on total activity and time to change strategy (Fig. 4C,E; Table S5), thus creating a partial phenocopy of the enduring effects of ketamine.

Discussion

Here, we first implemented a new approach using automated FST analysis to objectively quantify subtle effects of antidepressant-like actions on both classical and novel FST parameters with high sensitivity. Our data indicate a role for hippocampal glutamate receptors as targets for the antidepressant action of ketamine both acutely and on the long-term. Finally, we provide evidence that prolonged effects of ketamine are mediated through an increased functionality of AMPARs that could result in neuronal and behavioral plasticity.

Novel forced swim test parameters:

The improved protocol and the automated analysis for the FST employed have the advantage that it combines the measurement of classical FST parameters, such as immobility, and novel FST parameters related to the intensity of activity and swim strategy. With low-dose imipramine using both an acute and chronic treatment, we showed that this

protocol has an improved sensitivity to detect changes in immobility and latency to first float, resulting in antidepressant-like effects that remained unnoticed previously[106,107].

Furthermore, by introducing the novel parameters of total activity, strategy and time to change strategy, we show that we can distinguish different types of effects that characterize both chronic and acute treatment. Specifically, the effect of chronic imipramine treatment at low doses could be measured using these parameters without having an effect on classical parameters like immobility. In addition, prolonged effects of ketamine affected strategy and time to change strategy but not activity. Acute ketamine also affected the latter parameter. Finally, automated analysis of digital images increases replicability and favors reduction of potential observer bias, and thus a more objective analysis of the data. Taken together, this protocol increases sensitivity and predictive value of the FST as a measure of antidepressant action.

NMDA/AMPA receptors

Impaired neural and structural plasticity is assumed to play a causal role in the development of mood disorders[94,115]. Furthermore, there is growing evidence from preclinical and clinical studies for altered NMDAR activity and a role for glutamatergic transmission in the pathology of depression, making glutamate receptors a promising potential therapeutic target. Clinical evidence comes from antidepressant effects of ketamine and the GluN2B subunit-selective antagonist CP-101,606, in a group of treatment-resistant patients[116]. Interestingly, NMDARs containing the GluN2B subunit are localized primarily in the forebrain, including the hippocampus, which is implicated in the pathology of mood disorders like MDD. The hippocampus is a crucial site for pathological alterations in neuronal plasticity resulting from various stressors[35,117].

Recently, the role of the GluN2A receptor subtype in the etiology of depression has been under investigation, with GluN2A knockout mice exhibiting a robust depression-resistant phenotype[118]. In line with this, we found that a higher abundance of NMDARs, specifically the GluN2A-containing receptor in the hippocampus of C57BL/6J mice, appears to be important for the depressive-like phenotype of this strain in the forced swim test.

In line with this, we show that ketamine injected into the CA1 region of the dorsal hippocampus alleviates the depressive symptoms in C57BL/6J mice not only acutely but also 9 days after infusion. The behavioral patterns differ slightly, and this is possibly related to the differences in molecular mechanisms taking place (see below, Fig. 5). Acutely, this antidepressant effect of ketamine is likely to be mediated by the well-described inhibitory effect of this drug on the ion-channel of the NMDARs, and concurrently its ability to increase

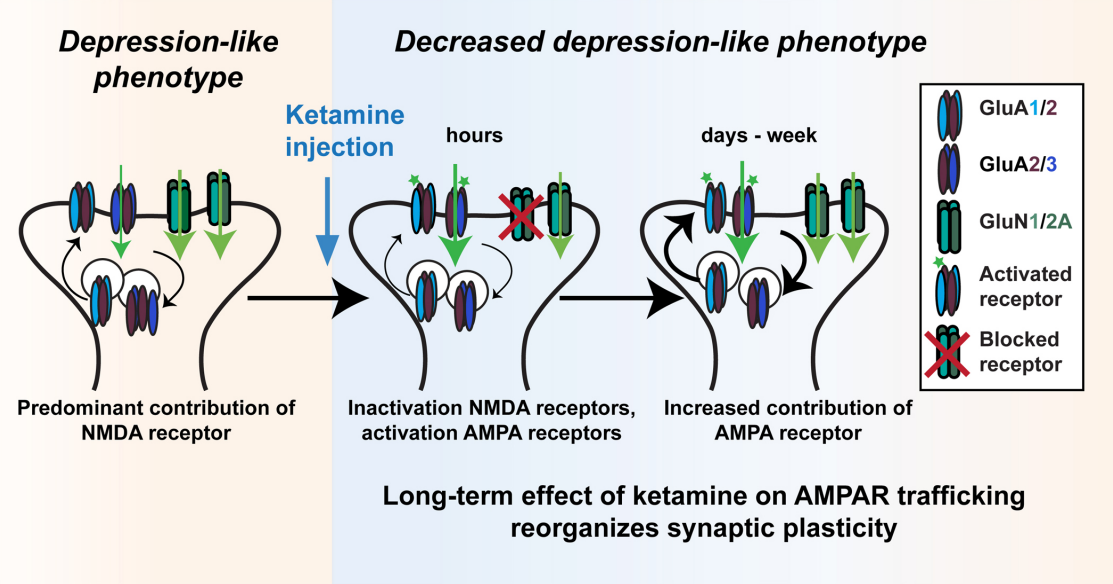

Figure 5. Model of hippocampal synaptic plasticity mechanism induced by ketamine. In individuals with a depression-like phenotype there is an imbalance in AMPARs and NMDARs, favoring the contribution of NMDARs (increased green arrow). Acutely, ketamine blocks NMDARs, and concurrently it increases pre-synaptic glutamate release thereby favoring AMPAR over NMDAR signaling. On the long-term, increased trafficking of AMPARs into the membrane might yield a higher proportion of functional AMPARs, thereby recovering the imbalance between AMPARs and NMDARs

pre-synaptic glutamate release at low non-anesthetic doses[32,89,112,119]. Both actions favor AMPAR over NMDAR transmission (Fig. 5). The acute antidepressant effect of ketamine was completely abolished when NBQX, an AMPAR antagonist, was given prior to ketamine infusion[32]. Similarly, NBQX blocked the ketamine-related induction of synaptic proteins in the PFC that are causal to the immediate antidepressant effects[86]. Classic antidepressants with a long therapeutic lag after treatment onset, like imipramine, do not share this mechanism of action as the acute antidepressant effect of imipramine was unaffected by NBQX. Increasing glutamate signaling via AMPARs relative to NMDARs is therefore the likely mechanism by which ketamine exerts its rapid effect[86].

Here, we show that the long-term effects of ketamine are also modulated by AMPAR-related mechanisms. Nine days after ketamine administration, there was an increase in levels of Ser845 phosphorylated GluA1, with no effect on total amounts of surface expressed AMPAR and NMDAR subunits. This is surprising, since acutely after administration, ketamine has been shown to decrease phosphorylation of GluA1 at Ser845[90]. However, this might be due to the fact that in this study the animals were tested directly after the forced swim test. Ser845 phosphorylation is known to prime GluA1 for membrane insertion and increases its open probability[96,114]. This finding of increased AMPA receptor functionality has important implication for the antidepressant effects of ketamine.

Depressive-like behavior induced by long-lasting stress is mediated by an impairment of AMPA-dependent hippocampal LTP[43]. The ketamine-induced increase in AMPA receptor functionality could therefore counteract these stress-induced deficits.

Even in the absence of increased levels of GluA1 on the membrane, it is likely that an increased turnover of AMPARs leads to increased functionality due to less desensitized receptors at the membrane (Fig. 5). Interestingly, increased phosphorylation of GluA1 at Ser845 has been found to underlie chronic treatment with classic and novel antidepressants, such as fluoxetine and tianeptine[70,120], and was linked to the antidepressant action of several AMPAR potentiators that improve glutamatergic transmission[121]. In addition, chronic treatment with riluzole, and lamotrigine increases the amount of P-Ser845, but also enhances AMPAR surface levels[70,122]. Thus, the molecular changes of a single ketamine infusion are shared with those observed after chronic treatment with several (classical) antidepressants.

In order to substantiate our hypothesis that increased hippocampal AMPAR functionality is key to the lasting effects of a single ketamine injection, we kept AMPARs at the synaptic membrane by blocking stimulated-endocytosis using a mimetic peptide. This intervention prevents induction of LTD[102], and likewise it mimics the enduring antidepressant-like effects of ketamine on immobility and latency to immobility (Fig. 4). Increased phosphorylation of GluA1 at Ser845 increases its availability at the active zone of glutamatergic synapses[123]. Since GluA2-regulated endocytosis is know to induce GluA1-containing heterodimeric AMPA receptor endocytosis[124], the GluA2-regulated endocytosis blocking peptide will most likely also increase GluA1 availability at the active zone of the synapse. Therefore, it will mimic the ketamine-induced effect of increasing AMPAR synaptic availability. Thus, we postulate that the described long-term effect of a single injection of ketamine on AMPARs causes a critical increase in synaptic strength by restoring the balance between AMPAR and NMDARs functionality, thereby generating a non-depressed state (Fig. 5). It is important to note that a single infusion of ketamine into the hippocampus has long lasting anxiolytic effects but has no deleterious side-effects on baseline locomotion and fear memory.

In conclusion, we have identified a molecular process in which AMPARs are involved in the long-term antidepressant effects of a single ketamine treatment. This mechanism might be common to classical antidepressants when given chronically. Importantly, we demonstrate that this effect takes place in the dorsal hippocampus. Our findings are of interest for development of antidepressants that have rapid and enduring effects, bypassing negative side effects and delayed onset of prototypical antidepressants.

Supplemental Material

Materials and Methods

Mice & intracranial delivery

Mice were housed individually in standard Macrolon cages (type II) on sawdust bedding, and for the purpose of animal welfare, the cages were enriched with cardboard nesting material and a curved PVC tube. Food (Teklad, Harlan, The Netherlands) and water was provided ad libitum. Housing rooms were controlled for temperature, humidity and light-dark cycle (7 AM lights on, 7 PM lights off). Experiments were performed during the light phase. All experimental procedures were approved by the local animal research committee and complied with the European Council Directive (86/609/EEC).

For intra-hippocampal injections, mice were chronically implanted with double guide cannulae (C235, Plastics One, Roanoke, VA) in a customized high precision stereotaxic system (10 µm resolution). Outer diameter of both guide cannulae is 0.46 mm (26 gauge), whereas the double injectors and the dummy cannulae that are normally placed in the guide cannula have an outer diameter of 0.20 mm (33 gauge). The double injectors and dummy cannulae protruded 1 mm beyond the tip of the guide cannulae. The surgical procedures were performed under aseptic conditions. Mice were anesthetized by intraperitoneal injection of avertin (1.2%, 0.02 ml/g) as described previously[125]. Each double guide cannula (with inserted dummy cannula and dust cap) was fixed to the skull by dental cement. The guides were directed towards both dorsal hippocampi targeting the area ventral of CA1. The coordinates were based on the stereotaxic plates of the mouse brain atlas[126] with anterior–posterior (AP) coordinates referred to bregma, lateral (L) coordinates to the midsagittal suture line, and ventral coordinates (V) to the surface of the skull: AP, –1.6 mm; L, +/- 1.03mm and V, –2.3 mm (injection site). The depth of injections was chosen to avoid micro-lesions of both dentate gyri by the cannula tips. Buprenorphine was injected subcutaneously at a dose of 0.1 mg/kg as analgesic. The mice were allowed a minimal of 7 days of recovery before the initiation of subsequent experiments. During this postsurgical period, mice were checked for signs of distress such as apparent behavioral abnormalities and profound loss of body weight.

A microinjection pump (CMA/100, CMA/Microdialysis, Solna, Sweden) was used for bilateral injections into the dorsal hippocampus (0.25 µl/hemisphere) at a rate of 0.33 µl/min as described before[99,125]. Two 25 µl gas-tight syringes were mounted onto the pump and connected by polyethylene tubing to the double injection cannula (Plastics One, Roanoke, VA). The injection cannula delivered the injection solution 1 mm below the tip of the guide cannula at the depth of 2.3 mm. Before injection, both dust cap and dummy cannula were removed. Injections into the dorsal hippocampus were applied during a 90 s isoflurane (Forene, Abbott, Wiesbaden, Germany) inhalation anesthesia. After the end of the injection, the injector remained in place for 15 s in order to prevent backflow into the cannula guides. Thereafter, the injector was replaced by the dummy cannula before the dust cap was screwed back onto the guide cannula. Injections were applied minimally 1 h before testing.

Tissue Preparation and Immunoblotting.

The hippocampus (strain differences) or the dorso-medial part of the (ketamine effect) hippocampus was dissected from fresh brains and immediately frozen (< –60 °C). Synaptic membranes were isolated (pool of 3 or 2 mice per sample) on a discontinuous sucrose gradient, as described previously[103]. Protein concentration was measured by a Bradford assay. For all groups 5 µg/sample was dissolved in Laemli lysis buffer and loaded on an 8% SDS-PAGE gel. Proteins were blotted onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA). After blocking and incubation of the first antibody, against GluA1 (1:1,000; Genscript, Piscataway, NJ), GluA1-Ser845 (1:1,000; Phosphosolutions, Aurora, CO) GluA2 (1:1,000; Neuromab, Davis, CA), GluN1 (1:1,000; Millipore, Billerica, MA), GluN2A (1:1,500; Abcam, Cambridge, UK) and GluN2B (1:1,000; Neuromab, Davis, CA) the blot was washed and incubated for 1 h at RT with AP-conjugated secondary antibody (1:10,000; GE Healthcare, Diegem, Belgium). Before being used for immunoblotting, all antibodies were checked for specificity, *i.e.,* whether they showed a band at the designated apparent molecular weight on Western blot. Immunodetection was performed using the ECF western blotting detection system (GE Healthcare, Diegem, Belgium) and blots were scanned with the FLA-5000 (Fuji Photo Film, Rotterdam, The Netherlands). Relative amounts of immunoreactivity were quantified using ImageJ (NIH, Bethesda, MD). For standardization of immunoblots, the lower half of the same gel was cut and stained for coomassie as shown previously to be a good control for general input. Quantification of immunoblots was performed using the program Quantity One® 1-D analysis software (Bio-Rad, Veenendaal, The Netherlands).

Open field (OF)

Open field box 1 – Mice were introduced into a corner of the white square open field (50 x 50 cm, walls 35 cm high) illuminated with a single white fluorescent light bulb from above (130 lx), and exploration was tracked for 10 min (12.5 frames/s; EthoVision 3.0, Noldus Information Technology, Wageningen, The Netherlands). Time spent in, and number of entries into the center square area (20 x 20 cm) was measured using EthoVision.

Open field box 2 – Mice were introduced into a Plexiglas chamber with a stainless steel grid floor with constant illumination (100-500 lx) and background sound (white noise, 68 dB SPL (sound pressure level)). The box contained 12 infrared beams on each side, allowing measurement of both activity and exploration (area covered within x time). Measurements (3 min) took place after a single habituation trial (3 min).

Elevated plus maze (EPM)

Mice were introduced into the same closed arm of an EPM (arms 30 x 6 cm, walls 35 cm high, elevated 50 cm above the ground), facing the closed end of the arm. The EPM was illuminated with a single white fluorescent light bulb from above (130 lx) and exploratory behavior was video tracked for

5 min (12.5 frames/s, EthoVision 3.0, Noldus Information Technology, Wageningen, The Netherlands). The border between center and arm entries was defined at 2 cm into each arm, producing the number of entries into the open arms, into the closed arms, onto the center platform, and time spent on the open arms. In addition, latency to explore was defined by the time between introduction into the maze and the first appearance in the maze center.

Contextual Fear Conditioning

Contextual fear conditioning - All experiments were carried out in a fear conditioning system (TSE-Systems, Bad Homburg, Germany), as described previously[127]. Briefly, training and testing was performed in a Plexiglas chamber with a stainless steel grid floor with constant illumination (100-500 lx) and background sound (white noise, 68 dB SPL). The chamber was cleaned with 70% ethanol prior to each session. Training consisted of placing mice in the chamber for a period of 180 s after which a 2 s foot shock (0.7 mA) was delivered through the grid floor. Mice were returned to their home cage 30 s after shock termination.

Contextual fear retrieval - Retrieval test consisted of placing the animals in the conditioning context for 180 s. The automatically measured percentage of inactivity[99] served as measure of learning-induced fear response.

Forced swim test

We used a rectangular tank since a round tank gives image distortions when recording movements by camera recording using a lateral view (Supplemental Fig. 1A). As mice have the tendency to swim in circles in a round tank by simply following the curvature of the tank, the image of the animal is distorted. The distortion depends on the position and will affect the magnitude of detected movements. The more lateral the position of the object, the larger the distortion and the lower the size of detected movements. In a rectangular tank the visible surface of the animal remains almost constant irrespective of its position especially since we slightly reduced the depth of the tank. Thus, a rectangular tank causes substantially fewer image artifacts. The side-view and top-view give highly correlated results. Tail movements are more prominent in the top-view setup, and leg movements more prominent in the side-view setup. However, the side view offered a slightly higher sensitivity and was therefore selected for our analysis.

The digital movie was used for automatic analysis, using a motion detection algorithm to determine whether a mouse is moving or not. The motion detection is based on the differences between two successive video frames. First, each frame is divided into small rectangles (grid; see Supplemental Figure 1B). The size of the rectangles determines the sensitivity to motion with smaller rectangles giving higher sensitivity. The program counts the number of rectangles where motion was detected given a specific detection threshold (noise filter). This measure yields the activity diagram (Supplemental Figure 1C). A threshold for mobility can be set manually for all digital movies to be analyzed batch-wise. Analysis was performed with 15 frames/bin, each bin representing 0.6 sec. As

the cumulative plot of activity yields non-stationary data due to changes in swim strategy, the separate stationary segments were fitted to line curves using Douglas-Peucker polyline fit[104] with an 8 pixel tolerance (Supplemental Figure 1D). Parameters as measured from digital images were:

Immobility – Time (%) spent inactive with activity detected below the threshold of 7 transitions per time bin.

Latency to immobility – Time (s) spent before a first bout of inactivity, lasting > 1 s.

Strategy plot – The coordinates of the average cumulative activity (y) and time (x) of each fitted line segment (each set of stationary data) was calculated and plotted. This parameter gives detailed information about the swim strategy over time. Because the swim strategies were mostly captured by at least 3 line segments for every mouse, only those segments were used for analysis.

Total activity – The sum of the activity during the 6 min swim session calculated by the quantitative motion-detection analysis.

Time to change strategy – Total time before activity was less than before. The latency to reach the fitted line segment of which the slope was lower than before, i.e. when the second derivative was at a minimum, was taken.

The program for automatic analysis, using a custom-developed motion detection algorithm, is made available upon contact (R.F. Jansen and O. Stiedl (oliver.stiedl@cncr.vu.nl). For more information, see http://www.falw.vu/~ngc/FST.html.

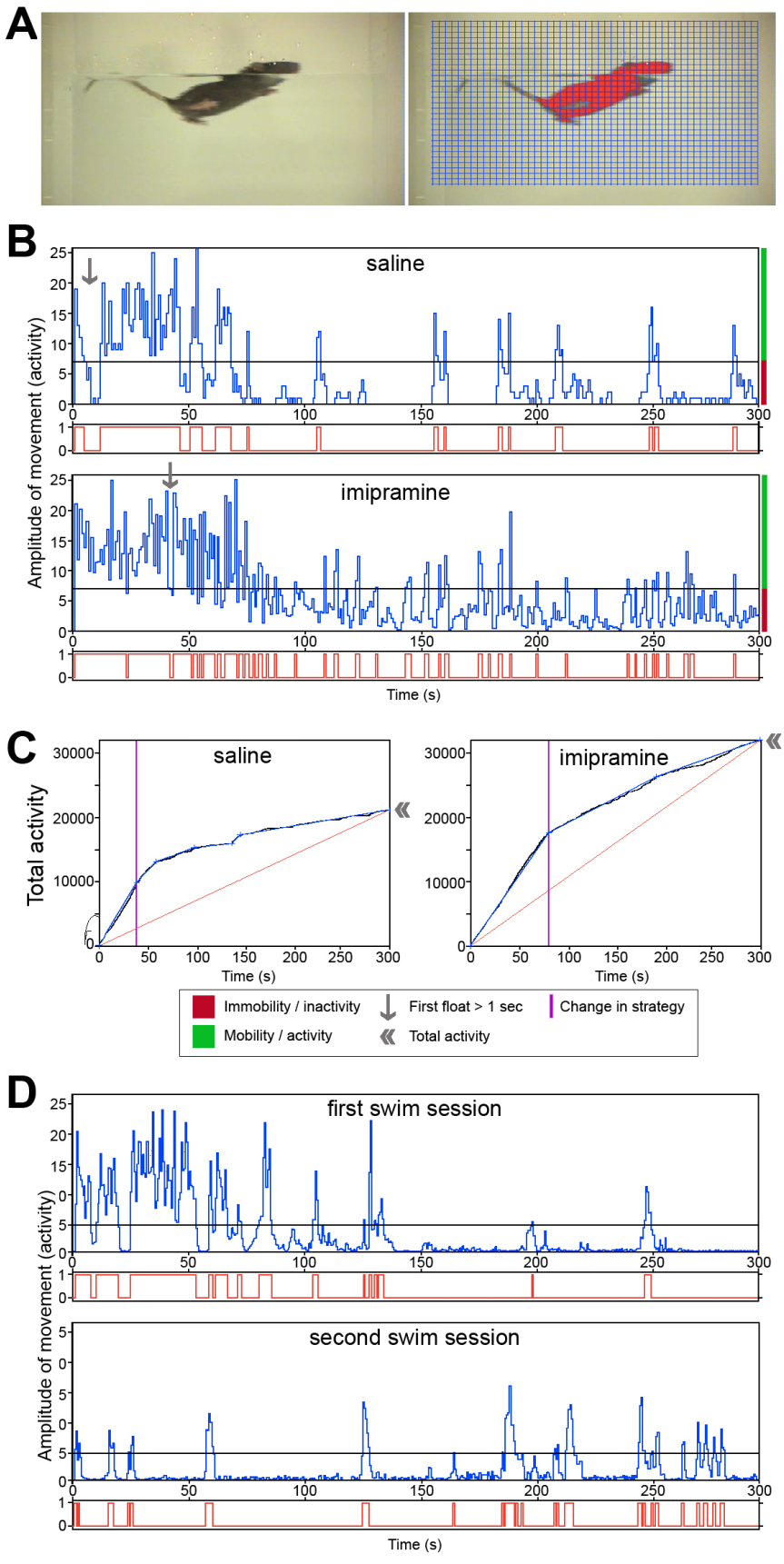
A
B
saline
imipramine
Amplitude of movement (activity)
Time (s)
C
saline
imipramine
Total activity
Time (s)
Immobility / inactivity
Mobility / activity
First float > 1 sec
Total activity
Change in strategy
D
first swim session
second swim session
Amplitude of movement (activity)
Time (s)

Figure S1. Analysis of classical and novel parameters for behavioral despair in the FST. (A) From digitized images (zoom in), using a grid structure to monitor activity, classical and novel parameters of the FST are captured. (B) Examples are given from the analysis window for 2 individual mice that received either saline or imipramine (8 mg/kg, i.p.) 30 min before the test. The most widely used classical parameter mobility/immobility (green/red) was extracted from the activity plot. The lower panel (red lines) displays the binned time of mobility and immobility, with imipramine-treated mice (i.p. injection 30 min before the test) showing more mobility both during the total period as well as during the last 4 min. In addition, the latency to float for ≥ 1 sec is indicated (>2 bins; 0.6 sec/bin). Naive and saline-treated C57BL/6J mice show a low latency to the first float (~10 sec), while imipramine-treated mice have a prolonged latency (~50-60 sec). (C) Novel parameters, such as strategy, total activity and time to change swim strategy can be extracted from the individual swim strategy plots, which shows the cumulative activity (number of rectangles in the detection grid with activity above the set threshold (horizontal black line) vs. time (seconds). Strategy plots are shown for the same two mice as in B. The blue lines indicate separate line fits to the non-stationary data, and the red line indicates a cumulative distribution with no change in activity (stationary data). Note that the latency to the first change in swim strategy, denoted by the first change towards less activity (purple vertical line), is longer in imipramine-treated mice. In addition, the total activity of imipramine-treated mice is higher than that of saline-treated mice. (D) A second swim session changes the swim strategy of mice (lower panel), and hence parameters typical for a first swim session (upper panel) cannot be used. Note the regular swimming bouts in the second session, lacking the vast amount of activity displayed during the beginning of the first session.

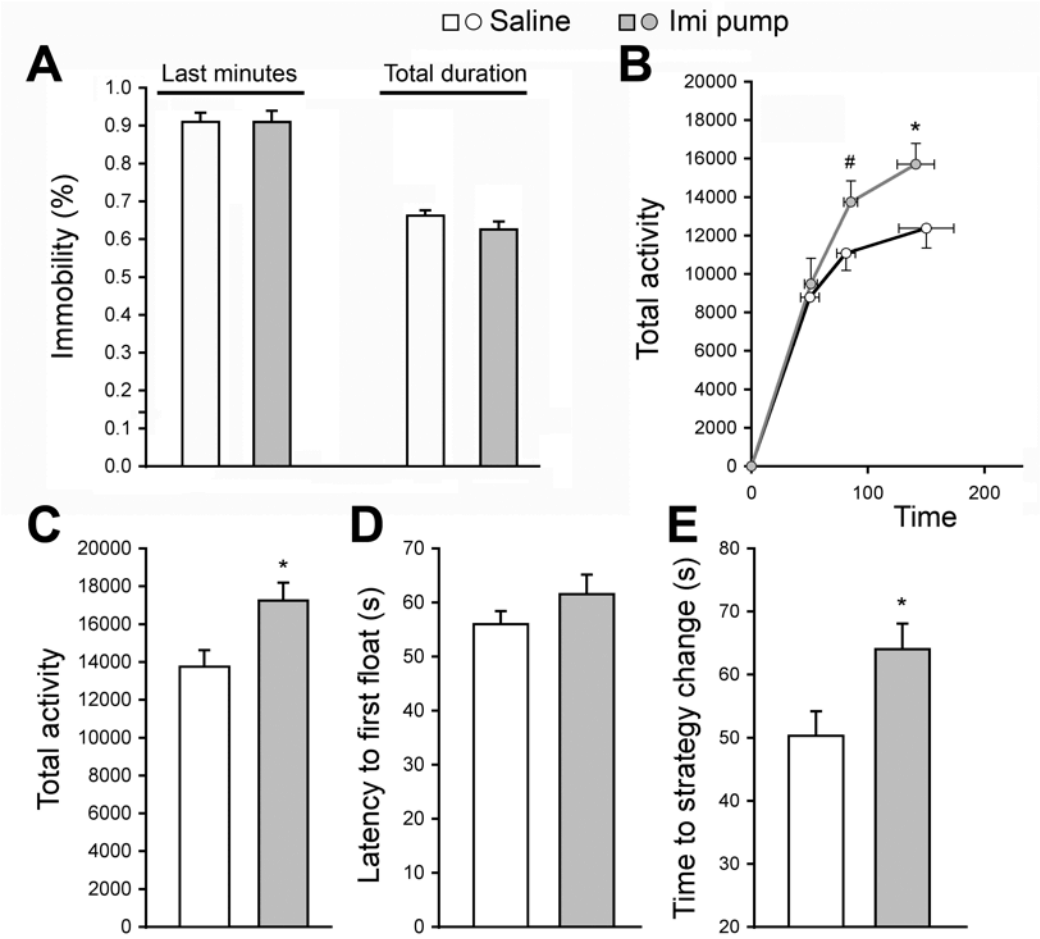

Figure S2. Effects of low chronic imipramine on FST performance of C57BL/6J mice. An osmotic minipump was placed subcutaneously 26 days before the test and delivered imipramine at a dose of 6-8 mg/kg/day for 28 days. Imipramine-treated mice (n=13) differed from control mice (saline; n=11) only in the novel FST parameters strategy (B), total activity (C), and time to change swim strategy (E), but not in classical parameters of behavioral despair, such as immobility (A), nor in the latency to first float (D). # P<0.1, * P<0.05.

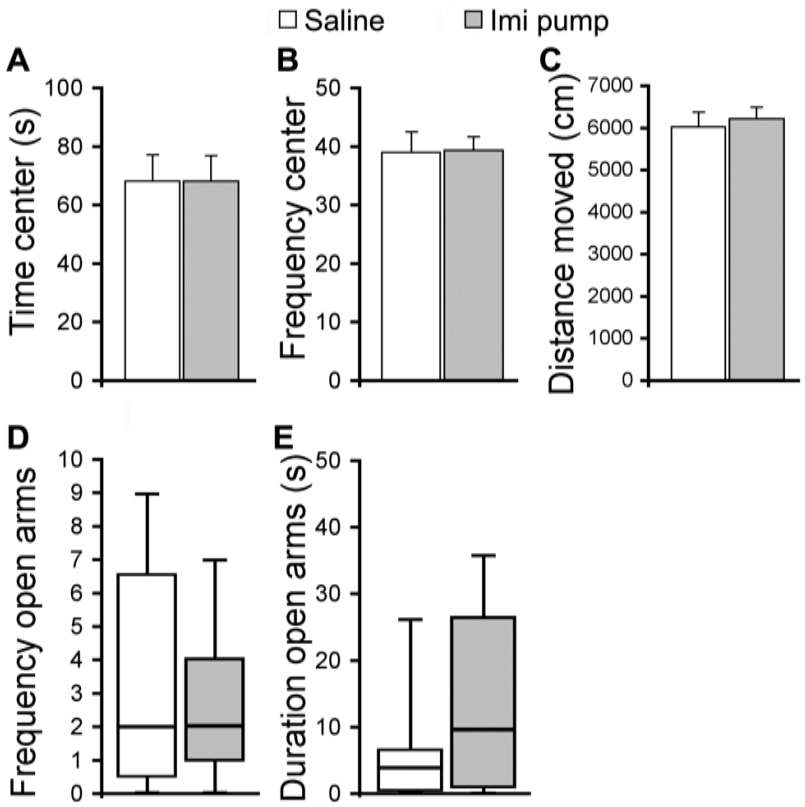

Figure S3. Effect of low chronic imipramine on locomotor and anxiety parameters of C57BL/6J mice. An osmotic minipump was placed subcutaneously 26 days before the test and delivered imipramine at a dose of 6-8 mg/kg/day for 28 days. Imipramine-treated mice (n=13) did not differ statistically ($P<0.05$) from control mice (saline; n=11) in open field behavior determined by the time spent in the center (A), frequency in the center (B), total distance moved (C), nor in elevated plus maze behavior such as frequency of the open arm visits (D) and duration on open arms (E). For the latter, only a trend was visible ($P=0.176$) towards reduced anxiety-like behavior.

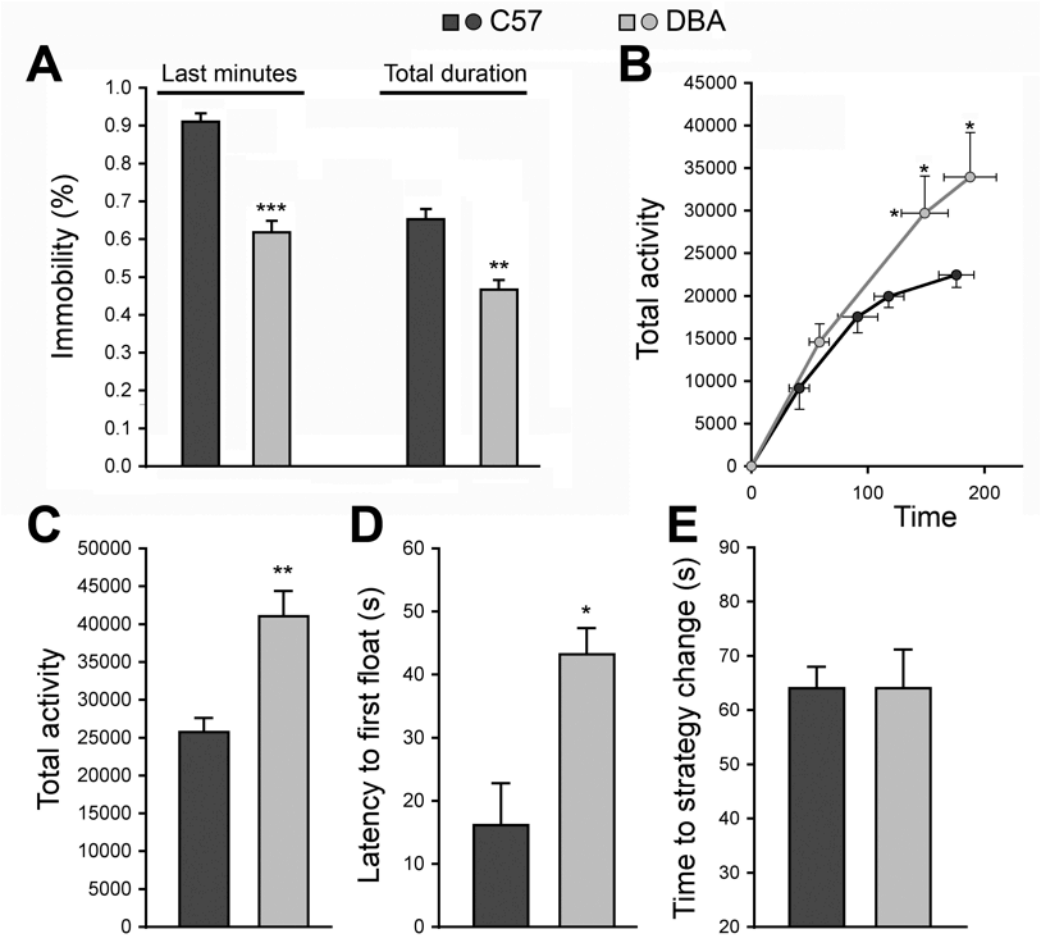

Figure S4. Mouse strain differences in FST performance. A strain-specific effect (C57BL/6J mice (n=11), DBA/2J mice (n=10)) was apparent for immobility as measured traditionally during the last 4 min (A, left), as well as measured during the total 6-min test time (A, right). Novel parameters resulting from our automated analysis like strategy (i.e. activity over time displayed for fitted line segments shared by all mice within a group (i.c. up to ~ 200 sec), see Methods; B), total activity (C) and latency to first float (D) showed strain-specific differences, but not time to change swim strategy (E). *** P<0.001, ** P<0.01 and * P<0.05 vs. C57BL/6J.

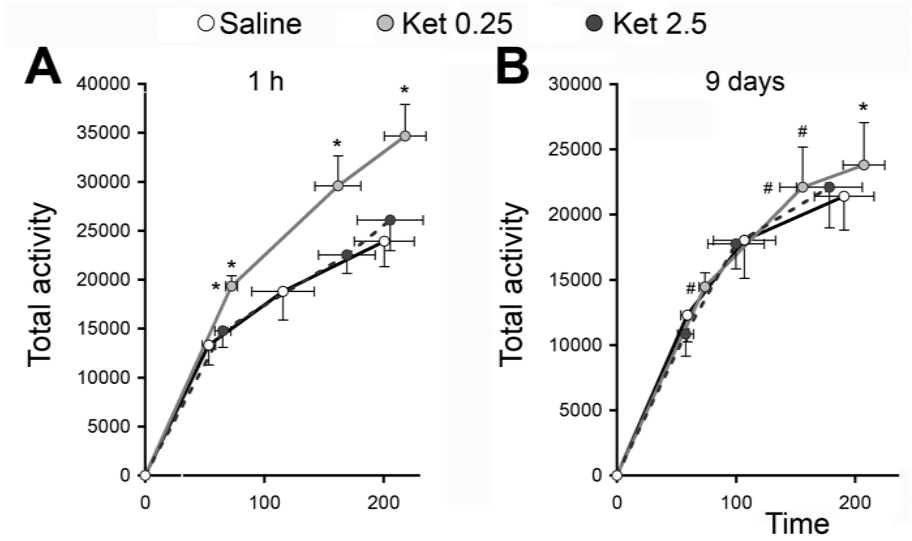

Figure S5. Acute and long-term effect of hippocampal ketamine on strategy of swim behavior. Bilateral dorsohippocampal injection of saline (n=6) or ketamine (0.25 µg, n=6-8; 2.5 µg, n=8) were given 1 h (A) or 9 days (B) before the test. Ketamine treatment caused differences from saline controls with respect to swim strategy only in the 0.25 µg dose. The effect was highest acutely after ketamine injection (A). * $P<0.05$, # $P<0.2$ vs. saline.

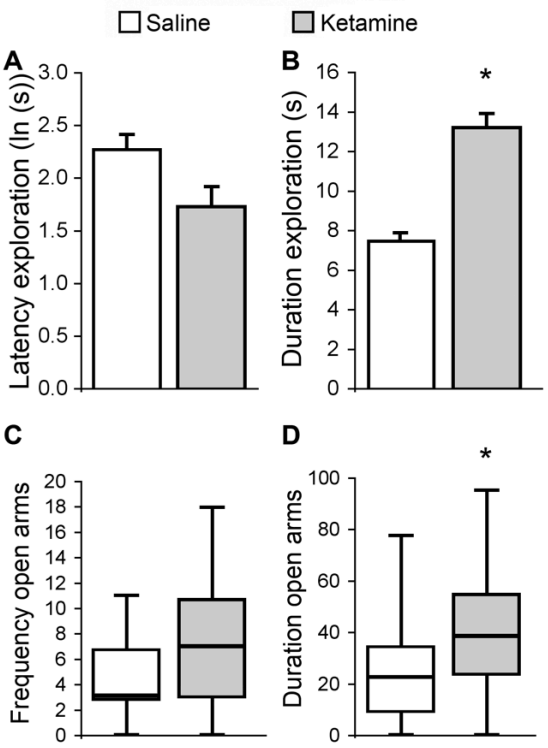

Figure S6. Long-term effects of hippocampal ketamine on exploration and anxiety-like behavior of C57BL/6J mice. Novelty exploration in the home cage was measured 6 h after dorsohippocampal injection of ketamine (0.25 µg; n=8) or saline (n=8) for log-normalized (ln) latency of exploration (A; P=0.443) and duration of exploration (B; * P=0.025). Two days after hippocampal injection of ketamine (0.25 µg; n=22) or saline (n=20), anxiety parameters as frequency open arms (C; P=0.116) and duration on the open arms (D; * P=0.044) were measured on the elevated plus maze.

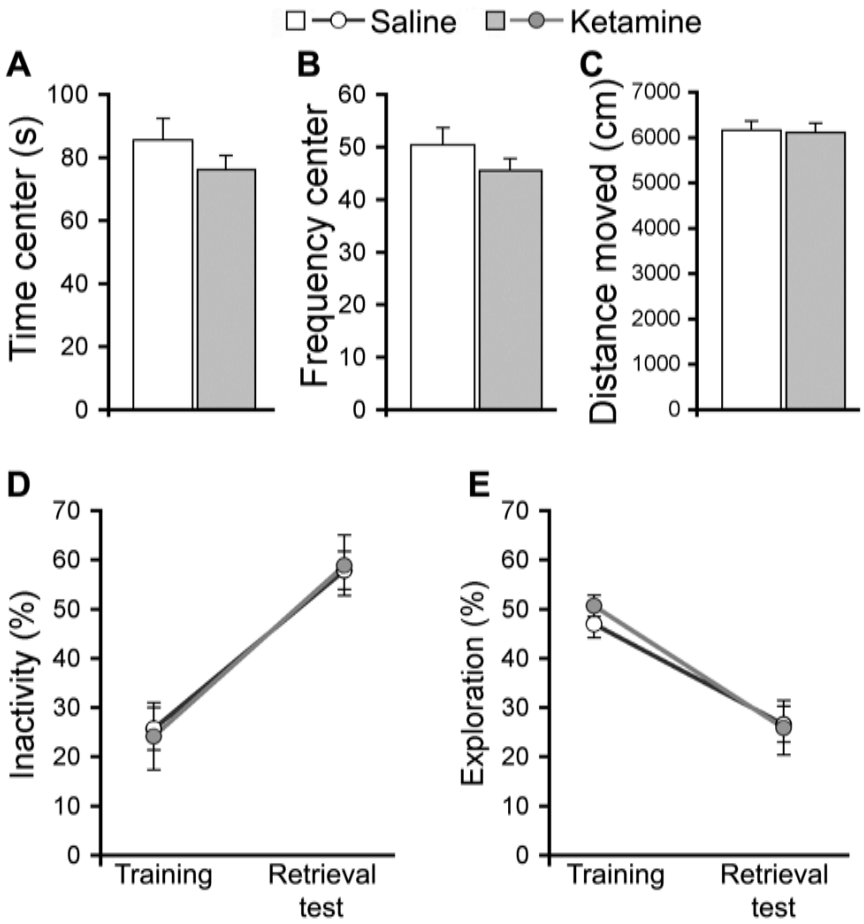

Figure S7. Long-term effects of hippocampal ketamine injection on locomotion and contextual fear conditioning in C57BL/6J mice. Open field behavior was measured 1 day after hippocampal injection of ketamine (0.25 µg; n=22) or saline (n=20) for time spent in the center (A), frequency in the center (B), total distance moved (C), with no significant differences in either of the parameters measured. Contextual fear conditioning took place 5 days after hippocampal injection of ketamine (0.25 µg; n=8) or saline (n=8), and 48 h later animals were tested for expression of fear memory. No significant differences were observed in inactivity (D) or exploration (E). Note that both groups expressed high levels of conditioned fear based on a substantially increased inactivity and reduced exploration.

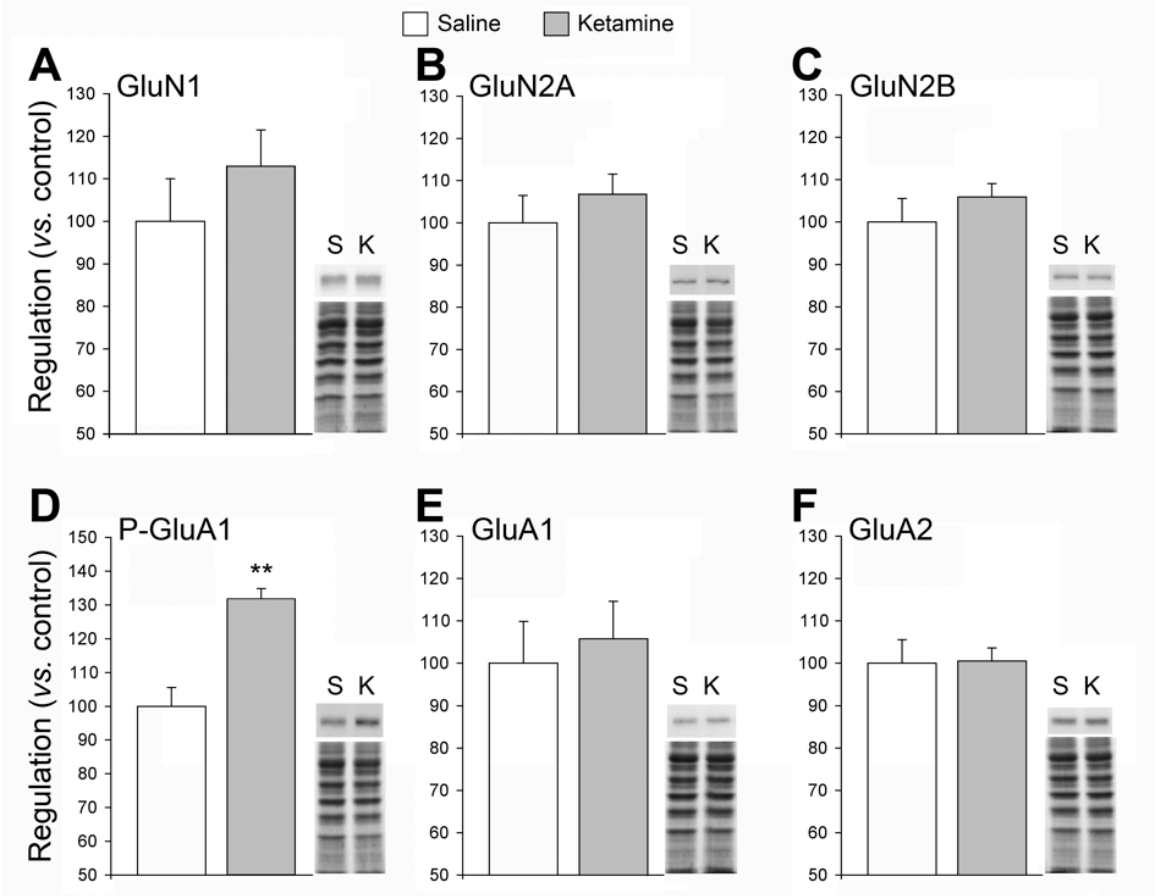

Figure S8. Long-term effects of hippocampal ketamine on synaptic hippocampal glutamate receptors. Dorso-medial hippocampal synaptosome fractions of mice with dorsohippocampal injections of saline or ketamine (0.25 µg, n=6) were used for immunoblotting with glutamate receptor subunit antibodies; GluN1 (A), GluN2A (B), GluN2B (C), P-GluA1 (D), GluA1 (E), GluA2 (F). Typical examples of the immuno-detected protein levels are shown for each treatment (saline, S and ketamine, K) mice, together with the coomassie-stained lower part of the gel to the right of each bar graph. ** P=0.007.

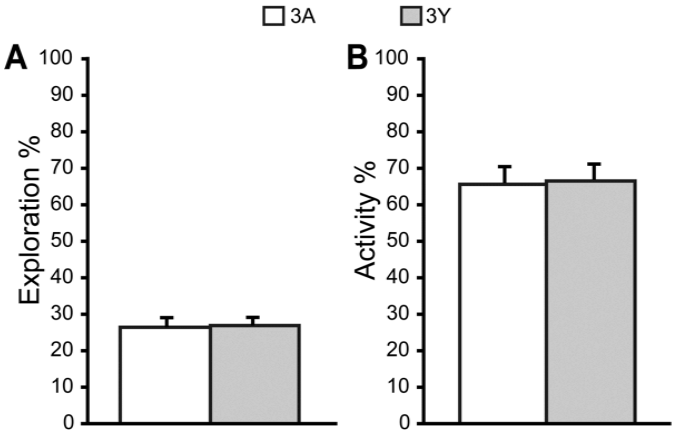

Figure S9. Effect of TAT-GluA2$_{3Y}$ peptide on locomotor activity. The GluA2 blocking peptide (3Y; n=9) and control peptide (3A; n=9) were injected 1 h before an open field test. The peptide had no effect on exploration (A) and activity (B).

Table S1. Statistical results of the FST performance of C57BL/6J mice with pharmacological interventions. ANOVA results (F- and P-value) and subsequent t-tests (P-value) for the acute effect of imipramine (8 and 16 mg/kg, i.p.), and the acute and long-term effect of local hippocampal infusion of ketamine (0.25 and 2.5 mg). Parameters measured (*cf*. Supplemental Figure 1) and P-values (<0.05, bold; <0.2, italics) are indicated. *Non-parametric test results (Kruskall-Wallis and Mann-Whitney U-test) are indicated. ‡Albeit significant or a trend towards significance, Kruskall-Wallis/ANOVA did not allow to perform post-hoc analysis.

Forced Swim Test	**ANOVA**		**t-test**	
30 min after Imipramine (i.p.)	F-value (2,24)	P-value	P-value (8 mg/kg)	P-value (16 mg/kg)
Immobility_last minutes	*	*0.064*	0.022‡	0.606
Immobility_total	*	**0.001**	**<0.001**	**0.001**
Total_activity	13.023	**<0.001**	**0.00018**	**0.00026**
Latency_first_float	11.058	**<0.001**	**0.00003**	**0.00284**
Time_strategy_change	5.850	**<0.001**	**0.00795**	**0.00537**
1 h after local ketamine	F-value (2,17)	P-value	P-value (0.25 µg)	P-value (2.5 µg)
Immobility_last minutes	4.041	**0.037**	**0.0433**	0.7025
Immobility_total	5.542	**0.014**	**0.0074**	0.7182
Total_activity	5.897	**0.011**	**0.0123**	0.4892
Latency_first_float	1.138	0.344	0.2402	0.2574
Time_strategy_change	4.957	**0.020**	**0.0166**	**0.0434**
9 days after local ketamine	F-value (2,21)	P-value	P-value (0.25 µg)	P-value (2.5 µg)
Immobility_last minutes	3.433	*0.051*	0.0370‡	0.0528‡
Immobility_total	3.523	**0.048**	**0.0123**	*0.1104*
Total_activity	0.672	0.521	0.2761	0.7887
Latency_first_float	4.633	**0.022**	**0.0364**	**0.0324**
Time_strategy_change	3.977	**0.034**	**0.0020**	0.2088

Table S2. Statistical results of the FST performance by imipramine and strain difference. Values of t-tests (P-value) for the long-term effect of imipramine (6-8 mg/kg/day, s.c. in C57BL/6J mice) and the strain difference between C57BL/6J and DBA/2J mice. Parameters measured (*cf.* Supplemental Figure 1) and P-values (<0.05, bold; <0.2, italics) are indicated.

Forced Swim Test	**Imipramine (minipump)**	**Strain difference**
	P-value	P-value
Immobility_last minutes	0.9876	**0.0001**
Immobility_total	*0.1984*	**0.0034**
Total_activity	**0.0170**	**0.0074**
Latency_first_float	0.2546	**0.0207**
Time_strategy_change	**0.0280**	0.9999

Table S3. Statistical results of anxiety tests and contextual fear conditioning performances after pharmacological intervention in C57BL/6J mice. T-test or Mann-Whitney U test (P-value) for the long-term effect of imipramine (6-8 mg/kg/day, s.c. by minipump) and long-term effect of local hippocampal infusion of ketamine (0.25 mg). Parameters measured, time after insertion of a minipump or local injection, and P-values (<0.05, bold; <0.2, italics) are indicated.

Anxiety & learning			t-test / MWU test
Imipramine minipump	days post minipump	test parameter	P-value
Novelty exploration	13	Latency exploration	0.8308
		Duration exploration	0.4012
Open field	15	Duration center	0.9979
		Frequency center	0.9321
		Duration center	0.6753
Elevated plus maze	16	Duration open arms	*0.1755*
		Frequency open arms	0.4321
Fear conditioning	19	Inactivity pre-shock	0.2312
	19	Exploration pre-shock	0.3147
	21	Inactivity 48 h post-shock	0.6858
Local Ketamine	days post Ketamine	test parameter	P-value (0.25 µg)
Novelty exploration	0.25	Latency exploration	0.4427
		Duration exploration	**0.0250**
Open field	1	Duration center	0.2547
		Frequency center	0.2232
		Duration center	0.8578
Elevated plus maze	2	Duration open arms	**0.0440**
		Frequency open arms	*0.1160*
Fear conditioning	5	Inactivity pre-shock	0.8749
	5	Exploration pre-shock	0.4299
	7	Inactivity 48 h post-shock	0.9056

Table S4. Immunoblot analysis for hippocampal synaptic membrane glutamate receptor subunits. Statistical analyses for strain difference between C57BL/6J and DBA/2J mice (cf. Figure 3), and the long-term effects of local ketamine injection (0.25 µg). P-values (<0.05, bold) are indicated.

Immunoblot analysis	t-test
Strain difference	P-value
GluA1	0.295
GluA2	0.125
GluN1	0.924
GluN2A	**0.013**
GluN2B	0.727
9 days post local Ketamine	P-value
GluA1	0.675
P-GluA1	**0.007**
GluA2	0.928
GluN1	0.398
GluN2A	0.522
GluN2B	0.585

Table S5. Statistical analyses of FST performance test after molecular intervention in C57BL/6J mice. T-tests (P-value) for the acute effect of local GluA2 endocytosis block using the $GluA2_{3Y}$ peptide and $GluA2_{3A}$ control peptide (15 pmol per side, 30 pmol total). Parameters measured (*cf.* Supplemental Figure 1) and P-values (<0.05, bold) are indicated.

Behavioral despair	t-test
1 h after local GluA2 endocytosis block	P-value (30 pmol)
Immobility_last minutes	**0.0331**
Immobility_total	**0.0210**
Total_activity	0.3508
Latency_first_float	**0.0004**
Time_strategy_change	0.9732

Chapter 3

Different mechanisms mediate hippocampal-dependent memory impairments after social defeat stress at the short and long term

Submitted

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Abstract

Cognitive functions, such as learning and memory are greatly affected by stress, but the synaptic changes causing these deficiencies are largely unknown. Therefore, we investigated hippocampus-dependent memory function and concomitant expression of synaptic glutamate receptors over time after social defeat stress in rats. Acutely, 24 hours after cessation of social defeat exposure (5 daily social defeat encounters), plasma corticosterone levels were increased 2.5 fold, and performance in a hippocampus-dependent object place recognition task was significantly reduced. Moreover, this impaired spatial memory was associated with decreased synaptic expression of glutamate receptor subunits GluN1, GluN2B and GluA2 in the dorsal hippocampus. In addition, we found that glutamate receptor levels were unaltered 3 months after social defeat, whereas spatial memory performance remained impaired. Therefore, we conclude that different synaptic molecular mechanisms underlie memory impairment at the short and long term after social defeat stress.

Introduction

Cognitive functions such as learning and memory are greatly affected by stress[128]. In humans, acute stress disrupts declarative memory retrieval for previously learned information[42,129]. Also, in stress-related psychiatric disorders, such as depression, decreased attention and spatial memory have been found[130]. In rodents, acute and chronic stress also disrupts spatial memory formation and retrieval[40,41,131].

The hippocampus is a medial temporal lobe structure implicated in the consolidation of declarative memory in humans and spatial memory in rodents[132]. Increased corticosterone after stress has been shown to alter hippocampal synaptic plasticity, and to affect spatial memory performance[133]. Stress and glucocorticoids have a profound influence on the two most well characterized forms of synaptic plasticity of the hippocampal CA1 region, i.e., long-term potentiation (LTP) and long-term depression (LTD), the proposed cellular substrates for learning and memory[134].

Stress hormones have been extensively studied and were shown to have facilitating or impairing effects on hippocampal physiology and memory[37]. Recent evidence shows that, on the short term, rapid, non-genomic effects of corticosterone are mediated via high-affinity mineralocorticoid receptors (MRs), which act to enhance alpha-amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid receptor (AMPAR) mobility[135] and miniature evoked post-synaptic current (mEPSC) frequency[136], and facilitate synaptic potentiation[137]. However, long-lasting effects (>1 hour) are mediated via genomic glucocorticoid receptors (GRs), which impair N-methyl-D-aspartate receptor (NMDAR)-mediated LTP[43] and facilitate LTD[43,133]. This hippocampal LTD has been shown to be necessary and sufficient to cause acute stress-induced impairment of spatial memory retrieval and is dependent on GluN2B-containing NMDARs[133]. Similarly, acutely (~24 h) after chronic stress (>20 days exposure) spatial recognition memory has been repeatedly demonstrated to be impaired[128].

Thus, the degree through which stress alters spatial memory is dependent on many factors among which the severity and duration of the stressor, and the time between stress experience and behavioral assessment[128,138]. A persistent finding is that the time frame for chronic stress to influence performance is critical; for instance, a wire mesh restraint over a relatively long period (21 days) impaired spatial recognition on the Y-maze, but this restraint over a shorter time frame (5 – 10 days) was ineffective[139].

Here, we used a social defeat paradigm to assess short- and long term effects of a natural type of stressor. Social defeat stress was inflicted over a short 5-day period, and tested for its effects immediately thereafter or after a period of 3 months; socially defeated rats were individually housed from the first day of defeat onward. The repeated social defeat

stress as used here has previously been shown to reduce hippocampal LTP, and to increase LTD on the long-term (up to 9 months)[140]. In this study, we investigated both the direct and long-term effect of social defeat on glutamate receptor synaptic membrane expression in conjunction with the associated spatial memory deficits.

Materials and methods

Animals

Male Wistar rats (Harlan, Horst, The Netherlands) 8 weeks of age and weighing 180–200 g at the time of arrival were initially socially housed (2 per cage) in Makrolon type IV cages (Tecniplast, Milan, Italy). Long-Evans male rats (Harlan, UK), weighing 300–350 g were used as residents for social defeat[141]. These animals were pair-housed with age-matched sterilized females in plastic cages (63 x 25 x 33 cm) located in a separate room. All animals were housed in a temperature-controlled room (21±1 °C) under regulated lighting conditions (lights on at 7:00 p.m. and off at 7:00 a.m.). Food and water were available *ad libitum*. All experimental manipulations were conducted during the dark phase (activity period) under a dim red light. All experiments were approved by the Animal Users Care Committee of the VU University Amsterdam.

Experimental design and social defeat procedure

Wistar rats (age ≥ 11 weeks) of the social defeat group were subjected to 5 days of social defeat stress and were then housed individually in macrolon class III cages from the first defeat onwards, as described before[140,142]. Control rats were housed in pairs. The social defeat procedure consisted of daily resident-intruder interaction sessions using dominant male Long-Evans rats. Control animals were daily handled. All behavioral, electrophysiological and molecular analyses were performed either 24 h (acute) or 3 months (long-term) after the last social defeat session.

Corticosterone assay

Corticosterone levels were measured as described previously[142]. In short, trunk blood samples were collected via decapitation between 9:00 am and 11:00 am. Samples were collected into a 7-mL heparin-coated tube (Greiner Bio-One, Monroe, North Carolina) and kept on ice. The samples were spun at 1000x *g* for 10 min. Plasma was decanted and stored at −80 °C until the assay was used. Levels of plasma corticosterone were assessed using a rat Glucocorticoid (GC) ELISA kit (Cusabio Biotech Co., LTD), according to the manufacturers instructions.

Recognition tests

Recognition memory testing was conducted based on two previously reported tasks relying on spontaneous exploration of objects in an open field[143,144]. All testing was carried out in a rectangular arena, 79 x 57 surface area with 42 cm tall black walls. The box was surrounded by visual cues: computer light coming from the west side; a white wall (north); a metal rack (east side); and an open space where the experimenter was sitting (south). The arena was always placed inside the room at the same location and in the same orientation. All rats were habituated to the empty arena twice for 30 minutes on the two days preceding behavioral testing, as well as on the test day for 10 min and 1 min prior to the sample phase.

Object-recognition task – Round or square aluminum bars were used as novel or familiar objects and were chosen using a pseudorandom protocol, balanced across treatment groups. In the 4-minute sample phase, rats were exposed to two identical objects (round or square metal bars) followed by a 15 min inter-trial interval (Supplemental Fig. 1). In the test trial, one object (chosen using a pseudorandom protocol, balanced across treatment groups) was replaced with a novel object and rats were allowed to explore both objects for 4 minutes (Supplemental Fig. 1). The familiar object was a third copy of the two objects seen in the sample phase, to prevent possible carry-over of olfactory cues. Each session was recorded by a video camera suspended above the field and interfaced with a computerized tracking system using the 'Viewer' software package (BIOBSERVE, GmbH, Bonn, Germany). The nose of the rats was tracked by the 'Viewer' software and during both trials, the object exploration (defined as the time of the nose spent within 2 cm from the object) was measured for each object. Only rats that had accumulated at least 15 s of exploration at each object within the sample phase were included in the analysis (all but one tested met criteria). To further analyze object discrimination during the test trial, the discrimination index ((novel-familiar)/(novel + familiar)) was calculated for each rat using the individual object-exploration times recorded.

Object-place task – For the 4-minute sample phase, rats always entered the testing box from the south, and were placed facing the south wall. Rats were exposed to two identical objects (round aluminum bars) followed by a 15 min inter-trial interval. During test trials, rats entered the box from the east or west side and were placed facing the east or west wall, respectively. Thus, for each trial, the entry point on the sample and test phases were different. In the test phase a third and fourth copy of the same object were placed back in the arena, one in a familiar corner and one in a novel corner. The location of the novel object-place configuration was counterbalanced such that on each trial different corners were used as familiar and novel locations. Rats were now only able to discriminate between

objects based on their location with respect to its surrounding. Further analysis of discrimination was the same as for the object recognition task.

Tissue preparation

Following decapitation, brains were removed and rapidly frozen in ice-cold isopentane and stored at –80 °C until further use. The dorsal hippocampus (Bregma: –2.56 till –5.0) was removed freehand at –20 °C from 1-mm-thick slices. Synaptic membrane fractions were isolated for every hippocampus (left and right pooled). Samples were homogenized in ice-cold 0.32 M sucrose (5% of homogenate was collected as total cell lysate) and then centrifuged at 1000 g for 10 min. The supernatant was loaded on top of a sucrose gradient consisting of 0.8 and 1.2 M sucrose. After centrifugation at 100,000 g for 2 h, the synaptosome fraction at the interface of 0.85/1.2 M sucrose was collected and then lysed in hypotonic solution. The resulting synaptic membrane fraction was recovered by centrifugation using the sucrose step gradient as stated above. The synaptic membrane fraction was collected from the 0.85/1.2 M interface and protein concentrations were determined using a Bradford assay (Bio-Rad, Hercules, CA, USA).

Immunoblotting

Synaptic membranes of the dorsal hippocampus were isolated from two independent groups of animals (n=6 each) per time point. Samples (3 – 5 μg) were lysed in Laemli lysis buffer, separated by electophoresis on a Criterion 10.5 – 14% Tris-HCl sodium dodecyl sulfate-polyacrylamide precast gel and blotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories) as described before[145]. All antibodies were checked for specificity, i.e. whether they showed a band at the designated height on Western blot. The following antibodies were used: mouse anti- PSD95, (NeuromAB; 1:5000), mouse anti-GluN1 (BD Biosciences; 1:1000), rabbit anti-GluN2A (Abcam, 1:1000), mouse anti-GluN2B (NeuroMab, 1:1000), rabbit anti-GluA1 (GenScript, 1:1000), mouse anti-GluA2 (NeuroMab, 1:500).

Statistics

Determination of corticosterone levels (n=8 acute; n=6 long-term), behavioral assessments (n=12), and molecular analysis (n=6 in both experiments) were performed in independent groups of animals for each of the time points addressed. Physiological parameters were assessed in all experimental animals (n=32 acute, n=24 long-term). Significant differences between stress and control groups was determined by unpaired, two-tailed, Student's T-tests.

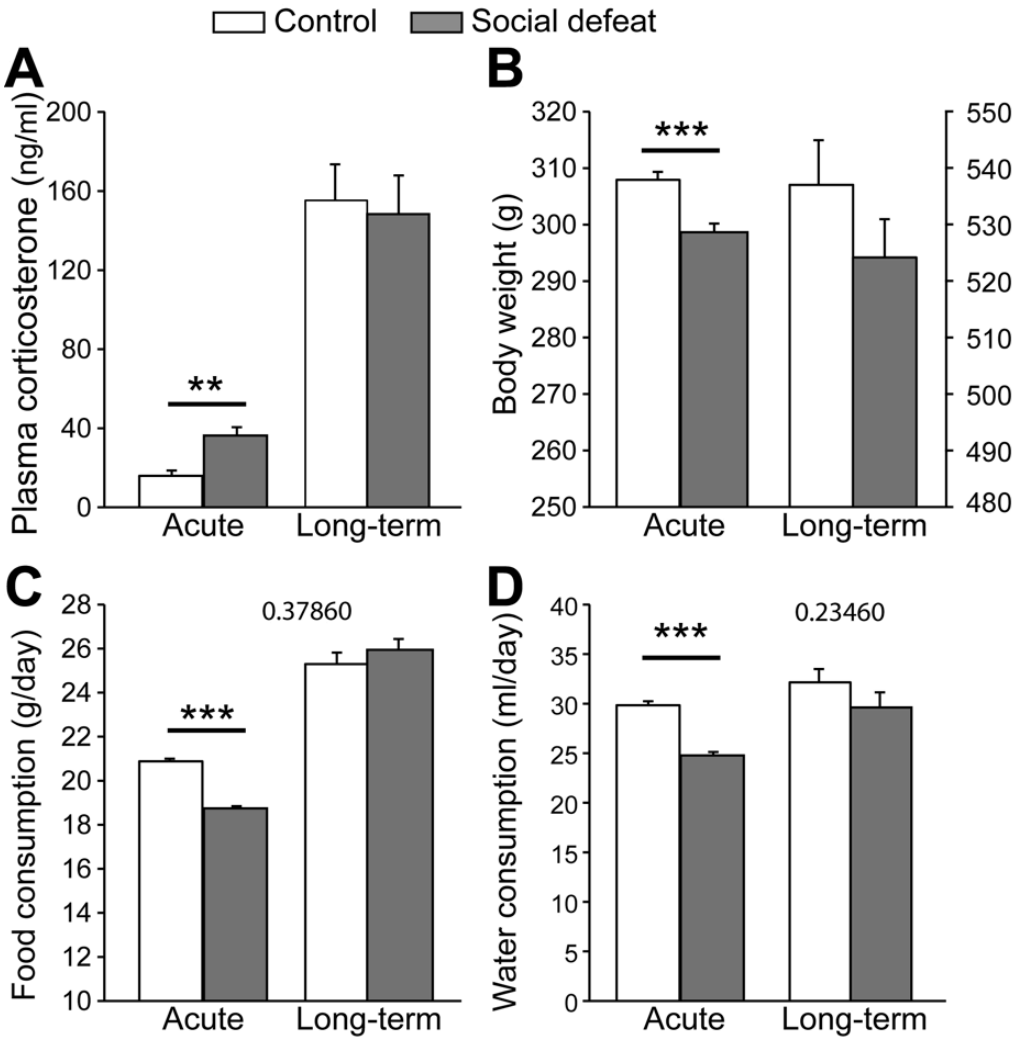

Figure 1. Social defeat stress affects physiological parameters. Either acute (24 h) or long-term (3 months) after the last social defeat encounter various physiological parameters were measured. Acutely after social defeat stress, plasma corticosterone levels (n=8) were increased (A), and body weight (B), food intake (C), and daily water intake (n=32) (D) were decreased. All these parameters were normalized at 3 months after social defeat (n=24). Data presented are mean+SEM. Unpaired, two-tailed, Student's t-test: ***P*<0.01, ****P*<0.001.

Results

Effect of repeated social defeat on physiological parameters

Social defeat stress resulted in a 2.5 fold increase in plasma corticosterone levels 24 hours after the last social defeat exposure (Fig. 1A, *P*=0.0023). These levels were back to normal at 3 months after social defeat, and there was no difference between treatment groups. The acute effect of social defeat was also evident by a decrease in body weight (Fig. 1B, *P*<0.001), and a decrease in food- (Fig. 1C, *P*<0.001) and water consumption (Fig. 1D,

P<0.001). All of these physiological parameters were back to normal at 3 months after social defeat. Previously described age-related differences in corticosterone-levels were observed (*P*<0.001)[146], as well as age-related increases in body weight (Fig. 1A,B).

Object-place memory is reduced short and long-term after social defeat stress

To test whether repeated social defeat stress affected hippocampus-dependent and/or hippocampus-independent memory, object place recognition and novel object recognition tests were performed, respectively (Langston and Wood 2009). For both tests, during the sample trials, two similar objects were explored equally for the different treatment groups. In the object-place recognition test, control rats showed place recognition by having a preference for the object placed in the novel location. This spatial memory was significantly reduced acutely after social defeat stress (Fig. 2A, *P*=0.038). Interestingly, this hippocampus-dependent cognitive deficit was still present 3 months after social defeat (*P*=0.0034). At this stage, depressive-like phenotypes in the affective domain were also present in this paradigm[33], indicative of a multi-layered phenotype. In contrast, in the object recognition test, both the acute and the long-term social defeat groups did not differ from their controls in the test-phase (Fig. 2B), suggesting that this form of hippocampus-independent memory was still intact. Interestingly, hippocampus-dependent processing in the object-place task seemed prone to ageing, although this effect was not significant (*P*=0.11).

Changes in synaptic expression of glutamate receptor subunits in the dorsal hippocampus short after social defeat stress

Reduced spatial memory performance is caused by altered synaptic plasticity at excitatory neurons of the dorsal hippocampus[43]. Therefore, it is of interest to investigate whether glutamate receptors, mediating hippocampal synaptic plasticity, are involved in the observed social defeat stress-induced impairment in spatial memory performance. We compared the expression of ionotropic glutamate receptors in dorsal hippocampal synaptic membrane fractions using quantitative immunoblot analysis (Supplemental Fig. 1). This revealed significantly lower levels of two NMDAR subunits, namely GluN1 (*P*<0.001) and GluN2B (*P*=0.048), acutely after social defeat stress (Fig. 3A). Synaptic expression of GluN2A was unaffected. Also, synaptic expression of the AMPAR receptor subunit GluA2 was lower (Fig. 3B, *P*=0.035), while GluA1 was unaffected. The change in expression of these subunits in synaptic membrane fractions was validated in an independent set of rats. The expression of the synaptic scaffold protein PSD-95 did not change after social defeat (Fig. 3B).

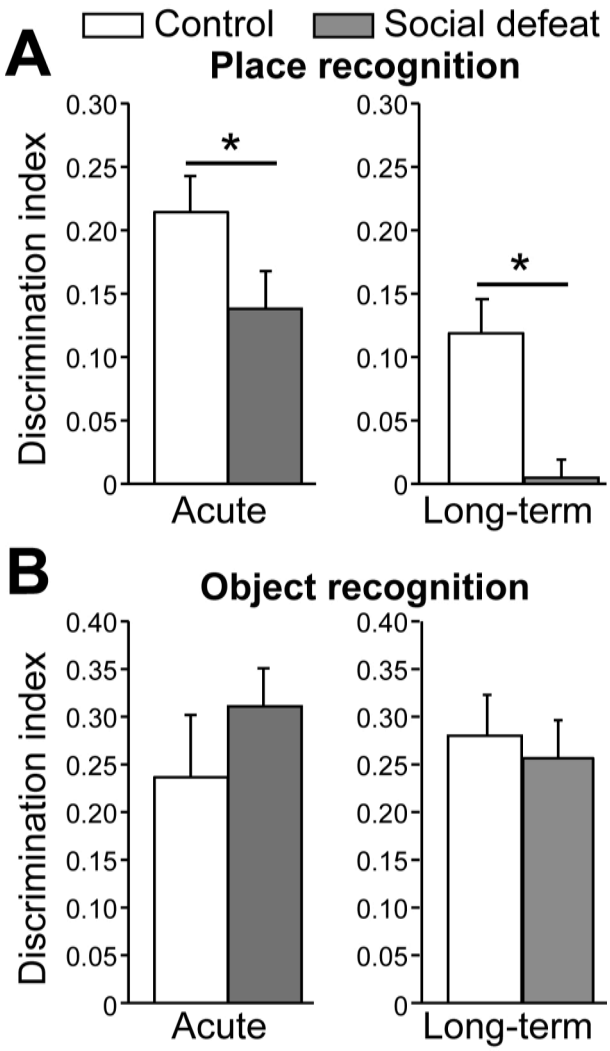

Figure 2. Repeated social defeat impairs spatial memory performance. Either acute (24 h) or long-term (3 months) after the last social defeat encounter object place recognition (A) and objects recognition (B) were assessed during the test phase. (A) Social defeat stress significantly suppressed object place recognition both acutely as well as long after social defeat. Note the difference in task performance between 3-month vs. 6-month old rats. (B) Social defeat stress did not affect performance on object recognition in either group. Data presented are mean+SEM of the discrimination index; n=12 for all groups. Unpaired, two-tailed, Student's t-test: **P*<0.05.

We then measured the expression of synaptic glutamate receptor subunits 3 months after social defeat stress. Despite the reported change in LTP at this moment[140], we did not find any change in levels of the glutamate receptor subunits, nor in the level of PSD-95 (Fig. 3).

Discussion

Here, we show that repeated social defeat stress for 5 consecutive days resulted in a hypothalamic-pituitary-adrenal (HPA) axis response, expressed by an increase in plasma levels of the stress hormone corticosterone 24 hours after cessation of the stressor. This 5-day social defeat exposure also reduced performance in a hippocampus dependent spatial memory task, and this was associated with reduced synaptic expression of the glutamatergic receptor subunits GluN1, GluN2B and GluA2. However, long-term after social defeat the

spatial memory deficit remained in the absence of elevated corticosterone levels and changed glutamate receptor expression.

Synaptic expression of GluN1, GluN2B and GluA2 is reduced acutely after social defeat stress, and associated with the deficit in spatial memory performance. Since GluA2 endocytosis underlies NMDAR-induced LTD[124,147], it is likely that the reduction in synaptic GluA2 expression acutely after repeated social defeat stress induces hippocampal CA1 LTD. This in turn might contribute to the observed memory impairments. The regulation of GluN1 and GluN2B levels acutely after social defeat stress is less straightforward to explain, especially since the number and subunit composition of synaptic NMDARs have long been considered to be quite static[148]. However, recent evidence shows that neuronal activity drives NMDAR synaptic targeting and incorporation, receptor retrieval, and lateral diffusion between synaptic and extrasynaptic sites[149]. An emerging concept is that activity-dependent, bidirectional regulation of NMDAR trafficking provides a dynamic and potentially powerful mechanism for the regulation of synaptic efficacy and remodeling. Indeed, it has been shown that alterations in NMDAR number and/or subunit composition contribute to the expression mechanisms of LTP (NMDAR-LTP)[150] and LTD (NMDAR-LTD)[151]. Low frequency stimulation and mGluR group I agonists induce NMDAR-LTD at CA1 synapses in hippocampal slices and the internalization of NMDARs in hippocampal neurons[152]. This might explain the reduced synaptic expression of GluN1 and GluN2B directly after social defeat stress. Alternatively, synaptic over-activation, accelerating the turnover rate of a subset of receptor subunits, such as GluN1 and GluN2B, may change the relative abundance of these subunits and decrease their levels in highly active synapses[153].

Stress-induced alterations in NMDAR-mediated synaptic strength are of great interest in that they involve potentiation of the plasticity trigger itself and, as such, would be expected to critically influence metaplasticity. This supports the current view[37] of stress-induced plasticity in which on the short term stress-induced hormones facilitate strengthening of contacts involved in the formation of memories directly associated to the stressor. As such a mild stressor enables avoidance learning of stressful associations. However, high levels of corticosterone and severe chronic stress suppress hippocampal LTP and promote LTD, with a delay of at least an hour. This would lead to an increased threshold for synaptic strengthening of input from other sources, thereby dampening the aversive learning process. Here, we show that repeated social defeat stress decreased expression of specific NMDA and AMPA receptor subunits. This might potentially underlie the process of metaplasticity and affect hippocampus-dependent learning.

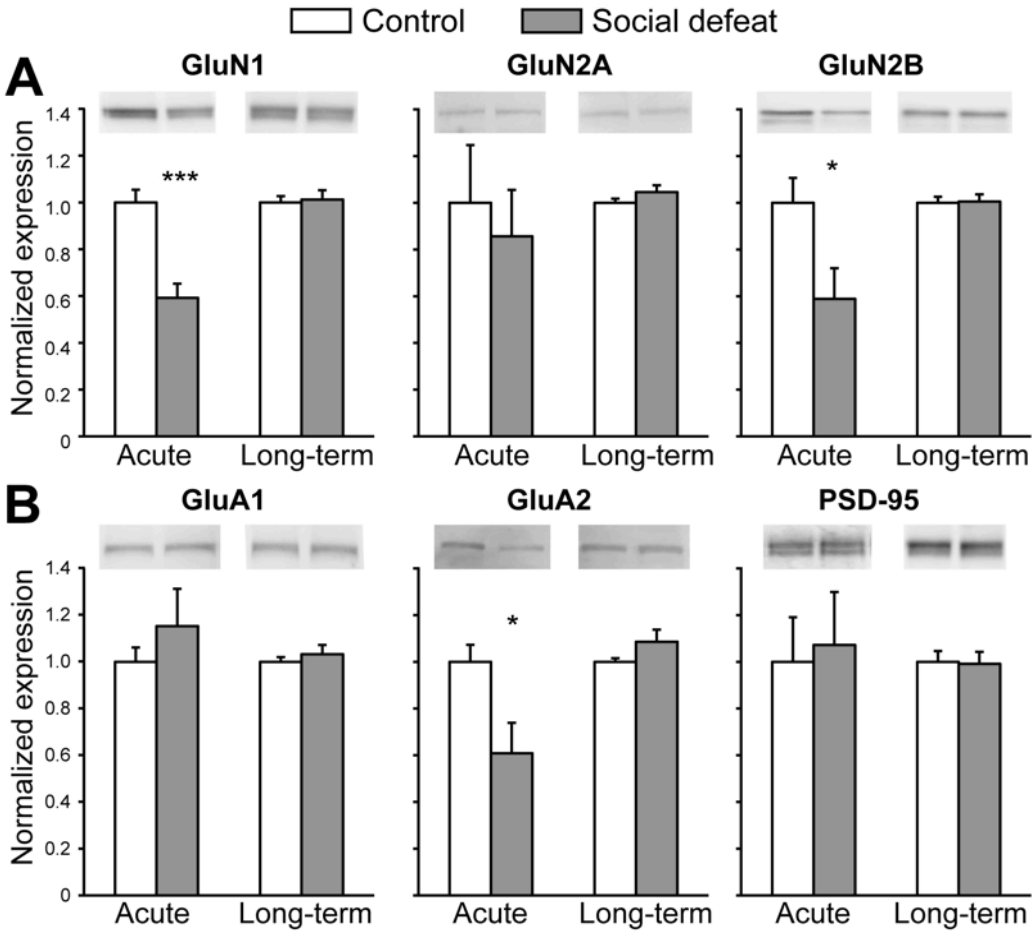

Figure 3. Repeated social defeat stress alters synaptic expression of Glutamate receptor subunits in the dorsal hippocampus. Either acute (24 h) or long-term (3 months) after the last social defeat encounter the dorsal hippocampus was dissected for synaptic membrane proteome analysis. (A – F) Quantitative Western blot analysis of glutamate receptor subunits revealed a downregulation of NMDA receptor subunits GluN1 (A) and GluN2B (C), while GluN2A was unaffected (B) acutely after social defeat stress, with no differences long-term after social defeat. In addition, GluA2 was downregulated (E), while GluA1 was unaffected (D) acutely after social defeat stress, with no differences long-term after social defeat. The marker of the postsynaptic density PSD-95 was unaffected at any time point after social defeat stress (F). Data presented are mean+SEM; n=6 for all groups. Unpaired, two-tailed, Student's t-test: **P*<0.05, ****P*<0.001.

We found that differences in levels of glutamate receptors were absent months after social defeat. However, at this time point hippocampus-dependent spatial memory function was still impaired. Also, previously it was shown that at this time point hippocampal LTP was affected[140]. Thus, despite the changes in hippocampal physiology and hippocampus-dependent task performance, the plasticity of ionotropic glutamate receptors expression at the synaptic membrane is not affected. There are several possible explanations for this outcome. For example, glutamate receptors, or their interacting proteins, may have undergone persistent posttranslational modifications that were not studied here. In particular, more in depth molecular analysis of the hippocampal synaptic proteome of rats 3 months

after social defeat may shed light on this. Alternatively, these effects could be caused by changes in hippocampal circuitry, such as altered neuronal morphology or reduced neurogenesis[18,36,142]). Taken together, these results show that different synaptic mechanisms are involved in deficits in hippocampal plasticity and spatial memory performance long and short after social defeat stress.

Chapter 4

Behavioral therapy rescues cognitive impairments, reduced hippocampal LTP and increased synaptic AT1B2 expression long after social defeat

Submitted

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Abstract

Depressed patients often persistently suffer from comorbid cognitive impairments, such as deficits in explicit memory. Here, we modeled persistent affective depression symptoms and memory deficits in a social defeat paradigm in rats. We found that hippocampus-dependent cognitive impairments were still apparent three months after social defeat stress. This maintenance phase of depression was characterized by increased levels of the Na,K ATPase subunit AT1B2 in hippocampal synapses and by a reduced potential to evoke hippocampal CA1 long-term potentiation (LTP). The antidepressant imipramine normalized the level of AT1B2 and rescued the hippocampal LTP deficit and cognitive decline. Most importantly, we found that behavioral therapy, consisting of daily housing in an enriched environment for one hour, thereby modeling positive activities and exercise, was just as effective as imipramine treatment in relieving all molecular, cellular and behavioral aspects of the depression-like state.

Introduction

Major depressive disorder (MDD) is a complex neuropsychiatric syndrome. Although MDD is most markedly typified as a mood disorder, with strong affective symptoms, most MDD patients suffer from associated cognitive impairments, such as disturbances in explicit and spatial memory[16,130]. Thus, MDD has debilitating properties both in the affective and cognitive domains, eliciting questions concerning underlying mechanisms and adequate therapies.

Stress is a potent causal factor in eliciting MDD. Over the years, compelling evidence has linked cognitive deficits in MDD to reduced hippocampal volume[12,154], and impaired hippocampal function[155]. In line with this, rodent models of depression involving acute stress showed morphological changes in the hippocampus, e.g., neuronal atrophy and reduced neurogenesis, plasticity changes, e.g., reduced long-term potentiation (LTP), and stress-induced reduction of dorsal hippocampus-dependent spatial learning (reviewed in[35,156]. However, paradigms with clinical validity, modeling and examining the maintenance phase of depression weeks to months after initial stress exposure, are scarce. To assess the long-term effects of stress exposure on cognitive function, to reveal its underlying synaptic mechanisms, and to investigate treatment options, we adopted a social defeat paradigm in rats, in which five daily encounters of social defeat were followed by individual housing for a period of 12 weeks[33]. This particularly enabled us to model long-term stress effects, as social isolation would appear to be particularly relevant to certain subtypes of human depression[157], and moreover, a combination of active and passive stress has strong precipitating effects on the development of the disease[84]. Importantly, the majority of stress stimuli that lead to MDD are of psychosocial nature[82],

This stress paradigm has been shown to cause insensitivity to rewards[33], resulting in impaired reward anticipation behavior indicative of an anhedonic state, a core symptom of depression, that can be counteracted by chronic antidepressant therapy[141]. In the present study, the long-term effects of social defeat stress on dorsal hippocampus-dependent cognitive performance were addressed. We examined the cellular plasticity mechanisms contributing to these impairments, and we investigated the changes in the synaptic proteome long after social defeat stress exposure. Since in humans, physical exercise and positive psychosocial activities can improve cognitive function, reduce depressive symptoms and increase stress resiliency[158], we questioned whether, next to pharmacotherapy, behavioral therapy would alleviate impairments in the social defeat paradigm at the molecular, cellular and behavioral level.

Materials and methods

Animals

Male Wistar rats (Harlan, Horst, The Netherlands) 8 weeks of age, weighing 180–200 g at the time of arrival were initially socially housed (2 per cage) in Makrolon type IV cages (Tecniplast, Milan, Italy) in a temperature-controlled room (21±1 °C) under regulated lighting conditions (lights on at 7:00 p.m. and off at 7:00 a.m.). Food and water were available *ad libitum*. Long-Evans male rats (Harlan, UK), weighing 300–350 g were used as residents for social defeat[141]. These animals were pair-housed with age-matched sterilized females in plastic cages (63 x 25 x 33 cm) located in a separate room. Housing conditions were the same as for Wistar rats. All experimental manipulations were conducted during the dark phase (activity period) under a dim red light. All experiments were approved by the Animal Users Care Committee of the VU University Amsterdam.

Experimental design and treatment

Wistar rats (age ≥ 11 weeks) of the social defeat group were subjected to 5 days of social defeat stress and were then housed individually for three months in macrolon class III cages from the first defeat onwards, as described before[140] (Supplemental Fig. 1). Control rats were housed in pairs. The social defeat procedure consisted of daily resident–intruder interaction sessions using dominant male Long-Evans rats for five consecutive days. Control animals were handled daily.

During the last three weeks of this social isolation, rats were treated by gavage administration of the antidepressant imipramine (20 mg/kg per 0.5 ml water; Sigma- Aldrich, Germany), behavioral therapy (BT), consisting of housing in an enriched environment for one hour every day, or water as control. This 2 x 3 design resulted in six experimental groups: (1) control rats with water (Control+H20), (2) control animals with chronic imipramine treatment (Control+IMI), (3) control animals with BT (Control+BT), (4) socially defeated animals with water (Defeat+H20), (5) socially defeated animals with chronic imipramine treatment (Defeat+IMI), and (6) socially defeated animals with BT (Defeat+BT). All behavioral, electrophysiological and molecular analyses were performed at the end of treatment, unless stated otherwise.

Reward anticipatory behavior

A classical Pavlovian conditioning setup was used to investigate anticipatory behavior, as described earlier[140]. To investigate the behavioral response to the conditioning stimulus

(repetitive sound (keyboard) and light flashes (three times)), animals were observed before training (trial 0) to determine baseline activity, and again after 35 training trials of pairing with a 5% sucrose-reward, using the computer program 'The Observer' (Noldus Information Technology, Wageningen, The Netherlands). The researcher who analyzed the behavioral data had no knowledge of the experimental groups. Differences in activity (reflected by frequency or transitions of behavioral elements) displayed before training compared with those after training were used as parameter for reward anticipation.

Sucrose preference

The preference for sucrose (5%) was measured in a two-bottle (sucrose and water) consumption test. Consumption was assessed after 24 h by reweighing the pre-weighted bottles. After 2 days, the consumption test was repeated. In case of social housing, consumption for each subject was set to half of the total consumption. Sucrose preference was expressed as the increase in consumption (gram) relative to water (gram), and this difference was represented as percentage of the total consumption (gram) [100% x (Δ sucrose–water)/total volume sucrose and water consumed].

Recognition tests

Recognition memory testing was conducted based on two previously reported tasks relying on spontaneous exploration of objects in an open field[143,144]. All testing was carried out in a rectangular arena, 79 x 57 in surface with 42 cm tall black walls. The box was surrounded by visual cues: computer light coming from the west side; a white wall (north); a metal rack (east side); and an open space where the experimenter was sitting (south). The arena was always placed inside the room at the same location and in the same orientation. All rats were habituated to the empty arena twice for 30 minutes on the two days preceding behavioral testing, as well as on the test day for 10 min and 1 min prior to the sample phase.

Object-recognition task – Round or square aluminum bars were used as novel or familiar objects and were chosen using a pseudorandom protocol, balanced across treatment groups. In the 4-minute sample phase, rats were exposed to two identical objects (round or square metal bars) followed by a 15 min inter-trial interval (Supplemental Fig. 2). In the test trial, one object (chosen using a pseudorandom protocol, balanced across treatment groups) was replaced with a novel object and rats were allowed to explore both objects for 4 minutes (Supplemental Fig. 2). The familiar object was a third copy of the two objects seen in the sample phase, to prevent possible carry-over of olfactory cues. Each session was recorded by a video camera suspended above the field and interfaced with a computerized

tracking system using the 'Viewer' software package (BIOBSERVE, GmbH, Bonn, Germany). The nose of the rats was tracked by the 'Viewer' software and during both trials, the object exploration (defined as the time of the nose spent within 2 cm from the object) was measured for each object. Only rats that had accumulated at least 15 s of exploration at each object within the sample phase were included in the analysis (all but one tested met criteria). To further analyze object discrimination during the test trial, the discrimination index ((novel-familiar)/(novel + familiar)) was calculated for each rat using the individual object-exploration times recorded.

'Allocentric' object-place task – For the 4-minute sample phase, rats always entered the testing box from the south, and were placed facing the south wall. Rats were exposed to two identical objects (round aluminum bars) followed by a 15 min inter-trial interval. During test trials, rats entered the box from the east or west side and were placed facing the east or west wall, respectively. Thus, for each trial, the entry point on the sample and test phases were different. In the test phase a third and fourth copy of the same object were placed back in the arena: one in a familiar corner and one in a novel corner (Supplemental Fig. 2). The location of the novel object-place configuration was counterbalanced such that on each trial different corners were used as familiar and novel locations. Rats were now only able to discriminate between objects based on their location with respect to its surrounding. Further analysis of discrimination was the same as for the object recognition task.

Long-term potentiation (LTP) measurements

Rats were sacrificed by decapitation. Subsequently, brains were rapidly removed, and placed in ice-cold artificial cerebrospinal fluid (ACSF; in mM: NaCl 124, KCl 3.3, KH_2PO_4 1.2, $MgSO_4$ 1.3, CaCl2 2.5, NaHCO3 20 and Glucose 10.0, constantly gassed with 95% O_2/5% CO_2). Horizontal hippocampal slices were cut on a vibrating microtome at 400 µm thickness and then placed in a submerged-style holding chamber in ACSF, bubbled with carbogen (95% O_2, 5% CO_2). Slices were allowed to recover for 1 hour following slicing. A planar multi-electrode recording setup (MED64 system, Alpha Med Sciences Co., Ltd, Tokyo, Japan) was employed to record the field excitatory post-synaptic potential (fEPSP), and to study LTP as described in[159](see supplemental materials and methods).

iTRAQ-based Proteomics

To analyze differential expression of hippocampal synaptic membrane proteins between experimental groups, quantitative iTRAQ proteomics was performed. To this end, tissue preparation, iTRAQ labeling, two-dimensional liquid chromatography, MS/MS and protein

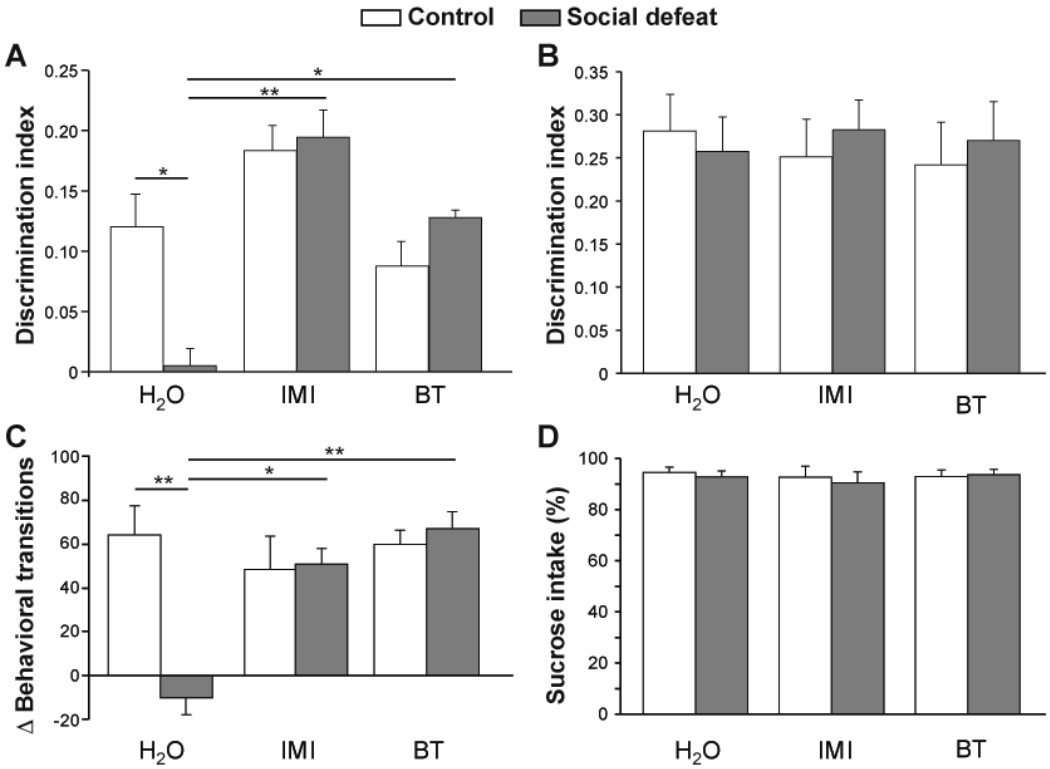


Figure 1. Social defeat impacts on cognitive and affective behavioral tasks. (A) Discrimination index during the test phase of an allocentric object place task. The social defeat paradigm (social defeat) significantly suppressed spatial memory performance, whereas behavioral therapy (BT) and imipramine treatment (IMI) reversed this long-term stress-induced effect (treatment F(1,47)=5.30, *P*=0.008;. n=12 for al experimental groups). (B) Discrimination index during the test phase of a novel object recognition task. Social defeat does not affect performance on this task. (C) Anticipation towards 5% sucrose expressed as the difference in activity in the CS-US interval post-training compared with pre-training. Social defeat significantly suppressed reward anticipation, whereas behavioral therapy and imipramine treatment reversed this effect (defeat x treatment interaction F(1,33)=5.48, *P*=0.0093). (D) Sucrose preference. Social defeat does not affect sucrose preference (sucrose intake – water intake)/total fluid intake (%). Data is presented as mean ± SEM. LSD *Post hoc*: **P*<0.05, ***P*<0.001.

identification and quantification were performed as described previously[145,160] (see supplemental materials and methods).

Immunoblotting

The total homogenate and synaptic membranes of the dorsal hippocampus was isolated from an independent group of animals (n=6) as compared to those used in iTRAQ. Samples (3 – 5 μg) were lysed in Laemli lysis buffer, separated by electophoresis on a Criterion 10.5–14% Tris-HCl sodium dodecyl sulfate-polyacrylamide precast gel (Bio Rad Laboratories), and blotted as described before[145]. The following antibodies were used: mouse anti-β-CaM Kinase II (ZYMED Laboratories; 1:1000), mouse anti-Sodium/potassium-transporting ATPase subunit beta-2 (Santa Cruz, 1:5000), mouse anti-Caseine Kinase IIβ (Santa Cruz,

1:1000), mouse anti-cAMP-dependent protein kinase catalytic subunit alpha (PKA) (Santa Cruz 1:1000).

Statistical analysis

The iTRAQ-based proteomics was performed in six biological independent experiments. Proper correction for multiple measurements was carried out in the R computing environment (1.81, Raqua) using the Linear models for Microarray data package[161] (Limma, 1.3.13), which is part of the Bioconductor project[162] (http://www.Bioconductor.org) with adjusted *P*-values[163]. For all other data, statistical analysis was performed using SPSS18.0. All results are expressed as group means ± SEM. Treatment effects were assessed with two-way analysis of variance (ANOVA), followed by Least-square difference (LSD) *post-hoc* analyses.

Results

Social defeat results in hippocampus-dependent cognitive impairments

Using a model of the maintenance phase of depression, in which 5 daily social defeat sessions were followed by individual housing for 12 weeks (Supplemental Fig. 1), we tested recognition memory based on spatial position and novelty of the object[144] (Supplemental Fig. 2). Social defeat decreased performance in the hippocampus-dependent object-place memory task (*P*=0.0034; Fig. 1A). Pharmacotherapy, in the form of imipramine treatment only during the last three weeks of the paradigm (Supplemental Fig. 1) was able to reverse this deficit, illustrating the predictive value of this model as antidepressants can also restore cognitive deficits in depressed patients[63]. Similarly, behavioral therapy (1 h daily enriched housing) was able to completely reverse these cognitive deficits (Fig. 1A), showing the potential of this type of treatment. Pharmacological and behavioral therapies had no effect in control animals. Socially stressed rats showed no difference in object-recognition, a task that is hippocampus-independent. Also there was no effect of treatment in control groups in this task (Fig. 1B). Thus novelty preference and discriminative ability were similar among all groups.

We confirmed that socially defeated rats display a deficit in the affective domain, i.e. an anhedonic phenotype[33,140,141], as shown by a reduced anticipation towards a 5 % sucrose solution compared with control rats (*P*=0.0034). This increase in anhedonia was reversed to control levels by behavioral therapy (Fig. 1C). In addition, a similar reversal was observed

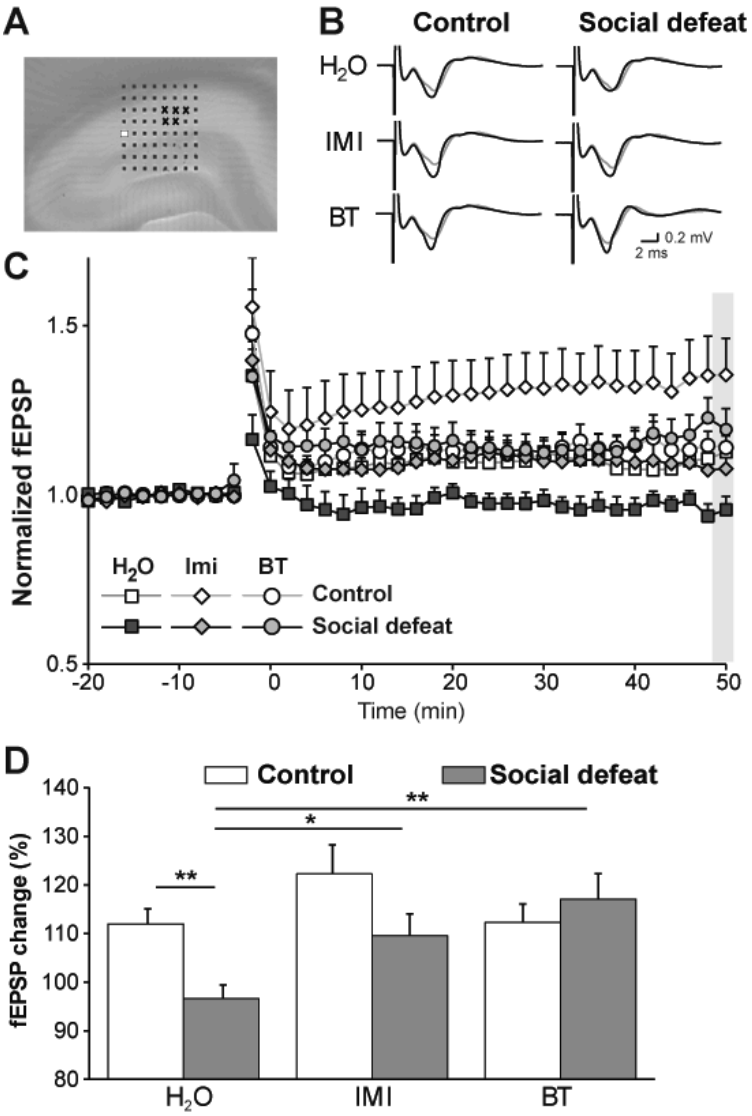


Figure 2. Effect of long-term social stress on LTP in the CA1 of the dorsal hippocampus. (A) Coronal section of the dorsal hippocampus on a MED64 electrode array. An electrode in the Schaffer collateral pathway (white) was used as stimulating electrode. Field potentials were recorded and averaged for the electrodes in the dendritic field of CA1 (marked x). (B) Representative traces from one experiment recorded before (gray) and after (black) LTP induction. (C) Time course of percentage change in fEPSP measured before and after HFS (arrow). (D) Change in fEPSP 50 min after LTP induction (gray indicated in C). Social defeat significantly suppressed CA1 LTP, whereas behavioral therapy (BT) and imipramine treatment (IMI) reversed this effect (defeat: $F(1,45)=4.08$, $P=0.049$; treatment: $F(1,45)=3.96$, $P=0.026$; n=6 for al experimental groups). Data is presented as mean ± SEM. LSD *Post hoc* $^{*}P<0.05$, $^{**}P<0.001$.

for the chronic administration of imipramine[141]. Neither treatment in control animals had an effect on anticipation towards 5 % sucrose. As reported previously, this long-term social defeat paradigm had no significant effect on sucrose preference[164], and no side effects were observed by behavioral therapy or imipramine (all $F(1,30)<0.2$, $P>0.80$, Fig. 1B). Also, no difference in plasma corticosterone levels was observed between any of the groups (all $F(1, 30)<0.2$, $P>0.90$, Supplemental Fig. 3), indicative of the absence of an acute stressor.

Reduction in LTP by long-term social stress is reversed by imipramine and behavioral therapy

As imipramine treatment and behavioral therapy were both capable of rescuing cognitive performance on a hippocampal-dependent memory task, we questioned whether behavioral therapy was able to recover reduced LTP in this long-term social defeat paradigm[140]. Using

a microelectrode array in the Schafer collateral pathway going from CA3 to CA1 subfields of the dorsal hippocampus (Fig. 2A) we applied a tetanus of field potentials. In control rats, average spike amplitude was increased to a response of 113% in CA1 at 50 min after LTP induction (Fig. 2C). LTP was significantly reduced after social defeat (*P*=0.019). This reduced potential to elicit full LTP in socially defeated rats was reversed to control levels by both imipramine and behavioral therapy. Imipramine treatment in controls slightly enhanced LTP, whereas behavioral therapy alone had no side effect on LTP maintenance.

LTP is well-known to be dependent on a the subunit-specific regulation of the AMPARs, during initiation and maintenance phases. In various paradigms, stress has been shown to impact directly on the basal levels of AMPAR subunits, thereby modulating LTP induction and maintenance[43,165,166]. Also, we find that immediately after social defeat AMPAR subunits are regulated (See Chapter 3, Fig. 3). Thus a straightforward explanation of the fact that, long after social defeat stress, LTP induction is impaired is that synaptic AMPAR levels are not at baseline. However, in contrast we find that synaptic expression of the glutamate receptor subunits is normal at this time point (See Chapter 3, Fig. 3). These results prompted us to further investigate the synaptic proteome long after social defeat stress and investigate alternative modes of synaptic modulation.

Reduced spatial performance and CA1 LTP are associated with an increased synaptic expression of AT1B2

To reveal the molecular basis of synaptic plasticity dysfunction that underlies decreased spatial memory after social defeat stress, we compared the proteomes of hippocampal synaptic membrane fractions of socially stressed and control rats. We used iTRAQ labeling of tryptic digests of synaptic proteins, separated these with 2D LC, and identified and quantified peptides using MS/MS (Supplemental Fig. 4). Previously, we used this method to detect subtle changes in synaptic membrane protein abundances in other animal models of disease[145,167]. In total, 382 proteins were identified with at least 3 distinct peptides with a confidence of ≥ 95% present among experimental groups (data not shown). To correct for multiple measurements *P*-values were adjusted to control for the false discovery rate[163]. Five proteins were significantly regulated (adjusted *P*-value <0.05) after social defeat stress (Supplemental Table 1). Differential expression of three of these proteins was confirmed by quantitative immunoblotting in the same sample set (Supplemental Fig. 5, middle panel); Sodium/potassium-transporting ATPase subunit beta-2 (AT1B2), cAMP-dependent protein kinase catalytic subunit alpha (PKAα), and Casein kinase II subunit beta (CSK2B). Of these three proteins AT1B2 was confirmed as upregulated (51%) by social defeat in a biologically

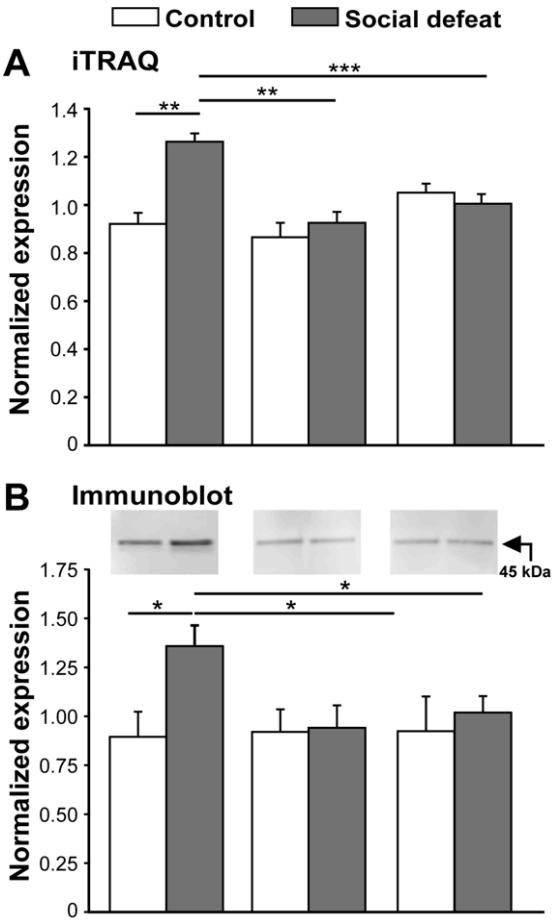


Figure 3. Long-term social stress increases synaptic expression of AT1B2 in the dorsal hippocampus. At the end of treatment, the dorsal hippocampus was dissected for synaptic membrane proteomics analysis. (A) Quantification of iTRAQ reagents revealed a significant higher levels of AT1B2 after long-term social stress (social defeat). Behavioral therapy (BT), and imipramine treatment (IMI) reversed the stress-induced increase in synaptic expression of AT1B2 (stress $F(1,33)=8.05$, $P=0.009$; treatment $F(1,45)=8.58$, $P=0.002$;, stress x treatment interaction $F(1,33)=8.97$, $P=0.001$; see Supplemental Table 1 for adjusted p-values; n=6 for al experimental groups.) (B) Regulated synaptic expression of AT1B2 was validated in an independent set of rats by quantitative immunoblot analysis (stress x treatment interaction: $F(1,45)=4.08$, $P=0.049$; n=8 for al experimental groups). *Insets:* representative example of immunoblots showing the regulated synaptic expression of AT1B2 (45 kDa). Data is presented as mean ± SEM. LSD *Post hoc*: $*P<0.05$, $**P<0.01$, $***P<0.001$.

independent set of animals ($P=0.007$; Fig. 3B, Supplemental Fig. 5, lower panel). At the level of gene expression there was a trend for downregulation of this transcript ($P=0.133$; 16%).

To substantiate that dysregulation of AT1B2 is associated with the animal's depressed state, we measured the effects of both imipramine treatment and behavioral therapy on AT1B2 levels. Indeed, we found that both treatments normalized the increased synaptic expression of AT1B2 after social stress, bringing it back to basal levels (Fig. 3A,B), with no additional effect of treatment alone.

Discussion

Cognitive deficits associated with human depression have been well-characterized[168], with reductions in hippocampus-dependent declarative and spatial memory[16,130]. Recently a link between these reported cognitive impairments and hippocampal function was shown[155], giving way to a hippocampus-dependent symptomatology of depression. In the present study, we showed that in a preclinical model of the maintenance phase of depression, impairments in the affective and cognitive domains are persisting over a long period of time. Because depressed patients still experience symptoms long after the cessation of stress

exposure[82], the long-term social defeat model has a high level of both etiological- and face-validity. Behavioral therapy was able to restore the social defeat-induced deficits from the molecular level all the way up to behavior, similar as achieved by pharmacotherapy using the tricyclic agent imipramine. This indicates that apart from having predictive validity, the present model might also have construct validity, i.e., at the level of the mechanism underlying cognitive core symptoms of MDD.

The reduction in hippocampus dependent memory long after social defeat was found associated with a reduction in the capacity to elicit hippocampal CA1 LTP. This form of synaptic plasticity is known to involve plasticity of glutamate receptors. On the short term, stress-induced effects on LTP are dependent on NMDA receptors[133] and insertion of AMPA receptors[165,169]. Also, stress hormones have been shown to regulate the mobility and postsynaptic membrane levels of AMPA receptors[135]. Accordingly, immediately after social defeat stress glutamate receptor levels are altered. However, here we show that the social defeat-induced impairment of LTP on the long term is not caused by pre-altered synaptic expression of glutamate receptor subunits, as their levels are not affected long after social defeat. Instead, our proteomics analysis showed the increased synaptic expression of a Na,K-ATPase subunit AT1B2 that might well influence the induction of LTP. Importantly, the reversal of AT1B2 to normal levels due to imipramine or behavioral therapy indicates that dysregulation of AT1B2 is indeed related to the disease phenotype. Taken together, our data suggests that on the long-term, different synaptic mechanisms are involved in causing LTP impairments when compared to acute stress.

One might wonder whether the observed changes in the cognitive domain are a consequence of individual housing per se, because social isolation is considered a social stressor by itself[83]. However, at the end of the social defeat paradigm, stress hormone levels have normalized, indicative of the absence of acute stressors. Also, the social defeat-induced changes in basal physiology, i.e. body weight and food/water intake, have already returned to basal levels during the isolation period (Supplemental Fig. 6). Moreover, we showed that hippocampal CA1 LTP is not affected by individual housing without previous social defeat (Supplemental Fig. 7). Thus individual housing after social defeat apparently does not result in a severe stress experience that affects basal physiology and neuronal plasticity.

The observation that reduced CA1 LTP is associated with increased synaptic expression of AT1B2 has interesting implications for the molecular mechanism underling the cognitive deficits observed after social defeat. AT1B2 is a β subunit of the P-type Na,K ATPase family. The protein complex is a heterodimer composed of two subunits: the

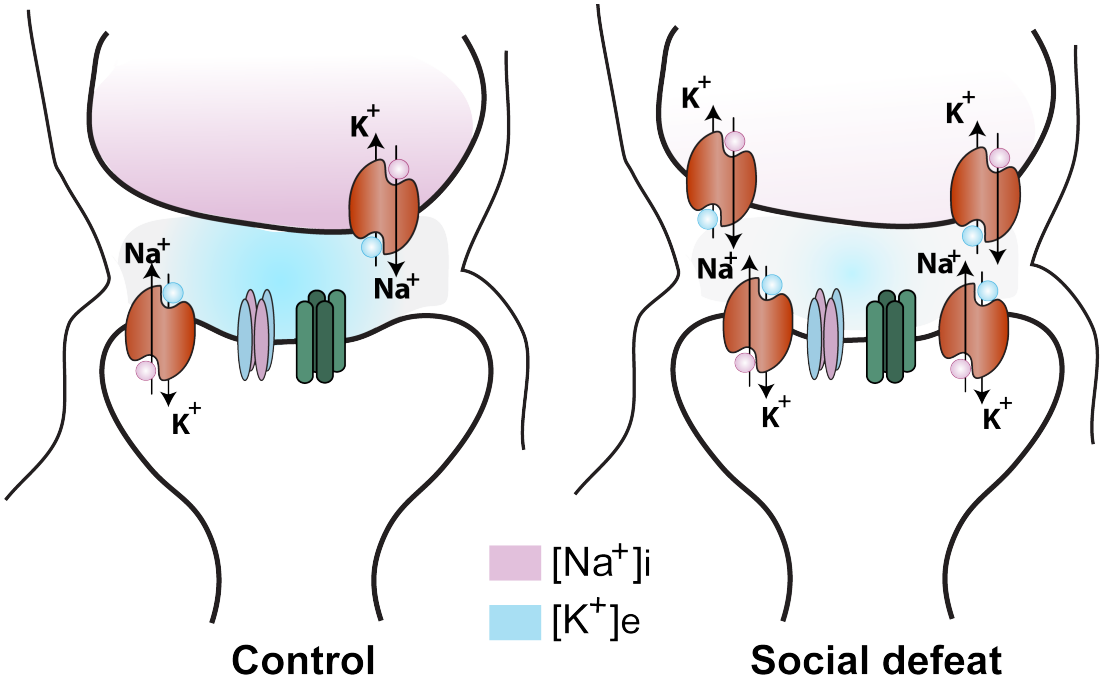


Figure 4. Model of the hippocampal synaptic adaptation after long-term social defeat stress associated with reduced LTP and impaired spatial memory. After social defeat stress and subsequent individual housing, the synaptic levels of subunit AT1B2 of the Na^+/K^+ transporter is increased by ~50%. This increased expression likely causes an increased transporter activity (shown by an increased number of ion pumps), thereby faster clearance of potassium from the synaptic cleft ($[Na^+]_i$), and of sodium from the synaptic compartments ($[K^+]_i$). This causes faster membrane re-polarization after firing of an action potential, and therefore less glutamate release and calcium influx. Together, increased levels of Na^+/K^+ transporter causes less plasticity in hippocampal synapses that might underlie impaired LTP.

catalytic α subunit that contains ATPase activity and the β subunit that regulates, through assembly of alpha/beta heterodimers, the number of sodium pumps transported to the plasma membrane[170,171]. Na,K ATPases are expressed throughout neurons, i.e., at neuronal cell bodies and dendrites, and are found enriched in synaptosomes[172]. They are membrane localized transporters responsible for active transport of Na^+ and K^+ ions across the plasma membrane, thereby generating a gradient responsible for the neuronal rest membrane potential, but also its repolarization after eliciting action potentials[173]. Increased levels of the regulatory subunit AT1B2 after social defeat on the presynaptic site, and hence increased numbers of Na^+/K^+ transporters, will lead to a faster clearance of Na^+ ions from the synaptic spine and influx of K^+ ions from the synaptic cleft (Fig. 4). Consequently, after generation of an action potential, increased pump activity will induce a faster repolarization, resulting in less glutamate release from the presynaptic site and longer after-hyperpolarization[174]. As a result, there will be less activation of postsynaptic glutamate receptors and thus less

induction of plasticity postsynaptically, reflected by reduced LTP. In line with presynaptic effects, increased numbers of Na^+/K^+ transporter post-synaptically will reduce building up a membrane depolarization, counteracting postsynaptic plasticity. The involvement of the Na^+/K^+ transporter in synaptic plasticity has been demonstrated in Leech and Drosophila[170,174,175]

Other subunits of the Na,K ATPase complex, e.g., AT1A1 subunits, have been implicated in antidepressant response, i.e., the effect of lithium[176] and in stress-induced animal models of depression[177]. In this perspective it is noteworthy that a trend of higher synaptic expression has been found for other subunits of this complex, e.g., AT1A2 (*P*=0.095), AT1b1 (*P*=0.170; Supplemental Table 2). Also, most of these subunits show decreased levels of expression due to behavioral therapy, and to a lesser extent due to imipramine, in socially defeated animals.

AT1B2 has been found regulated at the level of gene expression both in clinical and preclinical studies. Lower levels of AT1B2 transcript were observed in the hippocampus of depressed individuals[178], as well as in the hippocampus of a genetic model of endogenous depression, i.e., the Wistar-Kyoto strains[179]), similar to the lower transcript levels observed in this study. However, we find the AT1B2 protein at higher levels in the synaptic membrane fraction, indicating that caution should be taken when translating gene expression data into synaptic protein levels and further into a clinical perspective.

Our finding that the beneficial effects on cognitive domains of both chronic imipramine treatment and behavioral therapy are associated with a recovery to normal levels of synaptic AT1B2 holds important promises for intervention at Na,K ATPase-related mechanisms in the maintenance of depressive symptoms and their treatment. Obviously, application by pharmacological intervention at Na,K ATPases is complicated considering that after social defeat its expression was found only increased at synaptic sites and not in total cell lysates. Moreover, its ubiquitous expression throughout the body further precludes direct pharmacological interference. Fine-tuning the level of these transporters might be crucial, as too low levels could induce anxiety symptoms and increases stress-induced memory impairments in mice[177].

Behavioral therapy, consisting of housing in an enriched environment for one hour daily, thereby modeling positive activities and exercise, was effective in restoring stress-induced long-lasting cognitive impairments and its underlying cellular and molecular deficiencies. Also, in the affective domain, behavioral therapy restored the social defeat-induced reduction in reward anticipation (anhedonia). These findings, when translated to the human condition have important implications for treatment strategies in the clinic. Several forms of

psychotherapy –in particular, cognitive and behavioral therapies– were shown effective for patients with mild to moderate MDD[180], and the combination of medication and psychotherapy can exert a synergistic effect. Our study implies that, in addition to pharmacological treatment, with often bearing negative side effects, another form of behavioral therapy, i.e. physical exercise, should be explored further as treatment option for depressed patients with cognitive impairments and anhedonia.

Supplemental material

Material and methods

Social isolation

Wistar rats (age ≥ 11 weeks) of the social Isolation group were housed individually for three months in macrolon class III cages. Control rats were housed in pairs.

Corticosterone assay

Trunk blood samples were collected via decapitation between 9:00 am and 11:00 am. Samples were collected into a 7-mL heparin-coated tube (Greiner Bio-One, Monroe, North Carolina) and kept on ice. The samples were spun at 1000 x *g* for 10 min. Plasma was decanted and stored at −80 °C until the assay was used. Levels of serum corticosterone were assessed using a rat Glucocorticoid (GC) ELISA kit (Cusabio Biotech Co., LTD), according to the manufacturers instructions.

LTP recording

A planar multi-electrode recording setup (MED64 system, Alpha Med Sciences Co., Ltd, Tokyo, Japan) was employed to record the field excitatory post-synaptic potential (fEPSP), and to study LTP. The methodology has been described in detail elsewhere [159]. Briefly, hippocampal slices were placed on special probes that were fabricated with 8 x 8 electrode arrays and pre-coated with polyethylenimine (PEI, Sigma). P210A probes (Alpha Med Sciences) with an inter-electrode distance of 100 µm were used. Correct placement of the electrodes at the CA3–CA1 region was done manually, monitored by a microscope (MIC-D, Olympus Ltd., Japan) (fig. 2A). Four slices per rat were studied simultaneously. Each slice was superfused by 100 ml oxygenated ACSF, which was recirculated at a flow rate of 2 ml/min. fEPSPs were recorded from multiple electrodes in the dendritic layer of CA1 neurons by choosing an electrode in the Schaffer collateral pathway as the stimulating electrode. Based on the stimulus–response curve, we chose a stimulation intensity that evoked the fEPSP with a magnitude of 50% of the maximum response (around 1 mV in most cases). We found that this setting was suitable for the induction of LTP in healthy slices in the setup. After allowing a stable baseline of 20 min, an induction protocol that evoked LTP was applied, which consisted of 2 trains of 100 Hz stimulus that lasted for 1 s, separated by 10 seconds. The field potential response was recorded for 1 h after the tetanus. LTP was quantified as % change in the average amplitude of the fEPSP taken from 50 to 60 min interval after LTP induction. LTP in al electrodes within the CA1 region was averaged, and average LTP of all 4 slices was defined as final LTP per individual rat.

iTRAQ-based Proteomics

Tissue preparation – Following decapitation, brains were removed and rapidly frozen in ice-cold isopentane and stored at –80 °C until further use. The dorsal hippocampus (Bregma –2.56 till –5.30)

was removed freehand at –20 °C from 1-mm-thick slices. Synaptic membrane fractions were isolated for every hippocampus (left and right pooled). Samples were homogenized in ice-cold 0.32 M sucrose (5% of homogenate was collected as total cell lysate) and then centrifuged at 1000 g for 10 min. The supernatant was loaded on top of a sucrose gradient consisting of 0.8 and 1.2 M sucrose. After centrifugation at 100,000x g for 2 h, the synaptosome fraction at the interface of 0.85⁄1.2 M sucrose was collected and then lysed in hypotonic solution. The resulting synaptic membrane fraction was recovered by centrifugation using the sucrose step gradient as stated above. The synaptic membrane fraction was collected from the 0.85⁄1.2 M interface and protein concentrations were determined using a Bradford assay (Bio-Rad, Hercules, CA, USA). For each sample, 75 μg of protein was used for iTRAQ labeling (see below), and synaptic membrane fractions were dried in a SpeedVac overnight.

iTRAQ labeling – Synaptic membranes were resuspended in 28 μL of dissolution buffer and 2 μL of cleavage reagent [iTRAQ reagent kit, with 0.85% RapiGest (Waters Associates, Milford, MA, USA)] to solubilize synaptic membranes. After incubation for 1 h, 1 μL of cys blocking buffer (Applied Biosystems, Carlsbad, CA, USA) was added and vortexed for 20 min. Next, 10 μL of trypsin (Promega) dissolved in water was added and incubated overnight at 37 °C. Trypsinized peptides from each experimental group were then tagged with iTRAQ reagents (113, CON+H_2O; 114, CON+IMI; 115, CON+BT; 116, Stress+H2O; 117, Stress+IMI; 118, Stress+BT) dissolved in 80 μL ethanol. After incubation for 3 h, the six samples were pooled and acidified with 10% trifluoroacetic acid (TFA) to pH 2.5 – 3.0. After 1 h, the final sample was centrifuged and the supernatant dried overnight in a SpeedVac.

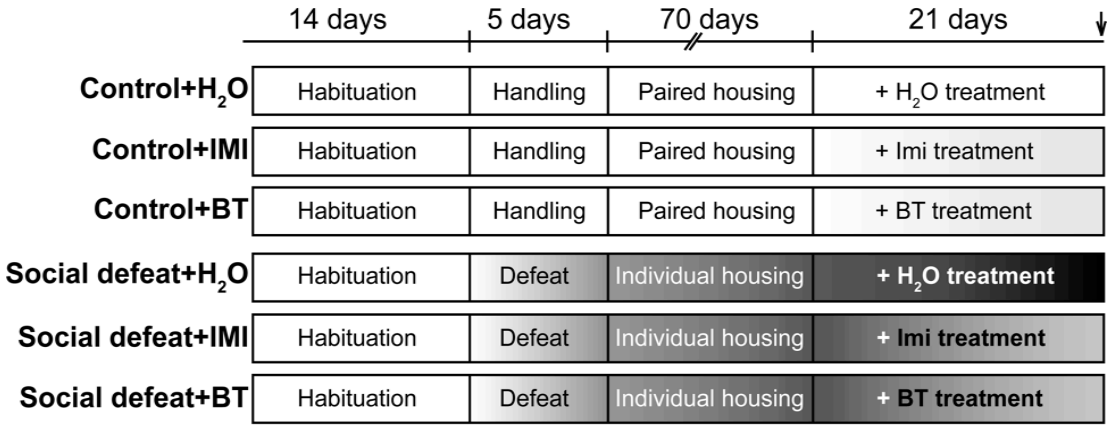
Two-dimensional liquid chromatography – The dried iTRAQ sample was dissolved in 300 μL of loading buffer (20% acetonitrile, 10 mM KH2PO4, pH 2.9) and loaded onto a polysulfoethyl A column (PolyLC, Columbia, MD, USA). Peptides were eluted with a linear gradient of 0–500 mM KCl in 20% acetonitrile, 10 mM KH2PO4, pH 2.9, over 25 min at a flow rate of 50 μL⁄min. Fractions were collected at 1-min intervals. In the second-dimensional liquid chromatography separation, peptides were delivered with a Famos autosampler (Dionex Corp., Sunnyvale, CA) at 30 μL⁄min to a C18 trap column (1 mm x 300 μm i.d. column) and separated on an analytical capillary C18 column (150 mm x 100 μm i.d. column) at 400 nL⁄min using the LC-Packing Ultimate system. Peptides were separated using a linearly increasing concentration of acetonitrile from 5 to 50% in 45 min, and to 90% in 5 min. The eluent was mixed with matrix (7 mg a-cyano-hydroxycinnaminic acid in 1 mL of 50% acetonitrile, 0.1% TFA, 10 mM dicitrate ammonium) delivered at a flow rate of 1.5 μL⁄min and deposited off-line to the Applied Biosystems metal target every 15 s for a total of 192 spots using a robot (Dionex, Sunnyvale, CA, USA).

Mass spectrometry – The Matrix-assisted laser desorption/ionization (MALDI) plates were analyzed on a 4800 proteomics analyzer (Applied Biosystems) and peptide collision induced dissociation (CID) was performed at 2 kV with nitrogen collision gas. MS⁄MS spectra were collected from 2500 laser shots. Peptides with a signal-to-noise ratio above 50 at the MS mode were selected

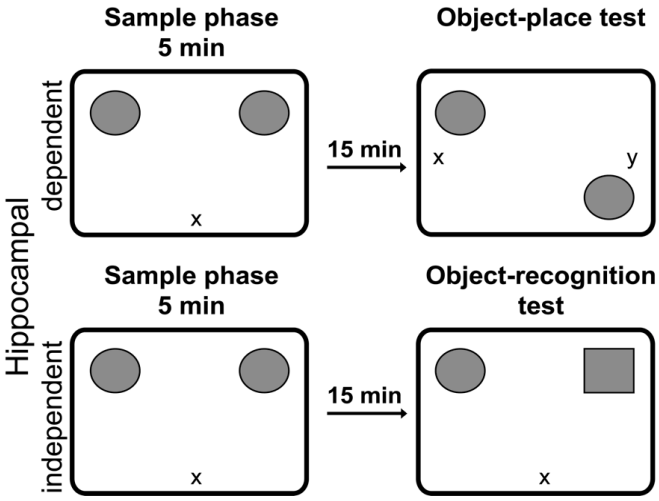
for MS/MS, at a maximum of 25 MS/MS per spot. The precursor mass window was set at 200 relative resolution full width at half maximum (FWHM).

Protein identification – The MS/MS spectra were searched against the rat database [Swissprot and National Center for Biotechnology Information (NCBI)] using GPS Explorer (Applied Biosystems) and Mascot (MatrixScience, Boston, MA, USA). A library was then generated containing all annotated peptides with a confidence interval score higher than 20%. Database redundancy and sequence redundancy were removed. Hence, quantification was performed only on those peptides that were annotated to a single protein, and are referred to as 'unique peptides'. Only proteins identified with ≥ 2 unique peptides, and of which at least one peptide had a confidence interval ≥ 95%, were selected for quantification and statistical analysis. All regulated proteins were analyzed with a large number of peptides, of which the majority was analyzed with high confidence.

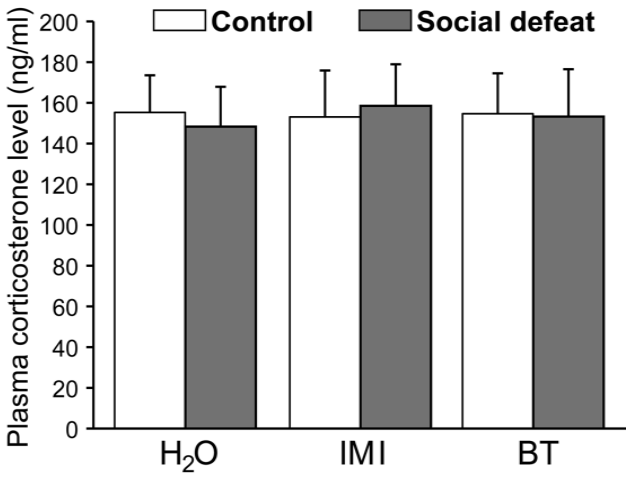
Protein quantification – The iTRAQ experiment was repeated six times with independent samples. The iTRAQ areas (*m/z* 113–118) were extracted from raw spectra and corrected for isotopic overlap using GPS Explorer. To compensate for the possible variations in the starting amounts of the samples, the individual peak areas of each iTRAQ signature peak were $\log_2$-transformed to obtain a normal distribution, normalized to the mean peak area for each sample, and finally standardized to the normalized peak average per peptide. The protein abundances in every experimental group were determined by taking the average normalized standardized iTRAQ peak area of all unique peptides annotated to a protein. In total, the iTRAQ-proteomics experiment was repeated six times with independent biological samples. Only proteins that were identified in five out of six replicates were included for further statistical analyses. For presentation of differential expression of synaptic proteins between groups, regulation was calculated by subtracting the average $\log_2$-transformed protein abundance of groups of interest. For presentation of absolute expression differences, exponents of these values were calculated.



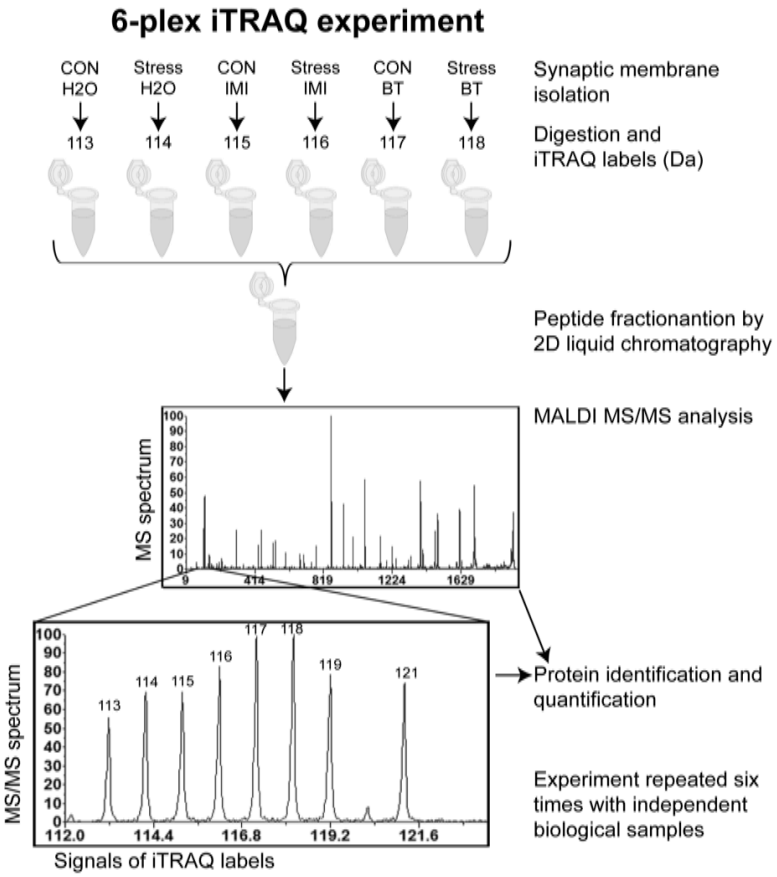
Supplemental figure 1. Experimental design and treatment groups. The social defeat paradigm (social defeat) in combination with behavioral therapy (BT) or imipramine treatment (IMI) was applied in 11-week old rats. After habituation to the new housing conditions, rats of the social defeat group received daily bouts (5 min) of social defeat during 5 days and subsequent individual housing (3 months). Control animals were handled daily for 5 days and were housed in pairs. Behavioral or pharmacotherapy was applied only during the last three weeks (or no treatment, H_2O) of the individual housing period. The length of each period is indicated. All behavioral and electrophysiological and biochemical analysis were performed at the end of the paradigm, at the end of treatment (arrow). Independent cohorts of animals were used for behavioral physiological and biochemical analyses.



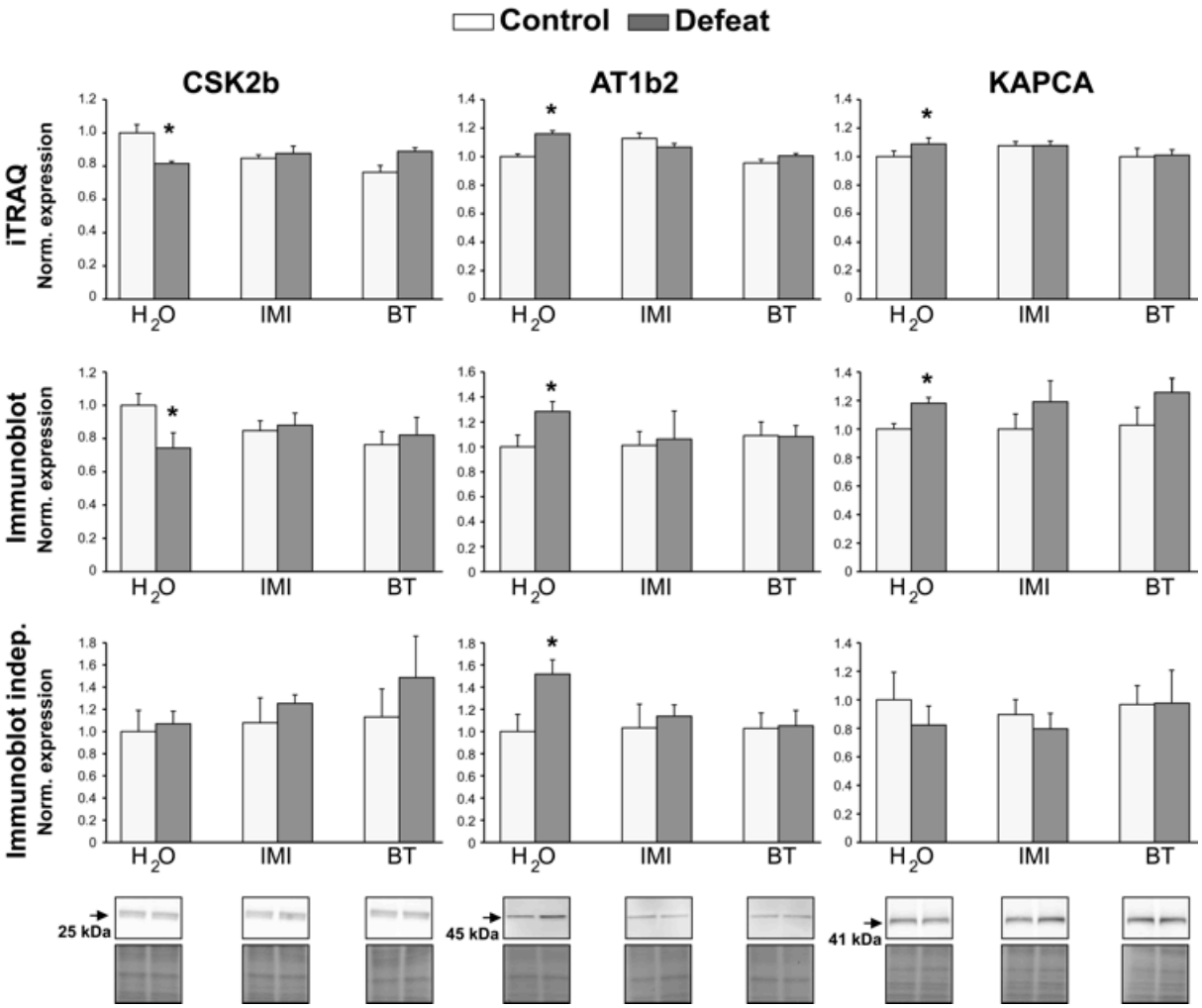
Supplemental figure 2. Experimental design for object-place and object-recognition tests. Schematic representation of the recognition tasks (Place, A; Recognition, B) and geometric arrangement of objects. The 'x' and 'y' indicate start locations of the rat in the tasks. In the object place test the start locations are counterbalanced between 'x' and 'y' over treatment groups. The circle and square represent the different objects.



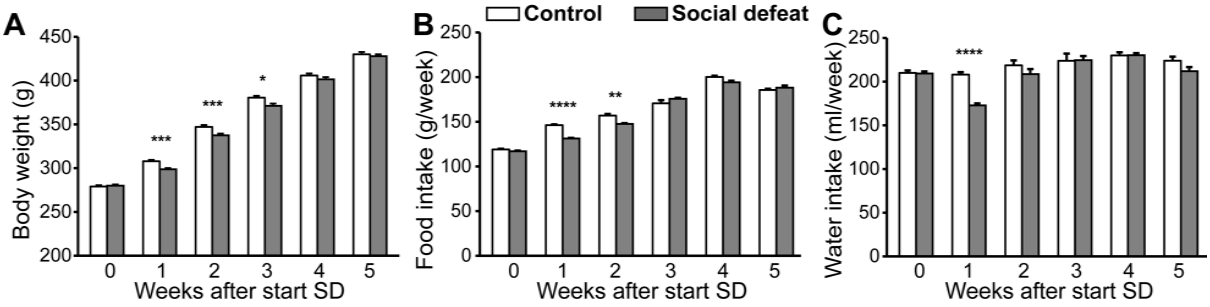
Supplemental figure 3. Mean levels of plasma corticosterone (± SEM), measured at the end of water (H_2O), imipramine (IMI) or behavioral therapy (BT) treatment. No difference between any of the groups was observed.



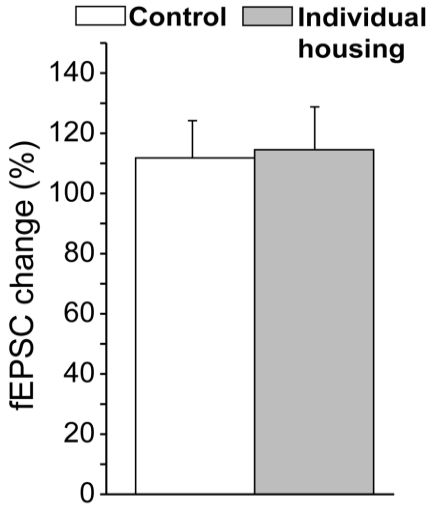
Supplemental figure 4. Set up of the iTRAQ experiments. Synaptic membrane fractions were isolated from one dorsal hippocampus (left and right) from one rat per experimental group. Tryptic digests of synaptic membranes from these samples were tagged with 6-plex iTRAQ reagents (one per experimental group). Peptides from each set of iTRAQ experiment were pooled together, fractionated by two-dimensional liquid chromatography and subjected to tandem mass spectrometric analysis. Protein identification and quantification were performed as detailed in the main text. The experiment was performed six times, each time with a biological independent set of samples.



Supplemental figure 5. **Synaptic expression of CSK2b, AT1b2 and KAPCA determined by iTraq and Western blot.** Protein expression in synaptic membrane samples was determined both by iTRAQ quantitative proteomics (upper panels), and by immunoblot (middle panels) on the same set of samples. Expression differences of these three proteins was tested in an independent set (indep.) by immunoblot (lower panels). Bar graphs show standardized average relative expression levels (vs. Control+H_2O) (±SEM) for CSK2b, AT1b2, and KAPCA (*cf.* Supplemental table 1). *P<0.05 *vs.* Control+H_2O. Regulated expression of only AT1b2 could be validated in the independent set of samples. Insets show representative examples of the immuno-detected band, as well as the coomassie-stained gel, used for correction of input material.



Supplemental figure 6. Basal physiological parameters tested *acutely* after social defeat and their normalization over time. Parameters were acquired in the first five weeks after the start of social defeat, before behavioral therapy and imipramine treatment had started. At this time point only two groups were present: social defeat, and controls. (A) Social defeat reduced body weight (A), food intake (B), and water intake (C). These parameters were normalized after 4, 3, and 2 weeks, respectively. Data is presented as mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001.



Supplemental figure 7. Effect of individual housing on LTP in the CA1 of the dorsal hippocampus. Average change (±SEM) in fEPSP 50 min after LTP induction. Individual housing had no effect on LTP.

Supplemental table 1. Synaptic protein changes after long-term social stress. Five proteins were significantly regulated (adjusted p-value < 0.05) after long-term social stress after adjustment for multiple hypothesis-testing using the Benjamini and Hochberg method. Expression of two of these proteins (AT1B2 and ANXA6) were rescued to control levels by imipramine treatment and three (AT1B2, KAPCA, and ANXA6) were rescued to control levels by behavioral therapy. Regulated expression of three of these proteins (CSK2B, AT1B2 and KAPCA; bold) could be confirmed by quantitative immunoblot analysis on the same samples as used for iTRAQ analysis. Regulated expression of AT1B2 (bold) could be confirmed by immunoblot in an independent set of biological samples. Indicated are protein accession number, protein name, average number of peptides (over six replicate experiments) used for quantification and identification, expression ratios (log2) of Stress+H_2O vs CON+H_2O and their adjusted-p-value for the iTraq data, of Stress+IMI vs CON+H_2O and Stress+BT vs CON+H_2O as a measure of rescue to control levels, of Stress+H2O vs CON+H_2O and their p-values (t-test) of the immunoblots on the samples used for iTRAQ quantification and on an independent set of biological replicates. NA: not available; no antibody suitable for quantification was available.

		iTRAQ					Western blot			
SwissProt Accession number	**Protein name**	**Nr unique peptides**	**Regulation SDH vs. CONH**	**Adjusted P-value**	**Regulation SDI vs. CONH**	**Regulation SDB vs. CONH**	**Regulation SDH vs. CONH**	**P-value**	**Regulation SDH vs. CONH independent**	**P-value indep.**
CSK2B_RAT	Casein kinase II subunit beta	4	-0.29	0.008	-0.19	-0.17	**-0.47**	**0.047**	0.21	0.557
AQP4_RAT	Aquaporin-4	6	0.30	0.008	0.23	0.17	NA	NA	NA	NA
AT1B2_RAT	Sodium/potassium-transporting ATPase subunit beta-2	8	0.21	0.008	0.09	0.01	**0.38**	**0.045**	**0.68**	**0.040**
KAPCA_RAT	cAMP-dependent protein kinase catalytic subunit alpha	7	0.12	0.049	0.11	0.01	**0.24**	**0.009**	0.00	1.00
ANXA6_RAT	Annexin A6	43	0.23	0.014	-0.08	-0.05	NA	NA	NA	NA

Supplemental Table 2. Several Na,K ATPase subunits are rescued by behavioral and pharmacological therapy in social defeated (SD) rats. Proteins as identified by iTRAQ proteomics are indicated by the protein accession number (SwissProt). Comparisons (regulation (log2), *P*-value) are made for the effect of water-treated social defeat (SD_H) vs control (Con_H) animals (upregulation), and the effect of behavioral therapy (BT) or imipramine (Imi) in defeated animals vs. defeated water-treated (SD_H) animals (down-regulation). Orange: *P*<0.05; yellow: *P*<0.1. Note that most of the ATPAse type 1 subunits are affected similarly.

	SD_H *vs.* Con_H		**SD_BT *vs.* SD_H**		**SD_Imi *vs.* Sd_H**	
Protein accession	**Regulation**	**p-value**	**Regulation**	**p-value**	**Regulation**	**p-value**
AT1A1_RAT	0.01	0.769	-0.09	0.127	-0.03	0.473
AT1A2_RAT	0.51	0.095	-0.60	0.015	-0.53	0.072
AT1A3_RAT	-0.16	0.498	-0.14	0.024	-0.13	0.056
AT1B1_RAT	0.09	0.170	-0.19	0.016	-0.11	0.120
AT1B3_RAT	0.08	0.372	-0.16	0.014	0.10	0.093
AT2A2_RAT	0.05	0.293	-0.10	0.045	-0.04	0.343
AT2B1_RAT	-0.23	0.790	-0.07	0.321	-0.04	0.573
AT2B2_RAT	0.04	0.460	-0.08	0.127	-0.03	0.376
AT2B3_RAT	0.01	0.825	-0.11	0.106	-0.04	0.410

Chapter 5

Reduction in hippocampal neurogenesis after social defeat is long-lasting and responsive to late antidepressant treatment

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Abstract

Major depressive disorder is a chronic disabling disease, often triggered and exacerbated by stressors of a social nature. Hippocampal volume reductions have been reported in depressed patients. In support of the neurogenesis theory of depression, in several stress-based animal models of depression, adult hippocampal neurogenesis was reduced and subsequently rescued by parallel antidepressant treatment. Here, we investigated whether repeated social defeat and subsequent individual housing for three months induces long-lasting changes in adult hippocampal neurogenesis in rats, and whether these can be normalized by late antidepressant treatment, as would match human depression. Neurogenesis was analyzed by stereological quantification of the number of immature Doublecortin (DCX) immunopositive cells, in particular young (class-I) and more mature (class-II) DCX^+ cells, to distinguish differential effects of stress or drug treatment on these subpopulations. Using this social defeat paradigm, the total DCX^+ cell number was significantly reduced. This was most profound for older (class-II) DCX^+ cells with long apical dendrites, whereas younger, class-I cells remained unaffected. Treatment with the broad-acting tricyclic antidepressant imipramine, only during the last 3 weeks of the 3-month period after social defeat, completely restored the reduction in neurogenesis by increasing both class-I and-II DCX^+ cell populations. We conclude that despite the lack of elevated corticosterone plasma levels, neurogenesis is affected in a lasting manner by a decline in a distinct neuronal population of more mature newborn cells. Thus, the neurogenic deficit induced by this social defeat paradigm is long-lasting but can still be normalized by late imipramine treatment.

Introduction

Exposure to stress forms an important risk factor for development of human psychopathologies such as major depressive disorder[181]. In particular chronic forms of stress, often psychosocial in nature, may predict precipitation of depression[10]. As patients who underwent a first episode, are at increased risk to develop recurrent or chronic depression, interest has been raised into the underlying mechanisms that determine particularly longitudinal aspects of the disorder and its maintenance[182].

Of the many brain regions affected in depression, the hippocampus is well known for its role in cognition and stress sensitivity. Volumetric studies have repeatedly found reductions in hippocampal volume in patients suffering from major depression, paralleled by alterations in various neuropsychological and cognitive measures[12,154]. In preclinical models, stress exposure in rodents causes mild volume reductions of the hippocampus as a whole, reduces dendritic complexity of neurons in the CA3 subregion, and impairs neurogenesis in the dentate gyrus (DG)[183]. Despite the absence of causal evidence that changes in adult DG neurogenesis are critical to the etiology of major depression, impaired hippocampal plasticity likely contributes to the cognitive symptoms of depression, as well as clinically effective antidepressant treatment[47,68,184]. Indeed, neurogenesis is affected by many factors, among which treatment with antidepressant drugs – an effect that is age-dependent[185] and is found both in stressed and naive animals[186-190].

Most studies examining the effect of stress as model for depression have measured neurogenesis shortly after exposure to either acute or chronic stressors that notably were often of a physical nature, like restraint[47,68]. Most stressors relevant for depression however, are chronic and psychosocial in nature[10], and depression may develop long after the initial stress exposure. Also, the effects of antidepressant drugs have been evaluated, but when used in conjunction with a stress model, they have been administered during or parallel to, and not after an extended period after stress exposure[68,186,191]. Therefore, we questioned (i) whether stress induced by social defeat has long-term effects on neurogenesis or corticosterone levels, and (ii) whether neurogenesis can still be normalized by antidepressant treatment starting at a late stage, when depressive-like symptoms are already manifest.

To address this, adult rats were subjected to a long-term social defeat paradigm, consisting of exposure to repeated severe social defeat stress followed by subsequent individual housing for three months. This type of paradigm models mainly the maintenance phase of depression as it results in increases in stress responsivity as well as decreases in social interaction and sensitivity to reward anticipation over time[33,141,192]. Social defeat stress

followed by subsequent individual housing for three months leads to impaired hippocampal long-term potentiation and this could be restored by late antidepressant treatment[140]. In view of the persistent changes in behavior and hippocampal function in this model, we investigated whether neurogenesis in the DG was affected on the long-term. As read-out we quantified the number of Doublecortin (DCX) immunopositive (DCX^+) cells. Doublecortin is a microtubule-associated protein selectively expressed in young, immature neurons from approximately 4 to 14 days after birth of a newborn cell[193,194]. During this period, the temporal course of dendrite maturation can be used to morphologically distinguish younger (class I) and older (class II) DCX-positive cells[195,196]. We used this classification to distinguish between differential effects of our social defeat paradigm or drug treatment on these DCX^+ subpopulations.

Materials and methods

Animals

Male Wistar rats (Harlan, Horst, The Netherlands) 8-9 weeks of age, weighing 180–200 g at the time of arrival, were initially socially housed (2 per cage) in Makrolon class IV cages (Tecniplast, Milan, Italy). Long-Evans male rats (Harlan, UK), weighing 300–350 g were used as residents for social defeat[141]. These animals were pair-housed with age-matched sterilized females in plastic cages (63 x 25 x 33 cm) located in a separate room. All animals were housed in a temperature-controlled room (21±1 °C) under regulated lighting conditions (lights on at 7:00 p.m. and off at 7:00 a.m.). Food and water were available *ad libitum*. All experimental manipulations were conducted during the dark phase (activity period) under a dim red light. The Animal Users Care Committee of the VU University Amsterdam approved all experiments.

Experimental design and treatment

Wistar rats (age ≥ 11 weeks) of the social defeat group were subjected to 5 days of social defeat stress and were then housed individually for three months in macrolon class III cages from the first defeat onwards, as described before[140] (Fig. 1). Control rats were housed in pairs. The social defeat procedure consisted of daily resident–intruder interaction sessions using dominant male Long-Evans rats for five consecutive days. Control animals were handled daily.

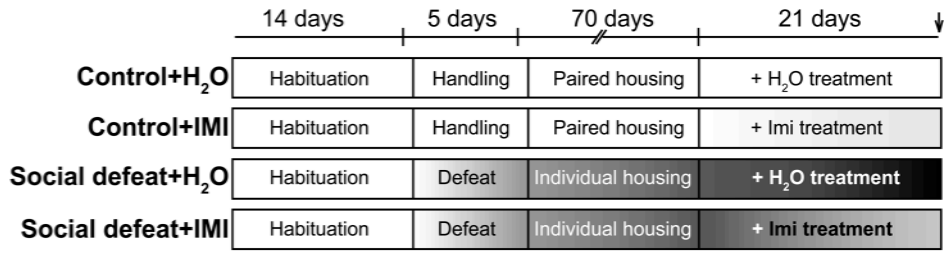

Figure 1. Experimental design and treatment groups. The social defeat paradigm (social defeat) in combination with antidepressant treatment was applied in 11-week old rats. After habituation to the new housing conditions, rats of the social defeat group received daily bouts (5 min) of social defeat during 5 days and subsequent individual housing (3 months). Control animals were handled daily for 5 days and were housed in pairs. Treatment with the tricyclic antidepressant imipramine was applied only during the last three weeks (IMI; or no treatment, H_2O) of the individual housing period. The length of each period is indicated. All behavioral and immunohistological tests were performed at the end of the paradigm, at the end of treatment (arrow). Independent cohorts of animals were used for behavioral and immunohistochemical analyses.

During the last three weeks of this three-month period, rats were treated by gavage administration of the antidepressant imipramine (20 mg/kg per 0.5 ml water; Sigma- Aldrich, Germany) or water as control (Fig. 1). Subsequently, four experimental groups were generated: 1) control rats with water (Control+H_20), 2) control animals with chronic imipramine treatment (Control+IMI), 3) social defeated animals with water (Social defeat+H_20), and 4) social defeated animals with chronic imipramine treatment (Social defeat+IMI). All behavioral and immunohistological analyses where performed at the end of the treatment period. Independent cohorts of animals were used for behavioral and immunohistochemical analyses.

Reward anticipatory behavior

A classical Pavlovian conditioning setup was used to investigate anticipatory behavior, as described earlier[140]. To investigate the behavioral response to the conditioning stimulus (repetitive sound (keyboard) and light flashes (three times)), animals were observed before training (trial 0) to determine baseline activity, and again after 35 training trials of pairing with a 5% sucrose-reward, using the computer program 'The Observer' (Noldus Information Technology, Wageningen, The Netherlands). The researcher who analyzed the behavioral data had no knowledge of the experimental groups. Differences in activity (reflected by frequency or transitions of behavioral elements) displayed before training compared with those after training were used as parameter for reward anticipation.

Sucrose preference

The preference for sucrose (5%) was measured in a two-bottle (sucrose and water) consumption test. Consumption was assessed after 24 h by reweighing the pre-weighted bottles. After 2 days, the consumption test was repeated. In case of social housing, consumption for each subject was set to half of the total consumption. Sucrose preference was expressed as the increase in consumption (gram) relative to water (gram), and this difference was represented as percentage of the total consumption (gram) [100% x (Δ sucrose–water)/total volume sucrose and water consumed].

Corticosterone assay

Trunk blood samples were collected at the end of the experiment via decapitation (between 9 – 11 AM) into a 7-mL heparin-coated tube (Greiner Bio-One, Monroe, North Carolina) and kept on ice before centrifugation at 1000 x *g* for 10 min. Plasma was decanted and stored at −80 °C until analysis. Levels of plasma corticosterone were assessed using a rat Glucocorticoid (GC) ELISA kit (Cusabio Biotech Co., LTD), according to the manufacturers instructions.

Tissue preparation and immunohistochemistry

Animals were anesthetized (between 9 – 11 AM) by injection of pentobarbital sodium salt (Nembutal; 1 mg/kg bodyweight; A.U.V. Cuijk) and perfused transcardially with saline followed by 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.4). Brains were post-fixed overnight in the skull at 4 °C, after which they were carefully removed, washed, and cryoprotected in 20% sucrose in PBS. Frozen sections (35 μm thick) were cut using a sliding microtome and collected in PB/azide.

The number of young, differentiating neurons was identified with an antibody against doublecortin (DCX; 1:800; polyclonal goat anti-DCX; Santa Cruz Biotechnology) as described before[187]. Amplification was performed with a biotinylated secondary antibody, donkey anti-goat (1:500; Jackson ImmunoResearch Laboratories)] and avidin-biotin complex (1:1000; Elite Vectastain ABC kit, Brunschwig Chemie) in combination with tyramide (1:500; 0.01% H_2O_2; kindly provided by Dr. I. Huitinga, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands). Subsequent chromogen development was performed with diaminobenzidine (20 mg per 100 ml of Tris buffer, 0.01% H_2O_2).

Stereological quantification and phenotypic analysis

Quantification of cell numbers was performed in every 10th coronal section along the entire rostrocaudal axis of the brain, in a total of 9 sections per animal as described before[196]. Total

numbers of DCX^+ cells per DG were quantified by systematic random sampling performed using the Stereo Investigator System (MicroBrightField) with optical fractionator settings of 180 x 150 grid size and 180 x 150 counting frame, resulting in 200 – 450 markers per animal. A single examiner unaware of the group codes performed the data collection.

We further distinguished morphologically different subclasses of DCX^+ cells, based on an adaptation of the stages of neuronal differentiation described before[195]; the most mature DCX^+ cells were named class-II; these were characterized by a primary dendrite that was orientated perpendicular to the subgranular zone and radially projecting up into the molecular layer. The younger cells were named class-I and were located in the SGZ, without a dendrite, or only a short dendrite reaching no further than the granule cell layer[196] (See Fig. 3).

Statistical analysis

Statistical analysis was performed using SPSS 18.0. Results are expressed as group means ± SEM. Treatment effects were assessed with two-way analysis of variance (ANOVA), followed by Student Newman's Keuls *post-hoc* analyses for further examination of group differences.

Results

Behavioral and physiological consequences of social defeat

In the present model, we studied consequences of repeated social defeat and subsequent individual housing for three months (Fig. 1), and assessed several pathological dimensions of depression[141,192]. We confirmed that socially defeated rats in this paradigm display a depressive-like phenotype[33,141], as shown by a reduced anticipation towards a 5 % sucrose solution compared with control rats (Fig. 2A). This was reversed to control levels by chronic administration of imipramine (IMI). Two-way ANOVA (stress x treatment) revealed a significant effect of SD stress ((F(1,20)=4.77, P=0.041), and an interaction of stress and treatment (F(1,20)=5.55, P=0.029). *Post hoc* comparisons showed a significant decrease after social defeat without treatment compared to controls (P=0.012), and for social defeat without treatment when compared with the imipramine-treated socially defeated group (P<0.029). In contrast, no difference was found for the social defeat group that was treated by imipramine when compared with either of the control groups. Imipramine treatment alone in control animals had no significant effect on anticipation towards 5 % sucrose. In contrast

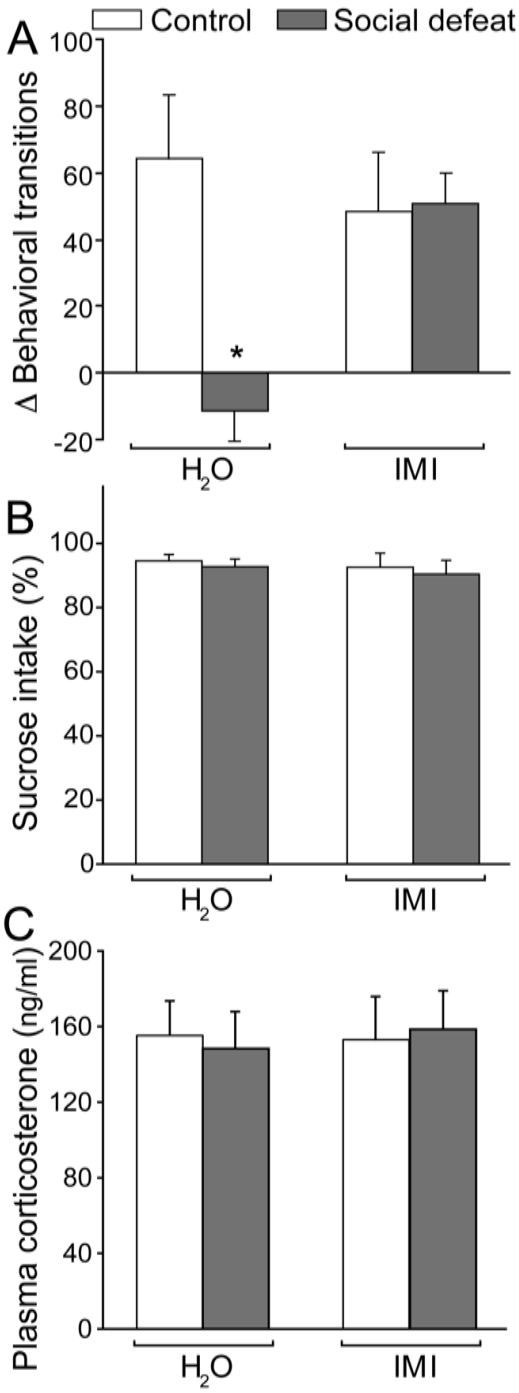

Figure 2. Behavioral and physiological consequences of long-term social stress. (A) Anticipation towards 5 % sucrose expressed as the mean difference in activity (# behavioral transitions) in the CS-US interval post-training compared with that during pre-training. The social defeat paradigm (social defeat) significantly suppressed reward anticipation, whereas imipramine treatment (IMI) reversed this stress-induced effect. *P=0.0034 *vs.* Control+H_2O. (B,C) Sucrose preference (sucrose intake-water intake)/total fluid intake (B) and plasma corticosterone level (C) were not affected. All data show mean ± SEM. n=6 for al experimental groups.

to sucrose anticipation, neither stress nor imipramine treatment had a significant effect on sucrose preference (Fig. 2B), which is an indicator of more acute stress.

Also, no difference was observed in plasma corticosterone levels between any of the groups (Fig. 2C), indicating that no lasting changes in stress hormone levels had been induced, nor that antidepressant treatment had been stressful.

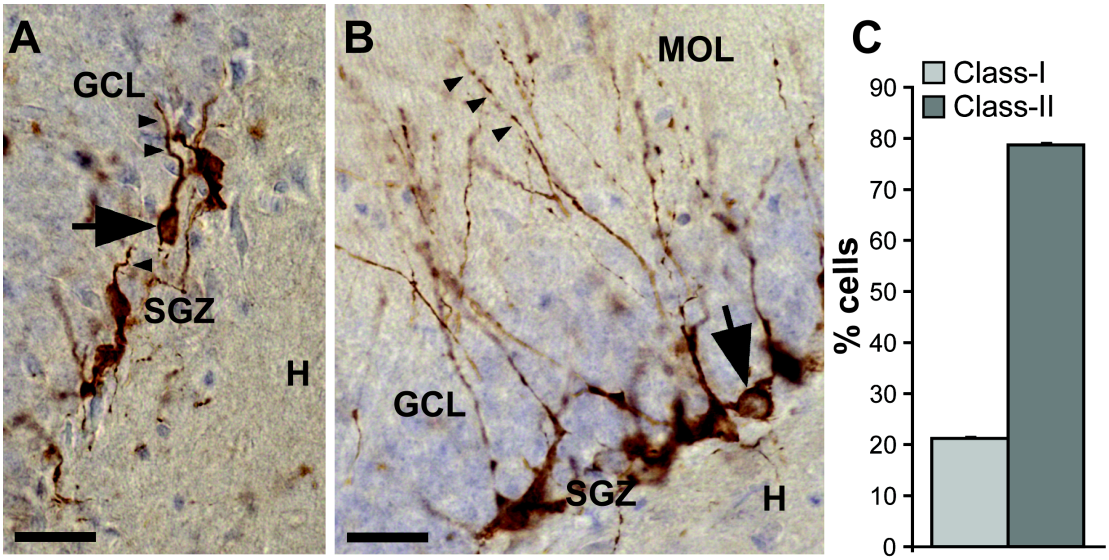

Figure 3. Class-I and class-II DCX-expressing cells in the DG subgranular zone. (A, B) High-power images of clusters of DCX^+ cells with different morphology. The granule cell layer (GCL) is seen as a purple cell layer. The hillus (H), subgranular zone (SGZ), and molecular layer (MOL) are indicated. Scale bar, 30 µm. (A) Class-I cells (arrow) with no or short processes reaching no further than the granule cell layer. Note how the initial segment of the dendrite grows parallel to the SGZ (arrow heads). (B) Class-II cells (arrow) with at least one dendrite reaching into the molecular layer (arrow heads) and occasionally showing delicate branching with few major branches. (C) Percentage of total amounts of class-I and –II cells in control animals (mean ± SEM).

Long-term social stress affects neurogenesis by reducing differentiation and survival of DCX-positive cells

Immunohistological staining of the adult hippocampus revealed numerous DCX^+ cell bodies located in the SGZ and only few DCX^+ cells within the granule cell layer itself. DCX-expressing cells were classified[195,197,198] into class-I (Fig. 3A), or class-II cells (Fig. 3B). About 70 % of all DCX^+ cells belonged to class-II (Fig. 3C). Hence, at any given time point, DCX identifies a majority of cells with a relatively mature phenotype.

To examine whether adult hippocampal neurogenesis was affected by the social defeat paradigm and/or antidepressant treatment, we first quantified the total population of DCX^+ neurons. Two-way ANOVA revealed a significant effect of defeat ($F(1,30)=6.17$, $P=0.019$) on total number of DCX^+ neurons (Fig. 4A). Social defeat significantly reduced the total number of DCX^+ cells ($P=0.004$). This effect was not present anymore after three weeks of imipramine treatment, as the social defeat group treated with imipramine differed significantly from the social defeat group ($P=0.048$) and was not different from both control groups (CON+H_2O, CON+IMI).

To further address which subset of the DCX cells was affected, i.e., the relatively younger or older cells, we quantified class-I and -II DCX^+ cells. No effect of defeat or treatment was

found on the number of cells belonging to class-I cells ($F(1,30) < 1$) (Fig. 4B). However, a significant effect of both defeat ($F(1,30)=10.41$, $P=0.003$) and treatment ($F(1,30)=4.96$, $P=0.034$) with no interaction was found on class-II DCX^+ cells. Social defeat significantly reduced the number of class-II cells ($P=0.002$), and three weeks of imipramine treatment reversed this effect ($P=0.026$; Fig. 4C). Imipramine treatment alone had no effect in control rats ($P=0.369$).

To confirm whether long-term social stress indeed enforced its strongest effect on class-II cells, the ratio of class-II cells over class-I cells was calculated. This ratio was significantly affected by social defeat ($F(1,30)=7.58$, $P=0.010$). *Post hoc* comparisons showed that social defeat significantly reduced the ratio of class-II/class-I cells ($P=0.004$). However, imipramine was not able to restore this ratio to basal levels. Together these data demonstrate that (1) social stress on the long term reduces neurogenesis by specifically reducing class-II DCX^+ cells, whereas class-I cells were unaffected, and (2) imipramine restored this reduction in neurogenesis by increasing both class-I and –II DCX^+ cells, thereby leaving their ratio unaffected.

Discussion

Here, we showed for the first time that adult hippocampal neurogenesis is still reduced at the end of a three-3 month individual housing period that followed a short period of severe social defeat stress. The reduction in neurogenesis was observed, despite that corticosterone levels were normal at the end of this paradigm, and was most profound for the class-II DCX^+ cells with long apical dendrites, arguing that neuronal differentiation and/or survival of newborn neurons is affected. Treatment with imipramine for the last 3 weeks completely restored this reduction by stimulating both class-I and -II DCX^+ cells.

Validity of the social stress model

The present social defeat model recapitulates several behavioral dimensions of depression[33,141]. We showed that the depressive-like phenotype of reduced anticipation towards sucrose is associated with a decrease in hippocampal neurogenesis, although sucrose consumption was not changed. This reward-related consummatory response is different from appetitive behaviors measured by anticipation[199]. Although consummatory behavior is known to be reduced shortly after stress exposure[200], we confirmed that on the long-term, appetitive behavior is specifically affected[33,141]. Since the mesolimbic dopamine reward system is primarily involved in appetitive behavior and not in the affective component of consumption, we hypothesize that reduced appetitive behavior is an adequate

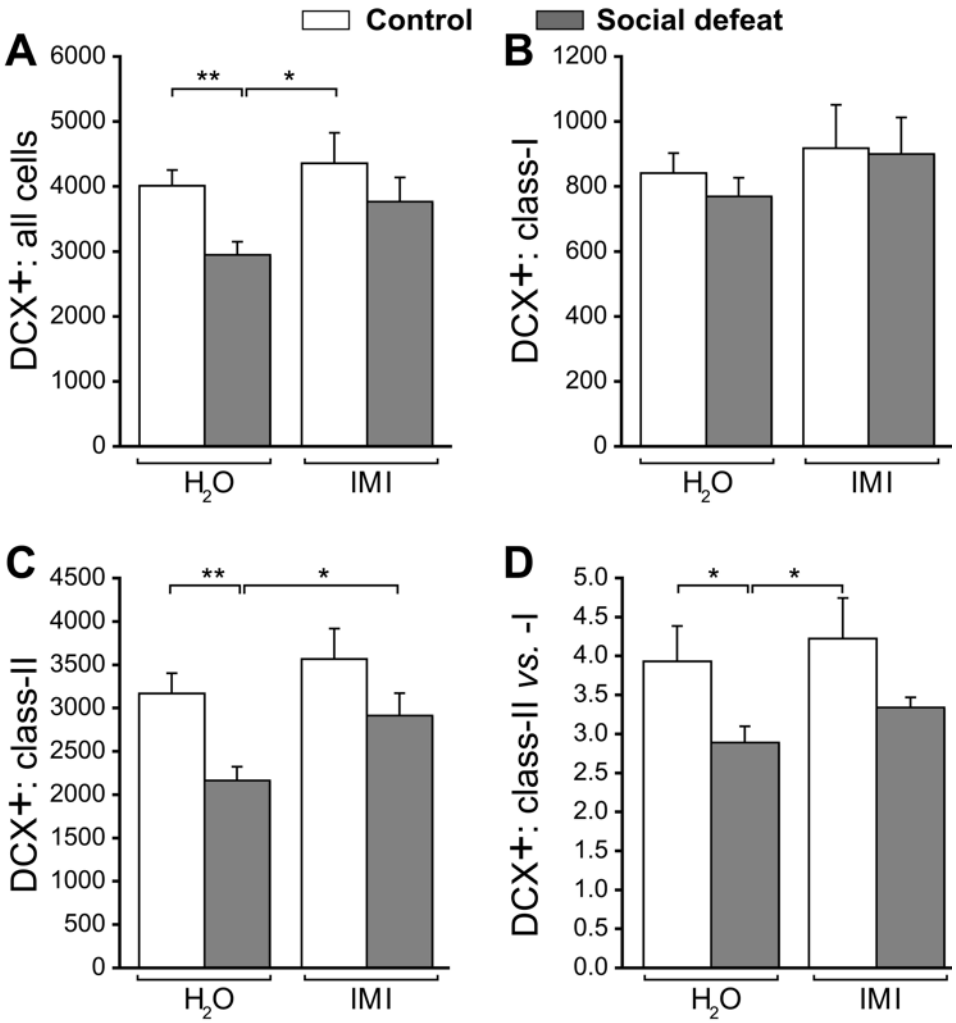

Figure 4. Differences in DCX-positive cells with different dendritic morphologies after long-term social stress and subsequent imipramine treatment. (A) Total number of DCX^+ cells in the SGZ per hemisphere. The social defeat paradigm (social defeat) significantly reduced total amount of DCX^+ cells, whereas imipramine treatment (IMI) normalized this stress-induced effect. (B) No effect of social defeat or imipramine was found on class-I DCX^+ cell numbers. (C) Social defeat significantly reduced amounts of class-II DCX^+ cells, whereas this reduction was not found in the imipramine treated group (IMI)(D). Social defeat significantly reduced the ratio of class-I cells over class-II cells. Imipramine treatment had no effect on this ratio. All data show mean ± SEM. n=9 for al experimental groups. ** $P<0.01$; * $P<0.05$.

representative of depressive-like behavior[141,199]. Moreover, anhedonia is commonly observed in depressive patients[201].

As the present results were obtained in rats 6 months of age, it is unlikely that interference with early postnatal development has played a major role[202]. The age at which rats experienced stress in our model (adult rats >11 weeks) is of importance given the protracted period of risk for development of affective disorders into young adulthood[203].

As compared with most other stress-induced models[34,186], the present paradigm was used

to study long-lasting effects of social stress on structural plasticity changes, that have been implicated in depressive symptoms and maintenance of depression, long after the initial exposure to active stress has occurred, and when the rise in stress hormone levels has normalized[10,12,204].

Neurogenesis – subclasses of DCX$^+$ cells

We quantified DCX$^+$ cell numbers in the DG to examine whether the persistent anhedonic phenotype present long after exposure to social defeat was associated with a reduction in neurogenesis. Doublecortin has been previously established as a reliable marker for young and migratory neurons in the adult DG[193,195,198,205]. It is expressed approximately from day 4 to day 14 after a new cell is born and most likely all DCX$^+$ young neurons originate from cell divisions during the past 3 – 4 weeks[198]. DCX expression is further selective for the neuronal lineage, as DCX-positive cells co-express early neuronal antigens like Tuj1, PSA-NCAM or pax-6[194], but lack specific markers for glia, undifferentiated, stem cells, or apoptotic cells, making it a reliable marker of newly generated neurons in the adult DG[193,198,206].

DCX immunoreactivity in dendrites allowed classification of these immature neurons. DCX is transiently expressed from the proliferative progenitor cell stage to a postmitotic phase with long dendrites[194,195]. Several subclasses of DCX$^+$ cells can be distinguished based on the presence and shape of the apical dendrites[195,196]. Smaller cells without a dendrite that penetrate the GCL are known to reflect progenitor cells (type-2b and type-3 cells, see[195], whereas longer cells with extensions into the molecular layer are considered immature postmitotic neurons.

Here, we used a simplified scheme of this subdivision[195] and defined class-I cells as young cells with no or short processes reaching no further than the molecular layer, and class-II cells as mature post proliferative cells with at least one dendrite reaching into the molecular layer. About 70% of DCX$^+$ cells belong to class-II and 30% to class-I, and it has been shown that of this latter 30%, about two thirds is in cell cycle[195]. Interestingly, the ratio of class-I and –II cells we found were similar to those in rodents 2 months of age, despite the fact that the total number of DCX$^+$ cells equals about 10% of what is observed in these younger animals. This implies that despite the lower overall DCX$^+$ cell numbers, different stages of the neurogenic process are present in similar proportions both in young adults as well as adult animals.

In a recent paper[207], DCX$^+$ cells were sorted using fluorescence activated cell sorting (FACS) and particularly the younger DCX$^+$ cells with low levels of DCX per cell, comparable with our class-I cells, were shown to be capable of dividing again, in contrast to the older

types with higher DCX levels. Although we cannot compare FACS sorted cells with the present populations in brain tissue, it is tempting to speculate that as predominantly the class-II DCX^+ cells were affected in our study, social stress may have affected progression through the cell cycle and thereby limited neuronal differentiation and/or newborn cell survival.

Neurogenesis – social defeat and reversal by imipramine

We found that at the end of the social defeat paradigm total DCX^+ cell numbers in the DG were reduced, which was due to a reduction in class-II cells, whereas the class-I cells remained unaffected. It has been proposed that neurogenic stimuli that act on precursor cells are different from those regulating dendritic maturation and survival of newborn cells. In contrast to chronic unpredictable (physical) stress exposure, after which a rapid recovery of neurogenesis occurs (Heine et al., 2004), our present results imply that social defeat reduces neurogenesis for a prolonged period of time and that it does so by inhibiting specifically the differentiation and survival stage, but leaves progenitor cells unaffected.

A remaining question however, is whether this long-term reduction in neurogenesis is caused by 1) long-term effects of social defeat, 2) by the individual housing or, –most likely– 3) by a combination of both. In adult rats, individual housing by itself is considered to be a social stressor. Adult individual housing induces changes in anxiety- and anhedonia-like behavior[83] as well as neurochemical alterations[208]. However, social isolation in male rats does not result in an increased, lasting expression of stress hormones[83,209,210], which is in line with our current findings. Also, in the absence of external factors, adult social isolation does not affect hippocampal neurogenesis[209,211]. However, individual housing does increase the stress response to external stressors, both on glucocorticoid levels[83,210] and neurogenesis[209,211], and it delays the positive effect of physical activity on neurogenesis[209]. This would argue that individual housing in our paradigm might be involved in the maintenance of the reduction in neurogenesis rather than in its onset.

This additive effect of social isolation is further supported by the observations that depressive-like behavior (e.g. reduced anticipation to sucrose) induced by social defeat is maintained during individual housing, but can be counteracted by social housing[33].

As social isolation itself cannot reduce anticipation towards 5% sucrose[33], the observed reduction in neurogenesis is most likely induced by repeated social defeat stress, and maintained on the long term due to a lack of social support. This would match human depression in which active stress is often involved in the onset of depression, while passive

stress, e.g., in the form of social isolation, has strong precipitating effects on the development of the disease[84].

The current reduction in hippocampal neurogenesis was associated with an anhedonic phenotype, which is in accordance with the concept of a role for neurogenesis in depression or antidepressant action. This concept originated from animal studies in which stress was shown to inhibit neurogenesis[188,212], and from studies in which various classes of antidepressants were found to promote newborn cell proliferation[20,46,189], survival and neurogenesis[187,189,213]. Furthermore, the 3- to 4-week therapeutic time lag of antidepressants coincides with the maturational time course of newborn neurons[214].

However, ablating neurogenesis does not result in a depressive-like phenotype per se[215]. Also, the enhanced survival of newborn cells that occurs upon administration of antidepressants to young mice, is age- and strain dependent, and is abolished when older mice are studied[185,216,217]. Similarly, in hippocampal tissue of depressive patients, the stimulatory effect of antidepressants[218] also appears to depend on age[184]. Hence, both neurogenesis-dependent and neurogenesis-independent mechanisms are likely to contribute to the reversal of depressive-like behaviors by antidepressants[47,216].

In this study, treatment with imipramine during the last 3 weeks of a 3-month individual housing period following exposure to severe social defeat stress restored total DCX^+ cell numbers back to control levels. Interestingly, whereas the social defeat paradigm affected specifically class-II DCX^+ cells, imipramine's action is not cell-type specific as it left the ratio of type-II over type-I cells unaffected. This is in accordance with previous studies in which imipramine increased several stages of the neurogenesis process[46,219], including proliferation, neuronal differentiation, survival as well as integration of adult-generated cells into existing neuronal circuits.

Overall, using a paradigm that models both temporal aspects of a social stress-mediated onset and the maintenance phase of depressive symptoms, we have shown that in absence of lasting changes in corticosterone levels at the end of the social defeat paradigm, neurogenesis is still significantly reduced. This is accompanied by a reduction of differentiation and/or survival of the newborn neurons, whereas younger cells are unaffected. An interesting outcome of our study is that this form of long-term social stress does not affect all domains of the adult neurogenic process but appears selective for the differentiation and survival stages. In addition, these neurogenic deficits can still be normalized by late imipramine treatment that increased both classes of DCX^+ cells.

Chapter 6

Genaral discussion

Major depressive disorder (MDD) is a multifaceted disease with a broad spectrum of symptoms. Evidently, the underlying causative factors of MDD are diverse in nature. Environmental factors exert a major influence on the initiation of MDD; a common factor causing the disease is stressful life events. Stress impacts on the individual thereby affecting mood- and cognition-related functioning. In particular, this disease-triggering factor can be modeled in animals, thereby providing the opportunity to investigate the molecular and cellular changes in distinct brain areas.

In this thesis, research focused on the impact of a natural occurring type of stressor, social defeat stress, on the functioning of the hippocampus in terms of learning and memory and the potential alleviating role of antidepressant and behavioral therapy. This thesis yielded several novel findings concerning the molecular and cellular correlates of MDD that originated from animal models of depression, and will be discussed below.

Major conclusions

First, we identified a mechanism by which ketamine may exert a direct and lasting antidepressant effect. We found that ketamine probably exerts its long-term antidepressant effects by increasing hippocampal synaptic membrane α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor function. This sheds new light on using ketamine in the treatment of depression.

Second, we found that impaired hippocampal function observed both short- and long-term after social defeat stress originated from different synaptic mechanisms. In particular, we noticed alterations in the glutamate receptor system only at the short-term, whereas on both time points hippocampal spatial memory performance was clearly affected.

Third, we showed that long after social defeat stress, hippocampal plasticity was severely affected. This translates into concomitant behavioral dysfunction. The long-term impairment of the hippocampal functioning can be alleviated by imipramine treatment and, much to our surprise, to the same extent with behavioral therapy.

Fourth, we found that neurogenesis in the hippocampal dentate gyrus was affected in a lasting manner after social defeat by a decrease in a distinct neuronal population of more mature newborn cells. Here, imipramine was able to rescue the neurogenic deficit.

Fifth, we were able to identify synaptic mechanisms that are regulated long-term after social defeat stress. A proteomics analysis turned out to be a useful approach to identify potentially new targets for MDD research.

Overall, in this thesis, observations are made concerning the molecular and cellular changes in the hippocampus related to depressive-like phenotypes. In the next sections I will discuss these findings in the context of ongoing MDD research.

Animal models of depression

Model validity

To appreciate the improvement of using animal models for depression based on long-term effects of stress over acute stress, it is important to compare how they meet criteria for a valid animal model.

At the very least, animal models must resemble the human condition in several respects[220], including (a) similarity in the symptom profile presented (face validity), (b) amelioration or attenuation by treatments effective in treating the human condition (predictive validity), (c) provocation by events thought to be important in eliciting the human disorder (etiological validity), and (d) involvement of similar neurochemical processes (construct validity) (Box 1).

An acute stress model to study antidepressant action

In this thesis several stress-induced animal models for depression have been used. The most basic and straightforward is the forced swim test (FST). This model has been proven efficient in predicting the clinical efficacy of antidepressants in a cost efficient way and therefore has good predictive validity[80]. However, this model has low etiological validity as acute swim stress is used to induce a depression-like phenotype. Also, its face validity is limited as only behavioral despair is accessed as readout parameter.

Using the predictive validity of this model, we showed in chapter 2 that ketamine, an N-methyl-D-aspartic acid (NMDA) receptor antagonist with acute and lasting clinically antidepressant effects, also showed acute and lasting efficacy in the FST and we revealed aspects of the contributing synaptic mechanisms. However, considering the limited validity of the test, care should be taken when interpreting the clinical relevance of these findings.

Using chronic stress to gain insight into mechanisms of depression

To gain insight in the molecular and cellular neurobiology of depression, animal models with the highest attainable validity should be used. In rodents, the social defeat paradigm is a good candidate. Since a natural type of social defeat stress is used, this model has good

Box 1. Validation criteria for evaluating animal models for psychiatric disorders

Category of validation criteria	Definition
Face validity	The phenomenological similarity between the behavior exhibited by the animal model and the specific symptoms of the human condition.
Predictive validity	The ability to predict changes in the human subject based upon changes in the model. This requires constant reality checking with clinical measures to make sure that the changes in the model correspond to those in the human. In terms of drug development, the special condition of predictive validity is usually determined through pharmacological validation that refers to clinically effective drugs showing activity in the test or model (pharmacological isomorphism).
Etiologic validity	The concept of etiological validity is closely related to the causes of the disorder in humans. When etiology can be established, the model becomes very useful. Unfortunately, the causes of behavioral disorders are often diverse. Therefore, this validity is limited to hypothesis regarding possible etiology.
Construct validity	Construct validity is closely related to the pathology and symptomatology of the disorder, and the accuracy with which changes in the model organism reflects that in the human. For example, close correspondence of changes in neurochemical or endocrinological parameters in depressed subjects and in the model systems used study depression endow the model with increasing construct validity.

etiologic validity, especially since chronic psychosocial stress is particularly effective in predicting human depression[10]. Moreover, social defeat has been shown to induce behavior relevant to depressive symptoms both acute[221] and long-term after defeat stress[33]. These symptoms include anhedonia (reduced sucrose preference or anticipation towards sucrose), behavioral despair, and reduced social interest, and therefore the model meets the criterium of face validity. Moreover, these symptoms were counteracted by chronic[141,222], but not acute[34] antidepressant treatment thereby establishing appropriate predictive validity. Finally, rodents subjected to the social defeat paradigm show neurobiological changes also observed in postmortem human studies, such as increased BDNF expression in the nucleus accumbens[81] and reduced hippocampal volumes[223] indicating that the paradigm has at least to some extent construct validity.

Here we adopted a social defeat paradigm, in which rats were subjected to severe social defeat stress once every day for five days. In chapter 3, we showed that this results in an overactive hypothalamic-pituitary-adrenal (HPA) axis response 24 hour after the last social defeat encounter, expressed by an increase in plasma levels of the stress hormone corticosterone. This further supports the construct validity of social defeat stress, as dysregulation in HPA axis responses are also observed in MDD patients[224]. As further shown in this chapter; this social defeat stress reduced performance in a hippocampus dependent spatial memory task, a phenotype relevant to the cognitive symptoms of depression.

Long-term effects of chronic stress

In humans, depressive episodes are still apparent long after the cessation of the stress exposure and depression is often considered as a chronic disease. Moreover, depression is a highly recurrent disorder; more than 75% of depressed patients have more than one depressive episode, often relapsing within two years of recovery from a depressive episode[225]. Indeed, between one-half and two-thirds of the people, who have ever been clinically depressed, will be in a relapse episode in any given year over the remainder of their lives[226]. Therefore, interest is in understanding the underlying mechanisms that determine particularly longitudinal aspects of the disorder and its maintenance[182].

To model the maintenance phase of depression rather than its induction phase, thereby increasing validity of the social defeat model, we adopted a social defeat paradigm in which five days of social defeat stress was followed by individual housing for 12 weeks[33]. In chapter 4, we confirmed face validity by showing that that this model induces depressive-like behavior by invoking anhedonic behavior, as indicated by reduced anticipation towards a palatable 5% sucrose solution, and by depression-associated cognitive impairments. Moreover, predictive validity was confirmed by showing that both behavioral and antidepressant therapy recovered both symptoms.

Novel neurobiological mechanisms of depression

Synaptic plasticity processes

Throughout this thesis, several novel mechanisms have been characterized concerning stress-induced depressive-like phenotypes and antidepressant action. These mostly concern synaptic plasticity processes. In chapter 2 of this thesis, we showed that ketamine exerts its antidepressant effects by interfering with glutamatergic signaling in hippocampal synapses. Ketamine is a non-competitive (NMDA) receptor antagonist that has been shown to have immediate antidepressant effects in treatment-resistant patients when administered at a subanesthetic dose[31,85]. Its primary mechanism of action is blocking the NMDA receptor at the phencyclidine site, i.e. in the pore of the ion channel. In addition, ketamine induces rapid increases in presynaptic release of glutamate, a process hypothesized to be mediated by NMDA autoreceptors, and/or mediated by activated GABAergic neurons[89]. We show that the long-term antidepressant effects of ketamine in the FST were paralleled by increased Ser-845 phosphorylation of GluA1 in hippocampal synapses. Moreover, a molecular blockade of regulated AMPAR endocytosis, using a TAT-$Glu2_{3Y}$ peptide in the CA1 region of the dorsal

hippocampus, mimicked this antidepressant effect. This substantiates the hypothesis that the antidepressant effects of ketamine are initiated by an increased AMPAR turnover and functionality. These results argue that ketamine exerts its antidepressant effects through glutamatergic signaling mechanisms. The role of glutamate signaling in MDD is supported by growing evidence showing that antidepressants ultimately converge to regulate AMPA and NMDA receptor-mediated synaptic plasticity[94], via a cascade of time-dependent signaling.

Using the social defeat paradigm, we found that after severe social stress synaptic plasticity mechanisms are affected. In chapter 3, we showed that reduced spatial memory after social defeat is associated with reduced hippocampal synaptic expression of the glutamatergic receptor subunits GluN1, GluN2B and GluA2. In particular, the regulated synaptic expression of NMDA receptor subunits provides a dynamic and potentially powerful mechanism for the regulation of synaptic efficacy and remodeling. Indeed, it has been shown that alterations in NMDAR number and/or subunit composition contribute to the expression mechanisms of LTP (NMDAR-LTP)[150] and LTD (NMDAR-LTD)[151]. This is another example in which synaptic glutamate signaling plays a role in generating a depressive phenotype. The reduced expression of NMDA receptor subunits is of particular interest since it might represent a mechanism of metaplasticity in the glutamatergic system[37], and most likely underlies the cognitive impairments in a hippocampus-dependent memory task.

Long after social defeat, synaptic changes are also apparent (chapter 4). Brain slice recordings of the hippocampus revealed a typical depressed state of the synapse. This reduced LTP in CA1 subfields probably affected hippocampus-dependent cognitive performance. We then used a proteomics analysis to identify synaptic alterations at the molecular level. The cognitive impairments and reduced LTP were associated with an increased hipocampal synaptic expression of the Na,K ATPase subunit AT1B2. Na,K ATPases are membrane localized proteins responsible for active transport of Na^+ and K^+ ions across the plasma membrane, thereby generating a gradient responsible for cell polarization and repolarization[173]. Increased expression and increased transporter activity could cause a faster clearance of K^+ from the synaptic cleft, and of Na^+ from the intrasynaptic compartment. This would result in a faster re-polarization after firing of an action potential, and therefore less glutamate release and calcium influx. Together, this might cause less plasticity in hippocampal synapses and underlie impaired LTP. Moreover, this Na,K ATPase dependent mechanism is a possible route through which stress can alter plasticity mechanisms, thereby inducing depressive symptoms.

Different mechanisms short and long-term after stress

Stress has well-established effects on spatial memory performance in rodents by affecting hippocampal LTP[40,41,43]. Since hippocampal LTP is dependent on subunit specific regulation of AMPA receptors, it is not surprising that synaptic expression of GluA2 is affected after social defeat stress. In line with this, the stress hormone glucocorticoid enhances AMPA receptor mobility in hippocampal synapses[135]. Our observation that also synaptic localization of two NMDA receptor subunits was affected provides another mechanism through which plasticity mechanisms are regulated acutely after stress.

Also, long after social defeat spatial memory performance and LTP in the CA1 subfields were reduced hinting towards altered glutamatergic signaling. However, in contrast to 24 hour after social defeat, none of the glutamate receptor subunits were differentially expressed in synaptic membrane fractions. Instead, we observed an increase in synaptic expression of AT1B2. The fact that this Na,K ATPase subunit was not differentially expressed 24 hours after social defeat (data not shown), further confirms that on the long-term different synaptic plasticity mechanisms are involved in causing reduced hippocampal LTP and memory. This argues that direct changes in levels of glutamate receptor subunits are involved in the installment of cognitive impairments immediately after stress exposure, while different synaptic mechanisms, e.g., changing the membrane potential, account for the maintenance of these symptoms.

Depressive-like phenotypes after normalization of corticosterone levels

The hypothalamic-pituitary-adrenal (HPA) axis has been found abnormal in depressed patients[224]. For example, a significant percentage of depressed patients have increased levels of cortisol in the saliva, plasma and urine, and increased size (as well as activity) of the pituitary and adrenal glands[227]. It is hypothesized that impaired hippocampal signal processing, due to damage by increased glucocorticoid levels, interferes with feedback inhibition of the HPA axis[35]. The resulting hyperactive HPA axis would then lead to an inappropriate stress response and, thereby, to the installment of depressive symptoms. However, here we show that despite hippocampal signaling being affected long after social defeat stress, corticosterone levels were normal. This argues that impaired hippocampal processing can induce depressive-like phenotypes, independent of HPA axis dysfunction. This supports the hypothesis that HPA axis hyperactivity is not a simple consequence or an epiphenomenon of depressive phenotypes, but on the contrary, that it is a risk factor predisposing to the development of depression[228].

Social defeat stress affects hippocampal neurogenesis

In chapter 5, we showed that after three months of individual housing, following a 5-day period of repeated social defeat, hippocampal neurogenesis is reduced indicated by a decrease in doublecortin postivie (DCX+) cells. This was most profound for older DCX+ cells with long apical dendrites, whereas younger cells remained unaffected. Chronic imipramine treatment subsequently increased both cell populations. Whether there is a causal relation between depressive phenotypes, cognitive impairments and a decrease in neurogenesis is still an active field of investigation[68]. However both neurogenesis-dependent and neurogenesis-independent mechanisms are likely to contribute to the reversal of depressive-like behaviors by antidepressants[47,216]. This is in agreement with the network hypothesis of depression stating that depressive-like behavior reflects problems in information processing within particular neural networks in the brain and that antidepressant drugs and other treatments that alleviate depression function by gradually improving information processing within these networks[229].

Behavioral therapy

Another interesting novel finding is the efficacy of behavioral therapy consisting of housing in an enriched environment for one hour daily. This type of therapy resembles activation and fysical execise therapy and aspects of behavioral therapy for depressed patients. Behavioral therapy was found equally effective in treating anhedonic symptoms, depression-associated cognitive impairments, as well as their underlying molecular and cellular correlates when compared with chronic imipramine treatment. Enriched environments produce functional and anatomical changes in neural networks that are reflected in the gradual improvement of natural behavior[230]. In analogy, behavioral psychotherapy might also have therapeutic effects on mood disorders through use-dependent neuronal plasticity. Therefore, behavioral and pharmacological therapies, might all lead to improved information processing and mood recovery through mechanisms that stimulate similar processes of plasticity. In this scenario, a combination of drug treatment and psychotherapy would be expected to be more beneficial than either treatment alone, and there is evidence that this might be the case[231].

Depression and neuroplasticity

Increasing evidence demonstrates that neuroplasticity, a fundamental mechanism of neuronal adaptation, is disrupted in mood disorders and in animal models of depression[156]. Chronic stress, which can cause depression, also disrupts neuroplasticity[35,44], whereas

several forms of antidepressant treatment induce opposing effects[67]. As discussed in the previous section, throughout this thesis, we made several novel observations supporting the neuroplasticity theory of depression. These findings hold throughout three different levels: structural plasticity, functional plasticity, and molecular mechanisms accompanying such changes. At the structural level we found that neurogenesis was affected after social defeat stress and imipramine treatment was able to restore the neurogenic process at this late time point, long after stress experience. This indicates that antidepressants might facilitate optimization of neuronal connectivity by increasing the choice of neurons available for selection through activity-dependent mechanisms. This process is expected to take time to develop and mature, which is consistent with the delayed appearance of the clinical effects of antidepressants[229].

Chronic stress impairs hippocampal LTP[232]. Short after social defeat stress, we found that hippocampal memory impairments were associated with decreased synaptic expression of glutamate receptor subunits. These are possibly caused by synaptic signaling pathways affected by social defeat stress. For example, several forms of chronic stress have been observed to increase the phosphorylation of MAPK[233]. The activation of MAPK appears to be critical for the effects of behavioral stress on hippocampal LTP[234]. Also, increased stress hormone levels and chronic stress can impair CREB activity[235].

The increased expression of AT1B2 that is associated with a decrease in LTP long after social defeat stress is a novel mechanism by which hippocampal plasticity is affected, and supports the neuroplasticity hypothesis of depression. However, care should be taken when projecting these findings to other brain regions. For example, in the amygdala[51] and nucleus accumbens[34] stress has opposing effects when compared with our data and increases neuroplasticity. Thus, our findings are brain region- and circuit-dependent and relate to the specific role of the hippocampus in MDD pathophysiology. The observation that both behavioral therapy and imipramine treatment (both effective treatments for MDD) have similar effects on plasticity and reverse of depressive-like phenotypes at the affective and cognitive domain argues that these changes are relevant for the disease phenotype of MDD.

Clinical relevance

Depression and cognition

The observed changes in neuroplasticity in our depression model hint to aberrant information processing. Indeed, we found reduced cognitive hippocampal performance

associated with plasticity changes both short- and long-term after social stress exposure. By employing the social defeat model, we were capable of modeling aspects of cognitive impairments after a severe stress experience. Socially stressed rats showed decreased spatial memory, a symptom also observed in MDD patients[130]. Our finding that increased plasticity induced by both pharmacological treatment and behavioral therapy were able to relieve symptoms in both the affective and cognitive domain argues that improved information processing contribute to their antidepressant effects.

There is a long history of research investigating the interaction of cognition and emotion in MDD. Clinicians and researchers alike have focused on cognitive processes and on the content of depressive cognition in trying to gain a more comprehensive understanding of MDD. These studies postulate that "associative networks" lead to cognitive biases on negative emotions in depressed individuals. Biases in cognitive processes, such as attention and memory, may not only be correlates of depressive episodes; they may also play a critical role in increasing the individuals' vulnerability for the first onset and recurrence of depression[236]. Most cognitive theories propose vulnerability-stress hypotheses that posit that the onset of this disorder is due to the interaction of a psychological vulnerability (e.g., certain cognitions or particular ways of processing information) and a precipitating stressor. Importantly, one of the most effective interventions for depression, cognitive-behavioral therapy, focuses on modifying biased interpretations and dysfunctional automatic thoughts[237].

Depressed people experience difficulties involving concentration and memory (Burt et al. 1995). A general accepted attempt to integrate these findings with cognitive biased processes is the resource-allocation hypothesis. This postulates that because cognitive capacity is reduced, depressed individuals have deficits in remembering and in engaging in other effortful cognitive processes[238]. Additionally, the amount of resources available for cognitive operations is limited and depression either occupies or functionally reduces these resources, for example, because resources are used by task-irrelevant emotional processing. Thus, deficits should become evident in effortful, resource-demanding components of memory tasks.

Given these still unsubstantiated views, the question remains how cognitive deficits are related to the hallmark feature of depression, the sustained negative affect. Therefore this interaction should be a focus of future depression research.

Implications for treatment strategies

Throughout this thesis, several observations were made with important implications for treatment strategies for MDD. Our social defeat paradigm models symptoms in the affective and cognitive domains. In this paradigm, depressive-like behavior is induced by social stress experience in adult rats and, therefore, models adult onset stress-induced depression with cognitive impairments seen in depressed patients[239]. Here, we found that behavioral therapy was just as effective as imipramine treatment in rescuing depressive phenotypes and their underlying cellular and molecular correlates. This argues that behavioral therapy should be explored further as treatment option for depressed patients with cognitive impairments, that do not need, do not respond to or have adverse effects from pharmacological treatment.

Furthermore, antidepressant effects of imipramine and behavioral therapy went hand in hand with an increase in hippocampal plasticity in glutamatergic systems as expressed by an increase in LTP after social stress. Also the acute and lasting antidepressant effects of ketamine were associated with a possible increase in AMPA receptor function in the hippocampus. This underscores that glutamatergic signaling pathways should be explored for potential therapeutic targets of MDD with faster therapeutic effects[54].

Finally, we showed that during the maintenance phase, likely different cellular and synaptic mechanisms are involved in stress-induced depressive phenotypes when compared with synaptic changes involved in the establishment of these symptoms directly after stress exposure. Since MDD patients are mostly treated (long) after cessation of the stress period, i.e., when depressive symptoms are evidently manifest, data originating from paradigms that model the maintenance phase of depression might be more relevant for implementation of treatment strategies. Therefore I argue for a shift in pre-clinical depression research, in which studies should focus increasingly on the maintenance phase of the disease rather that the induction phase of depression.

Nederlandse samenvatting

Moleculaire en cellulaire neuroplasticiteit in diermodellen voor depressie

Depressie is een veelvoorkomende psychiatrische aandoening die vaak diep in het leven van mensen ingrijpt. Depressie wordt gekenmerkt door een gedeprimeerde stemming en een verlies van belangstelling in aangename activiteiten. De oorzaak van depressie is grotendeels onbekend, maar in het algemeen wordt aangenomen dat een combinatie van genetische- en omgevingsfactoren hieraan ten grondslag ligt. Van de omgevingsfactoren is stress de meest voorkomende factor.

Neurobiologisch gezien zijn er verschillende hersengebieden betrokken bij het tot stand komen van depressie symptomen. Eén van de gebieden waarin duidelijke veranderingen waarneembaar zijn bij depressieve patiënten is de hippocampus. Deze is betrokken bij verschillende cognitieve processen waaronder het semantisch geheugen en ruimtelijke oriëntatie. Daarnaast staat de hippocampus in verbinding met verschillende andere limbische hersengebieden die eveneens betrokken zijn bij gemoedstoestandregulerende processen.

In het afgelopen decennium is duidelijk geworden dat er een verband bestaat tussen depressie en neuroplasticiteit. Deze relatie is duidelijk geworden door het gebruik van verschillende preklinische diermodellen voor depressie. Hiermee is aangetoond dat stress – één van de belangrijkste oorzaken van depressie – verschillende mechanismen van neuronale plasticiteit aantast. Ook is duidelijk geworden dat antidepressiva en niet-farmacologisch gebaseerde therapieën deze processen juist stimuleren, hetgeen neuroplasticiteit meer in het focus van aandacht heeft gebracht.

In dit proefschrift heb ik gebruik gemaakt van verschillende diermodellen in knaagdieren om de relatie van depressie met neuroplasticiteit te bestuderen. Naast de directe effecten van stress heb ik ook de lange termijn effecten van stress bestudeerd. Dit verdiend bijzondere aandacht omdat bij mensen de depressieve symptomen nog lang na een stressvolle levenservaring tot utdrukking komen.

Hoofdsuk 2: Het antidepressieve effect van ketamine door middel van regulatie van AMPA receptor activiteit

Ketamine is een N-methyl-D-aspartic acid (NMDA) receptor antagonist en wordt doorgaans gebruikt als verdovingsmiddel. Onlangs is gebleken dat deze stof, wanneer deze in lage

(sub-anesthetische) concentraties wordt toegediend, zowel een acute als een langdurige antidepressieve werking heeft in patiënten die verder niet reageren op klassieke antidepressiva, die hun eerste aangrijpingspunt hebben op het monoaminerge systeem. Van de klassieke antidepressiva is bekend dat ze pas effectief worden na chronische toediening van tenminste drie weken. De acute (binnen een uur) en langdurige werking van ketamine zijn daarmee erg interessant omdat dit impliceert dat de stof via, tot nog toe onbekende, monoamine-onafhankelijke mechanismen werkt. Om deze mechanismen te achterhalen heb ik gebruik gemaakt van de 'forced swim test'. Dit is een preklinische test in muizen die effectief is in het voorspellen van de mogelijke antidepressieve werking van een stof. Met behulp van deze test heb ik ontdekt dat locale injectie van ketamine in de CA1 regio van de dorsale hippocampus een acute en langdurige (negen dagen na toediening) antidepressieve werking heeft. Door middel van immunoblot analyse op het synaptisch proteoom van de dorsale hippocmapus, heb ik vervolgens aangetoond dat negen dagen na toediening van ketamine de phosphorylatie van Ser-845 van α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor subunit GluA1 verhoogd is. Dit duidt op een verhoogde 'turn-over' snelheid – en daardoor op een mogelijke verhoogde receptor functie – van AMPA receptoren. Om aan te tonen dat het antidepressieve effect van ketamine wordt veroorzaakt door een verhoogde AMPA receptor activiteit in de hippocampus, hebben we deze situatie nagebootst door toediening van een TAT-$Glu2_{3Y}$ peptide dat de gereguleerde endocytose van AMPA receptoren blokkeert. Toediening van dit peptide in de dorsale hippocampus had een antidepressieve werking in de 'forced swim test'. Hiermee hebben we aannemelijk gemaakt dat de antidepressieve werking van ketamine in ieder geval deels wordt veroorzaakt door een verhoogde AMPA receptor functionaliteit in de hippocampus.

Hoofdstuk 3: 'Social defeat' stress induceert veranderingen in de synaptische expressie van glutamate receptoren.

Om een beter inzicht te krijgen in de moleculaire neurobiologie van depressie moet gebruik worden gemaakt van diermodellen met een sterke validiteit voor de ziekte in mensen. Het 'social defeat' model in ratten is hiervan een goed voorbeeld. In dit model wordt gebruik gemaakt van een hevige sociale stressor, één maal per dag, voor vijf dagen achter elkaar. Hierdoor heeft het model een sterke etiologische validiteit, aangezien psychosociale stress in mensen de vatbaarheid voor depressie sterk verhoogd. Met dit model heb ik aangetoond dat 24 uur na de laatste sociale stresservaring, het niveau van het stresshormoon coritcosteron in het bloedplasma 2.5 keer zo hoog is in vergelijking tot controle ratten. Deze sociale stress vermindert ook de prestatie van deze ratten in een hippocampus afhankelijke

cognitieve gedragstaak gebaseerd op het ruimtelijk geheugen. Bovendien heb ik aangetoond dat dit verminderde ruimtelijke geheugen gepaard gaat met een verlaagde synaptische expressie van de NMDA receptor subunits GluN1 en GluN2B en de AMPA receptor subunit GluA2 in de dorsale hippocampus. Vervolgonderzoek moet uitwijzen in welke mate de veranderde expressie van deze receptor subunits leidt tot elektrofysiologische veranderingen in synaptische plasticiteit.

Hoofstuk 4: Gedragstherapie herstelt verminderde cognitieve prestaties na 'social defeat' stress, die samengaan met een verlaagde LTP en verhoogde synaptische expressie van AT1b2 in de hippocampus.

In mensen komen depressieve symptomen meestal nog lang na een stressvolle levenservaring tot uitdrukking. Om een beter inzicht te krijgen in de neurobiologie van depressie en om op zoek te gaan naar moleculaire targets voor antidepressiva is het van grote meerwaarde om juist de lange termijn effecten van stress te onderzoeken. Om deze aanhoudende fase van depressie te modelleren hebben we ratten na 'social defeat' stress voor drie maanden in sociale isolatie gehouden. Door deze behandeling werden (hippocampusafhankelijke) cognitieve prestaties van ratten sterk verminderd. Dit zou overeen kunnen komen met de cognitieve disfuncties, zoals een verminderd geheugen, die vaak worden waargenomen in depressieve patiënten. De verminderde cognitieve capaciteit van deze ratten gaat gepaard met depressieve symptomen, zoals een verminderde anticipatie ten opzicht suikerwater. Bovendien konden zowel de verminderde cognitieve prestaties als de anhedonische symptomen hersteld worden door zowel gedragstherapie – bestaande uit dagelijkse huisvesting in een verrijkte omgeving gedurende een uur – alswel door een farmacologische behandeling, bestaande uit chronische toediening van het tricyclisch antidepressivum imipramine.

Verder heb ik aangetoond dat deze verminderde cognitieve capaciteit lang na social defeat stress, gepaard gaat met een verlaagde lange-termijn-potentiatie (LTP) in CA3-CA1 synapsen in de dorsale hippocampus. Deze plasticiteitsverandering is te herstellen middels de gedrags- en farmacologische therapie. Om te achterhalen welke moleculaire veranderingen in de synaps hieraan ten grondslag zouden kunnen liggen, werd met behulp van iTRAQ proteomics de samenstelling van het synaptische 'proteoom' in de dorsale hippocampus geanalyseerd. Dit is een zeer gevoelige techniek, die het mogelijk maakt om synaptische eiwitten te detecteren en kleine veranderingen in eiwitexpressie te kwantificeren. Hiermee heb ik gevonden, dat lang na 'social defeat' stress, de synaptische expressie van de Na,K ATPase subunit AT1b2 met ~40% verhoogd is ten opzichte van

controle ratten. Zowel gedragstherapie als chronische toediening van imipramine zijn in staat om de verminderde LTP en verhoogde AT1b2 expressie te normaliseren. Of de verhoogde expressie van AT1b2 ook de oorzaak is van de verminderde cognitieve prestaties zal in vervolgexperimenten onderzocht moeten worden.

Hoofstuk 5: Neurogenese in de hippocampus is verminderd lang na social defeat stress

In verschillende stress geïnduceerde modellen voor depressie in knaagdieren is in voorgaande studies een verminderde neurogenese in de hippocampus waargenomen. Bovendien zijn verschillende soorten antidepressiva in staat om deze verminderde neurogenese te herstellen. Deze bevindingen hebben geleid tot de neurogenese theorie van depressie. In dit hoofdstuk heb ik gekeken of neurogenese ook aangetast is in de aanhoudende fase van de depressieve symptomen, dus lang na de laatste 'social defeat' ervaring. Hiervoor heb ik door middel van stereologische technieken het aantal doublecortin (DCX) immunopositieve cellen bepaald. DCX is een marker voor nieuwgeboren immature neuronen. Op basis van de morfologie van de dendritische bomen van deze cellen heb ik specifiek het aantal jonge (klasse-I) en meer volgroeide (klasse-II) DCX positive (DCX^+) cellen bepaald. Het aantal DCX^+ cellen was sterk verminderd lang na 'social defeat' stress, hetgeen vooral werd veroorzaakt door een afname van klasse-II cellen, terwijl het aantal klasse-I cellen onveranderd was. In tegenstelling tot verhoogde niveaus stresshormoon corticosteron direct na de 'social defeat', was op de lange termijn het niveau genormaliseerd tot dat van controle dieren. Verder heb ik laten zien dat chronische behandeling met imipramine de vermindering in neurogenese herstelt door een toename van zowel klasse-I als klasse-II cellen te bewerkstelligen. Hieruit kunnen we concluderen dat, ondanks dat de stresshormoon niveaus genormaliseerd zijn, neurogenese blijvend is aangetast na 'social defeat' stress door een afname van klasse-II cellen. Het is opmerkelijk dat imipramine de verminderde neurogenese in dit late stadium nog kan herstellen.

Conclusie

In dit proefschrift heb ik laten zien dat in verschillende diermodellen voor depressie, diverse plasticiteitsprocessen in de hippocampus zijn aangetast. Interessant daarbij is dat deze processen niet direct gekoppeld zijn aan het monoaminerge systeem. Dus met dit onderzoek zijn nieuwe mechanismen gekarakteriseerd, die mogelijk kunnen worden gebruikt voor farmacologische interventies. Bovendien heb ik aangetoond dat andere moleculaire processen betrokken zijn bij de lange termijn effecten van stress, in vergelijking tot de acute

effecten die betrokken zijn bij de inductie van depressieve symptomen. De karakterisatie van deze lange termijn effecten is interessant voor ontwikkeling van nieuwe antidepressiva, aangezien depressieve patiënten over het algemeen pas lang na een stressvolle levenservaring worden behandeld. Een andere interessante bevinding is dat gedragstherapie een vergelijkbare efficiëntie heeft in het verminderen van stressgeïnduceerde depressieve symptomen in vergelijking tot chronische behandeling met imipramine. Bovendien was de gedragstherapie net zo efficiënt in het herstellen van aangetaste plastische processen in de hippocampus. Deze bevinding heeft mogelijk een implicatie voor de behandelingsstrategie van depressie, die in de toekomst wellicht meer op activiteitentherapie gericht zou kunnen worden.

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dankzij jullie al wel hoe je een depressie kan voorkomen. Ik adviseer een intensieve omgang met de volgende personen:

Mijn drie maten: Paul, Heck en Sté: grollen in het huis, kijken of er een bandje speelt in Dadas, Hein stompjes geven als hij die verdient (eigenlijk altijd wel), rennen over het spoor, vier frikadellen. Wie wil er een Bator? Rik, praten over voetbal, wetenschap en vrouwen. Wat wil een mens nog meer. De koningen van de Regulierstraat en Krista, er is voor mij geen mooier moment in het jaar dan het moment waarop er op een vrijdagavond in mei 'Moonshadow' uit de speakers komt. Again my colleagues. Scheldeplein 6: Veer, Eef, Sexma en Jeroen, Brabantse gezelligheid in Amsterdam. Chaan, ooit zullen onze telefoongesprekken tot radioprogramma gepromoveerd worden. We bellen (smiley). Maart, we hebben lang naar de Buurvrouw gezocht en veel later ook gevonden. Dat hebben ze geweten. Lisa, wij weten elkaar ongevraagd te motiveren en hebben veel plezier, ook samen met Lau en Janna. Anneke vd Bijgaart, jij hebt het in genetisch opzicht wel zo goed voor elkaar. Daardoor ben je zo chill. Dalila, nergens eet ik zo lekker uit ergonomisch en esthetisch volmaakte bakjes. De Venmilie, een bont gezelschap van warmte en vooral gezelligheid.

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Tot slot, mijn familie. Ik denk dat het afgelopen jaar duidelijk is gebleken hoe sterk onze band is. Mam en Jack, jullie zijn er altijd voor me geweest. Ook al maak ik soms andere keuzes dan jullie voor ogen hebben, ik word altijd door jullie gesteund. Een steun die rust geeft, maar ook motiveert. Jullie hebben me enthousiast weten te maken voor alles wat er in deze wereld speelt.
Mijn zus, Anneke; onbegrensd, onbezonnen, doelgericht, gepassioneerd, alom geliefd en vrij van geest. En dan ook nog super lief.

Pieter

Curriculum Vitae

Pieter van Bokhoven werd geboren op 7 januari 1982 in Helmond. In 2000 behaalde hij cum laude zijn VWO diploma aan het Dr. Knippenberg College. In dat zelfde jaar begon hij zijn studie Natuurwetenschappen aan de Radboud Universiteit Nijmegen. Tijdens zijn doctoraal deed hij twee neurobiologische onderzoeksstages. Tijdens zijn eerste stage aan de afdeling Cellulaire Dierfysiologie van prof. Eric Roubos en dr. Bruce Jenks onderzocht hij het effect van 'Brain-Derived Neurotropic Factor' op de plasticiteit van melanotrope cellen in *Xenopus laevis*. Zijn tweede stage voerde hij uit aan de afdeling Cancer Research and Developmental Biology van The Hospital for Sick Children in Toronto, Canada. Hier onderzocht hij de rol van Plexin signaling op de uitgroei van neuronen. In 2006 studeerde hij cum laude af in de natuurwetenschappen met als specialisatie biochemie. Van 2007 tot 2011 deed Pieter zijn promotieonderzoek onder begeleiding van prof. Smit en dr. Spijker naar de neurobiologische achtergrond van depressie. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. Sindsdien werkt hij samen met collega Jochem Cornelis aan de ontwikkeling van applicaties en is hij sinds juli 2011 werkzaam als Associate Publication Manager bij Excerpta Medica te Amsterdam.