Homer has the blues

Involvement of Homer1 in stress-induced psychopathology

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If something's hard to do,
then it's not worth doing!

Homer Simpson

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Abstract

Excitatory glutamate signaling has received growing attention with respect to the emergence of mood disorders such as depression. To understand the involvement of this system in psychopathology, it is important to elucidate the molecular mechanisms that mediate potentially detrimental effects. In the current thesis, I investigated the role of Homer1, a postsynaptic scaffolding protein that links group I metabotropic glutamate receptors to intracellular target effectors, in stress-associated psychiatric disorders. I employed a model of social stress in mice that induces lasting and reproducible alterations on the behavioral and neuroendocrine level. I could show that Homer1 is dynamically regulated by acute and chronic social defeat stress and provided evidence that these regulations are directly connected to detrimental behavioral effects induced by stress. Activity of the hypothalamic-pituitary-adrenal axis, one of the major physiological systems involved in the stress response, was also modulated by Homer1, thereby further indicating the importance of Homer1-mediated signaling pathways during stressful challenges. In addition, I could show that Homer1 is critically involved in reward associated learning and behavior, especially with respect to the role of hippocampal Homer1, which has not been shown previously. I could also demonstrate the efficacy of novel metabotropic glutamate receptor 5 antagonists with respect to their antidepressant properties and their ability to reverse stress-induced behavioral alterations, both in acute and chronic treatment setups. The findings presented in this thesis provide a strong basis for further research investigating the mechanistic action of novel, glutamate-transmission based compounds that possess antidepressant-like properties.

Zusammenfassung

In den letzten Jahren wurde eine Reihe von Studien veröffentlicht, die den Einfluss des stimulierenden Neurotransmitters Glutamat auf die Entstehung von psychiatrischen Erkrankungen beschreiben. In diesem Kontext ist es außerordentlich wichtig, dass die molekularen Grundlagen dieses Signalsystems aufgeklärt werden. In der vorliegenden Arbeit untersuche ich die Rolle von Homer1, einem postsynaptischen Gerüstprotein, welches metabotrope Glutamat-Rezeptoren mit intrazellulären Signalkaskaden verknüpft, im Kontext von Stress-induzierten Veränderungen im Verhalten und auf neuroendokriner Ebene. Dazu habe ich ein Modell für sozialen Stress in Mäusen genutzt, welches wichtige phänotypische Aspekte von psychiatrischen Erkrankungen gut widerspiegelt. Ich konnte zeigen, dass Homer1 dynamisch von Stress reguliert wird und dass diese Änderungen in der Transkription direkt mit negativen Änderungen auf der Verhaltensebene verknüpft sind, die durch Stress hervorgerufen werden. Die Regulation der Hypothalamus-Hypophysen-Nebennieren-Achse kann ebenfalls von Homer1 moduliert werden, was die Bedeutung dieses Signalweges während Stress zusätzlich unterstreicht. Weiterhin konnte ich zeigen, dass Homer1 eine wichtige Rolle bei Verhaltensformen spielt, die mit Belohnungs-Lernen assoziiert sind. Hierbei konnte ich besonders die Beteiligung des Hippocampus in diesen Prozessen weiter hervorheben. Ebenfalls konnte in dieser Arbeit nachgewiesen werden, dass Substanzen die die Funktion des metabotropen Glutamat-Rezeptors 5 unterbinden, auf präklinischer Ebene ähnlich wie Antidepressiva wirken. Die vorliegende Arbeit bietet daher eine exzellente Basis für weitere Forschungsarbeiten, die sich mit der Rolle von Glutamat in psychiatrischen Erkrankungen beschäftigen.

List of abbreviations

AAV	adeno-associated virus
ACTH	adrenocorticotropic hormone
AdCx	adrenal cortex
ADHD	attention deficit hyperactivity disorder
AdM	adrenal medulla
AMP	adenosine monophosphate
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid
Amy	amygdala
AnPi	anterior lobe of the pituitary
ANOVA	analysis of variance
AVP	arginine vasopressin
BGH	bovine growth hormone
BSA	bovine serum albumin
CA1	cornu ammonis region 1
CA2	cornu ammonis region 2
CA3	cornu ammonis region 3
CCDS	consensus coding sequence
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
CoIP	coimmunoprecipitation
Cort	corticosterone
CRH	corticotropin-releasing hormone
cRNA	complementary RNA
CSDS	chronic social defeat stress
CTEP	2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine
CUMS	chronic unpredictable mild stress
Da	Dalton
DEX	dexamethasone

DG	dentate gyrus
DNA	deoxyribonucleic acid
EAAT	excitatory amino acid transporter
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EPM	elevated plus maze
EVH1 domain	enabled/vasodilator-stimulated phosphoprotein homology 1 domain
ExE	environment by environment
FKBP51	FK506 binding protein 51
FR	fixed ratio
FST	forced swim test
FUST	female urine sniffing test
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCs	glucocorticoids
GKAP	guanylate kinase-associated protein
GR	glucocorticoid receptor
GxE	gene by environment
GxExE	gene by environment by environment
HC	hippocampus
HPA axis	hypothalamic-pituitary-adrenal axis
IEG	immediate early gene
IP_3	inositol-triphosphate
IRES	internal ribosome entry site
ITI	intertrial interval
KO	knockout
LC	liquid chromatography
LSD	least significant difference
LTD	long-term depression
LTP	long-term potentiation
Lzip	leucine zipper
MAP	mitogen-activated protein

mGluR	metabotropic glutamate receptor
MPEP	2-Methyl-6-(phenylethynyl)-pyridine
mRNA	messenger RNA
MR	mineralocorticoid receptor
MS	mass spectrometry
MT-1	metallothionein-1
MTEP	3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine
MWU	Mann-Whitney-U
NDRI	norepinephrine-dopamine reuptake inhibitor
NMDA	N-methyl-D-aspartate
OE	overexpression
OF	open field
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Phe	phenylalanine
PND	postnatal day
PFC	prefrontal cortex
POMC	pro-opiomelanocrotin
PPi	posterior lobe of the pituitary
PR	progressive ratio
Pro	proline
PSD	postsynaptic density
PSD-95	postsynaptic density protein 95
PTSD	post-traumatic stress disorder
PVC	polyvinyl chloride
PVN	paraventricular nucleus of the hypothalamus
qPCR	quantitative reverse transcription PCR
RNA	ribonucleic acid
S/N	signal to noise
SA	social avoidance
SARI	serotonin antagonist and reuptake inhibitor
SDS	sodium dodecyl sulfate

SEM	standard error of the mean
shRNA	short hairpin RNA
SNRI	serotonin-noradrenaline reuptake inhibitor
sOR	spatial object recognition
SSC	saline sodium citrate
SSRI	selective serotonin reuptake inhibitor
TRP	transient receptor potential
UTP	uridine triphosphate
UV	ultraviolet
VGLUT	vesicular glutamate transporter
VR	variable ratio
WPRE	woodchuck post-transcriptional regulatory element
WT	wild type

1. General introduction

1.1. Conceptual and molecular basis of stress

The concept of stress

Modern language use is readily referring to the term stress when describing for example social and economic challenges, which are affecting individuals. Many people have established a rather clear picture of what it feels like *to be stressed*, and consequently aim for *stress-relieving* activities to counterbalance their perceived daily hassles. Interestingly, the concept of stress had not been used in physiological contexts until the 1930s, when the Canadian-Hungarian endocrinologist Hans Selye (1907 - 1982) formulated the hypothesis of the general adaptation syndrome (Selye, 1936). Selye was initially inspired by the work of the American physiologist Walter Cannon, who defined the term homeostasis as a balanced state of the body's physiological parameters, such as pH and glucose levels, that are essential for survival (Cannon, 1932). A disturbance in these parameters by exterior influences consequently leads to the general adaptation syndrome that is initiated by an alarm reaction in which the organism tries to restore homeostasis and is followed by a resistance stage, where adaptation to the challenge is optimally sustained. Selye also identified a third stage of exhaustion, where the organism is no longer able to respond adequately to its environment which ultimately leads to illness. Further developing his concept, he later defined the term stress as *"the state manifested by a specific syndrome, which consists of all the nonspecifically induced changes within a biologic system."*(Selye, 1956), thereby marking the beginning of the modern stress theory. In light of the scientific advancements on the field of stress research over the last decades, it became apparent that stress itself is not necessarily detrimental, but enables the individual to readily adapt to environmental challenges and prepare for future exposures (McEwen, 1998; McEwen, 2003). Successive work by Bruce McEwen further developed this concept of adaptation to physiological or behavioral challenges by the term allostasis which is defined as *"the adaptive processes that maintain homeostasis through the production of mediators such as adrenalin, cortisol and other chemical messengers."* (McEwen, 2005).

Molecular pathways of the stress response

There are two major systems involved in the stress response. On the one hand, the sympathetic arm of the autonomic nervous system is triggered within seconds following a stress exposure and results in increased energy mobilization, accelerated heart rate and elevated blood pressure (Koolhaas et al., 1999; Ulrich-Lai and Herman, 2009). This “fight or flight” response is mediated by the release of adrenaline and noradrenaline and enables the organism to quickly react to a threat. However, these alterations are of short duration due to the concomitant parasympathetic activation, which counteracts the sympathetic effects (Goldstein, 1987).

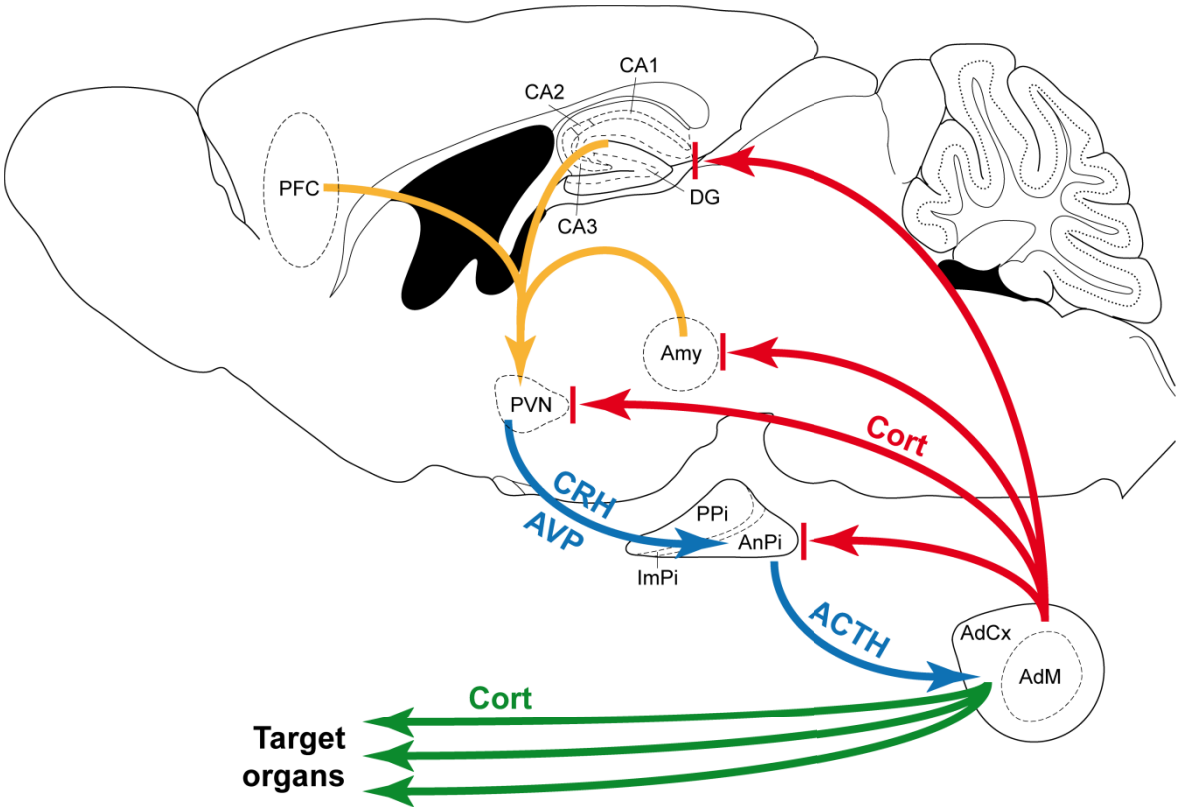

Figure 1.1: Signaling pathway of the hypothalamic-pituitary-adrenal axis in the mouse. Upon stress exposure, a signaling cascade originating from the paraventricular nucleus of the hypothalamus (PVN) (Blue arrows) results in the peripheral release of corticosterone (Green arrows). At the same time, cort inhibits the continuous release of stress hormones by a negative feedback loop, which targets the pituitary and several sites in the central nervous system, including the hippocampus and the amygdala (Red arrows). These brain regions, together with the prefrontal cortex, have been shown to exert modulatory effects on the PVN via neuronal connections, which are both direct and indirect via other nuclei (Yellow arrows). PFC, prefrontal cortex; CA1, CA1 region of the hippocampus; CA2, CA2 region of the hippocampus; CA3, CA3 region of the hippocampus; DG, dentate gyrus; Amy, Amygdala; CRH, corticotropin-releasing hormone; AVP, arginine vasopressin; PPi, posterior lobe of the pituitary; ImPi, Intermediate lobe of the pituitary; AnPi, anterior lobe of the pituitary; AdCx, adrenal cortex; AdM, adrenal medulla; Cort, corticosterone. See text for further details.

On the other hand, the hypothalamic-pituitary-adrenal (HPA) axis provides a more long-term reaction to a stressor, but the complex signaling cascades involved in the activation take some time to exert their effects on the individual (De Kloet et al., 1998) (Figure 1.1). Upon stress exposure, corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are released from parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) into the hypophyseal portal system and subsequently act on the anterior pituitary, where the synthesis of pro-opiomelanocortin (POMC) is stimulated (Sapolsky et al., 2000). POMC, in turn, is processed to adrenocorticotropic hormone (ACTH), which is then secreted from the anterior lobe of the pituitary and stimulates the release of glucocorticoids (GCs) from adrenal cortex cells of the adrenal glands. GCs, predominantly cortisol in humans and corticosterone in most rodents, then exert multiple effects on the individual, including cardiovascular activation as well as suppression of immune and digestive functions (Strehl et al., 2011). Furthermore, they are mainly responsible for the effective shutdown of the HPA axis activation in response to a stressor by acting in a negative feedback loop. In the PVN and the anterior pituitary, but also in other brain regions directly or indirectly connected to the PVN, GCs bind to their respective steroid receptors and thereby inhibit the secretion of the aforementioned hormones (De Kloet et al., 1998).

The mineralocorticoid receptor (MR) is mainly expressed in limbic regions such as the hippocampus, the septum and the amygdala (Kolber et al., 2008) and has a high affinity for GCs, which suggests its prominent role in the regulation of basal HPA axis activity (De Kloet et al., 1993; Joëls and De Kloet, 1994; ter Horst et al., 2012). The glucocorticoid receptor (GR) is widely expressed throughout the brain and its binding affinity for GCs is 10-fold less than MR, which results in increased GR occupancy when GC levels rise in response to HPA axis activity. Besides their role as mediators of the negative feedback on the HPA axis activity on the level of the PVN and the pituitary, GRs have also been shown to modulate the stress response in other brains regions such as the hippocampus and the amygdala (Joëls and Baram, 2009). Both receptors function as transcriptional regulators but also have recently been shown to modulate fast GC-dependent effects of the cell membrane.

Pathological impact of stress

It is a well-established concept that an inappropriate or overextended stress response comes with energetic costs referred to as allostatic load, which in turn produces wear and tear on the regulatory systems of the body (McEwen, 2003; de Kloet et al., 2005). Such perturbations of the HPA axis have been strongly linked to the emergence of psychiatric disorders and render them a severe risk factor for pathology (Ising et al., 2007; El Hage et al., 2009). Since chronic exposure to stress is constantly activating the stress system of the organism, it comes as no surprise that individuals that are exposed to stress suffer from a higher risk to develop depression (Brown et al., 2004; Monroe and Reid, 2008). In this context, it has also been demonstrated that normalization of disturbed HPA axis activity is crucial for symptom improvement in depression (Ising and Holsboer, 2006). Starting already in early life, traumatic events have been associated with an increased risk for depression, depending on the number and intensity of the stressor (Heim and Nemeroff, 2001; Heim et al., 2008; Klengel et al., 2013). Similarly, chronic stress exposure in adulthood can shift the individual's response from adaptive to detrimental and may subsequently lead to psychiatric pathologies, such as post-traumatic stress disorder or depression (Mehta and Binder, 2012). In this context, the genetic makeup of the individual has also been demonstrated to play an important role in the emergence of such diseases, with strong evidence for interactions between the genotype and the environment that is experienced (Dunn et al., 2011; Heim and Binder, 2012). Further reviewing this line of argumentation, recent evidence also proposes that individuals adapt their physiology to the perceived environment, and thus a mismatch between early life and adult environment increases disease risk (Schmidt, 2011; Nederhof and Schmidt, 2012). Nonetheless, the underlying molecular mechanisms of stress in the emergence of depression, both detrimental and beneficial, have remained largely elusive and constitute a major obstacle in the development of novel pharmaceutical treatment options.

1.2. Depression and the current state of treatment

The lifetime prevalence of depression in the United States has been estimated at 20%, a number that emphasizes the impact that mood disorders, with depression leading the way, exert on western societies (Kessler et al., 2012). Next to the personal consequences for the individual concerned, depression also has major implications on the economic level, with e.g. long-term disability or suicide attempts causing costs in the range of billions (Wittchen et al., 2011).

Based on these observations, the improvement of treatment options for mood disorders has become one of the most important scientific research avenues. Current widespread therapies apply psychotherapy as well as pharmacotherapy. Nevertheless, pharmacological treatment options suffer from severe side effects, mediocre response rates and unsatisfactory relapse rates (Rush et al., 2006; Thase, 2006; Trivedi et al., 2006). Pharmacological intervention to-date has mainly focused on the monoamine hypothesis of depression, which emphasizes a deficiency of monoaminergic neurotransmitters such as serotonin and noradrenaline as a major molecular point of origin for the development of depression (Hirschfeld, 2000). Indeed, chemical compounds such as monoamine oxidase inhibitors, tricyclic antidepressants or selective serotonin reuptake inhibitors (SSRIs) have been shown to positively affect mood in depressed patients (Hirschfeld, 2012). Further research led to the development of various compounds based on the same molecular approach, such as Serotonin-norepinephrine reuptake inhibitors (SNRIs), Norepinephrine-dopamine reuptake inhibitors (NDRIs) or Serotonin antagonist and reuptake inhibitors (SARIs), which incorporated increased efficacy with reduced side effects. The main mechanistic effect of these drugs is to increase the amount of available monoamines in the synaptic cleft and these molecular actions take place within hours after application of the drug. However, a mood-alleviating effect is usually not detected until several weeks of treatment (Hyman and Nestler, 1996). Extensive research in this field led to the hypothesis that several neuroplastic mechanisms, including changes in gene expression, synaptic transmission and neurogenesis, are induced by pharmacotherapy and, over time, produce the beneficial effects that were previously ascribed to increased monoamine availability (Racagni and Popoli, 2008). These findings illustrate that modulation of monoaminergic signaling alone is not sufficient to explain the complex pathology of depression. Consequently, recent investigations have

concentrated on targeting other neurotransmitter systems, with glutamatergic signaling becoming one of the most promising candidates in depression research (Sanacora et al., 2012).

1.3. The glutamate system

Organization of the glutamate synapse

It is estimated that glutamatergic synapses make up more than 70% of all synapses, indicating that this neurotransmitter system is by far the most prevalent in the brain (Orrego and Villanueva, 1993; Douglas and Martin, 2007). Initially, glutamate is synthesized from α-ketoglutarate and then packaged in vesicles in the presynapse, one part of the tripartite glutamatergic synapse, which furthermore consists of the postsynaptic density (PSD) as well as glia (Machado-Vieira et al., 2009; Machado-Vieira et al., 2012). Since overexposure to high glutamate levels has toxic effects on the synapse, glutamate signaling is tightly regulated on different levels. In response to a presynaptic stimulus, glutamate is released from presynaptic vesicles into the synaptic cleft, where it binds to and activates both postsynaptic iono- and metabotropic receptors. The neurotransmitter is then taken up by astrocytes, converted to glutamine and transported back to the presynaptic compartment (Sanacora et al., 2008).

The two major ionotropic receptors of the glutamate synapse are N-methyl-D-aspartate (NMDA) receptors and 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) receptors. Kainate receptors are also expressed at glutamate synapses, but are less frequent in the brain and therefore supposed to exert only minor effects on synaptic signaling and plasticity compared to AMPA receptors (Huettner, 2003). Furthermore, there are eight G-protein coupled receptors that have been identified in glutamate signaling, which are subdivided into three functional subgroups (Table 1.1). All metabotropic glutamate receptors (mGluRs) consist of seven transmembrane spanning domains and a large N-terminus extracellular domain, which includes a ligand-recognition site as well as an intracellular carboxy terminal region that mediates postsynaptic signaling (Witkin et al., 2007). Group I mGluRs positively modulate phospholipase C activity, thereby increasing phosphoinositide turnover while group II and III mGluRs negatively regulate adenylyl cyclase activity, thereby inhibiting cyclic AMP formation (Cartmell and Schoepp, 2000). These effects, in conjunction with their localization on all three parts of the synapse, make mGluRs important, predominantly indirect modulators of glutamatergic signaling. In addition to receptors, glutamate transporters such as excitatory amino acid transporters (EAATs) and vesicular glutamate transporters (VGLUTs)

directly impact excitatory neurotransmission by regulating the availability of glutamate in the synaptic cleft and may therefore also represent potential therapeutic targets (Sanacora et al., 2008; Machado-Vieira et al., 2012).

Group	Receptor	CNS expression	Synaptic localization	Signaling pathways
Group I	mGluR1	Widespread in neurons	Predominantly postsynaptic	Phospholipase C stimulation Stimulation of adenylyl cyclase (some systems) MAP kinase phosphorylation
	mGluR5	Widespread in neurons, astrocytes		
Group II	mGluR2	Widespread in neurons	Presynaptic and postsynaptic	Inhibition of adenylyl cyclase Activation of K^+ channels Inhibition of Ca^{2+} channels
	mGluR3	Widespread in neurons, astrocytes		
Group III	mGluR4	Widespread in neurons, high in cerebellum	Predominantly presynaptic	Inhibition of adenylyl cyclase Activation of K^+ channels Inhibition of Ca^{2+} channels Stimulation of cGMP phosphodiesterase (mGluR6)
	mGluR6	Retina	Postsynaptic in ON-bipolar retinal cells	
	mGluR7	Widespread in neurons	Active zone of presynaptic terminal	
	mGluR8	More restricted expression than mGluR4/7	Predominantly presynaptic	

Table 1.1: Overview of different metabotropic receptor subtypes. CNS, central nervous system. Adapted from Niswender and Conn, 2010

Glutamate signaling and mood disorders

Dysfunction of glutamate signaling has been described in the context of various disorders, including Alzheimer's disease, epilepsy, schizophrenia as well as anxiety and mood disorders (Parsons et al., 1998; Francis, 2003; Cortese and Phan, 2005). In individuals suffering from mood disorders, abnormal levels of glutamate have been shown to be present in the plasma, serum and the brain (Francis et al., 1989; Mauri et al., 1998), strongly suggesting that disturbed glutamate signaling may be a key mechanism in these disorders. Furthermore, a number of studies provide evidence that altered NMDA receptor levels and binding properties are associated with the emergence of mood disorders (Choudary et al., 2005; Beneyto and Meador-Woodruff, 2008). This is

accompanied by genetic studies that identified single nucleotide polymorphisms in NMDA receptor subunits, which are linked to psychopathology (Mundo et al., 2003; Martucci et al., 2006).

A multitude of NMDA receptor modulating agents have been tested in both clinical and preclinical setups for their mood-alleviating efficacy (Sanacora et al., 2008; Fricker et al., 2009; Burgdorf et al., 2013). The most prominent potential treatment option emerging from these studies is ketamine, a non-competitive NMDA receptor antagonist. Ketamine has been demonstrated to exert strong antidepressant and anxiolytic effects in animal models (Garcia et al., 2008a; Garcia et al., 2008b), which are also dependent on AMPA receptor activation (Maeng et al., 2008). In clinical trials, ketamine demonstrated rapid antidepressant effects in patients with treatment resistant major depression (Zarate, Jr. et al., 2006) and was also able to reduce suicidality (Price et al., 2009). However, the severe side effects after repeated exposure, including cognitive deficits and psychotomimetic effects, render ketamine largely unsuitable for chronic treatments (Tsai, 2007). According to studies that elucidated the molecular mechanism of ketamine's antidepressant effect, a blockade of NMDA receptors subsequently leads to increased AMPA signaling relative to NMDA-mediated throughput (Maeng et al., 2008). Positive AMPA receptor modulators have therefore also been proposed to act as antidepressant treatment, and animal studies support this hypothesis (Li et al., 2001; Bai et al., 2003; Black, 2005). Interestingly, AMPA receptor function potentiators have also been demonstrated to prevent chronic stress-induced cognitive deficits (Schmidt et al., 2010). Although clinical studies implicate an association between decreased AMPA receptor levels and mood disorders (Meador-Woodruff et al., 2001; Beneyto and Meador-Woodruff, 2006), well-controlled clinical trials have only recently begun testing therapeutic agents for their possible antidepressant potential (Nations et al., 2012a; Nations et al., 2012b).

Although the molecular mechanics of mGluRs are relatively well understood, their role in the pathology of mood disorders has not been as clearly demonstrated as the previously described ionotropic receptors. There are only few reports that correlate differences in mGluR expression with psychopathology, therefore evidence for their involvement has been demonstrated largely preclinical, as various mGluR ligands show a certain level of efficacy in animal models of mood disorders (Palucha and Pilc, 2007; Krystal et al., 2010).

Group II mGluRs are mostly located at the presynapse, and have a regulatory influence on glutamate release, thereby making them ideal targets for potential antidepressant treatments. Indeed, mGlUR2/3 ligands have been demonstrated to exert both antidepressant and anxiolytic effects, which are hypothesized to be mediated by AMPA signaling (Karasawa et al., 2005).

By contrast, mGluR1 and mGluR5 are mostly expressed at the postsynapse and have been shown to strongly modulate postsynaptic excitability by interacting with NMDA receptors (Pilc et al., 2008). In particular mGluR1/5 antagonists such as 2-Methyl-6-(phenylethynyl)-pyridine (MPEP) and 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine (MTEP) have been tested in preclinical settings and exhibited strong anxiolytic effects as well as potential antidepressant-like properties (Marino and Conn, 2006). Fenobam, another mGluR5 antagonist, has also been tested in a clinical study, where it proved to have anxiolytic effects as well (Porter et al., 2005). However, the antidepressant-like effects of these drugs have mostly been derived from results obtained in the forced swim test (FST). Although this test is one of the most popular screenings for antidepressant-like properties in animal models, further lines of investigation are needed to support these findings (Li et al., 2006; Molina-Hernández et al., 2008). As a mode of action, the modulation of NMDA receptor signaling by mGluR5 via postsynaptic scaffolding proteins has been proposed, but the precise molecular mechanisms of these behavioral effects are yet to be revealed (Krystal et al., 2010).

The protein complex in the PSD that links the intracellular part of mGluRs to NMDA and AMPA receptors is an intricate aggregate of several proteins, including PSD-95, guanylate kinase-associated protein (GKAP), Shank and Homer (Tu et al., 1999; Naisbitt et al., 1999). Several additional regulatory factors that modulate the function of this complex have been identified and further increase the complexity of this signaling pathway (Hu et al., 2012; Gao et al., 2013). Understanding the molecular underpinnings of this postsynaptic machinery may tremendously increase our knowledge on the genesis of psychiatric disorders such as depression as well as contribute to the development of novel treatment strategies.

1.4. The postsynaptic scaffold Homer1

Structure and function of Homer family proteins

One of the major scaffolds in the glutamatergic PSD belongs to the Homer protein family. There are three major members (Homer1, Homer2, and Homer3) and each of them are expressed as a different isoform that originates from alternative splicing. Interestingly, although their protein structure is similar, their respective genes are located on different chromosomes, indicating a certain independence from one another.

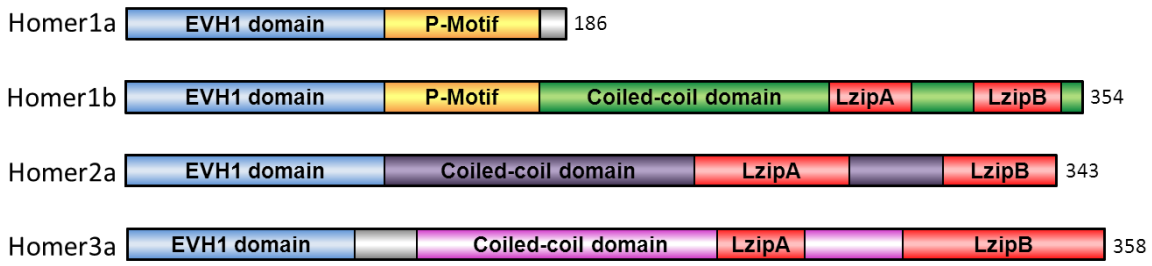

Figure 1.2: Schematic primary structure of different Homer proteins. All Homers consist of an enabled/vasodilator-stimulated phosphoprotein homology 1 (EVH1) like domain (Blue), which is highly conserved. Homer1 isoforms also show a P-motif (Yellow). Homer1a misses a coiled-coil domain with two leucine zipper (LzipA/B) sites (Red), which is present in all long-form Homer proteins, yet their sequence similarity is only 30% (Green, purple, pink). The total number of amino-acids is depicted to the right. For additional transcription variants, also refer to Shiraishi et al., 2007.

All members of the Homer protein family share two main structural features (Figure 1.2): The amino-terminal domain is highly conserved and shows 80% sequence similarity between isoforms (Xiao et al., 1998; Kato et al., 1998). The carboxy-terminal domain contains a coiled-coil structure and two leucine zipper motifs that have been shown to mediate multimerization (Hayashi et al., 2006), but its structure only shows 30% similarity across the protein family (Sun et al., 1998; Tadokoro et al., 1999). The ligand-binding amino-terminal domain is highly similar to the enabled/vasodilator-stimulated phosphoprotein homology 1 (EVH1) domain and interacts with amino-acid sequences of the form Pro-Pro-x-x-Phe (Brakeman et al., 1997; Tu et al., 1999), which enables Homer to bind to a number of proteins, including mGluR5, Shank and inositol-triphosphate (IP_3) receptor types 1 and 3 (Tu et al., 1998; Tu et al., 1999). Homer proteins are widely expressed both in the brain and the periphery (Shiraishi-Yamaguchi and Furuichi, 2007). Studies in mice have also shown that Homers play an important role in the postnatal

development of mice, where distinct expression patterns in different brain regions occur (Shiraishi et al., 2004). The major isoforms of Homer proteins, consisting of both an EVH1-like and carboxy-terminal domain, are constitutively expressed in several brain regions. However, there is also an isoform of Homer1 labeled Homer1a that is missing the coiled-coil structure required for multimerization, which is induced upon environmental manipulations (Brakeman et al., 1997; Kato et al., 1997). Homer1a can therefore compete for EVH1-like binding sites with long-form Homer1 isoforms like Homer1b and Homer1c, which are usually detected concurrently (Shiraishi-Yamaguchi and Furuichi, 2007).

The specific isoforms of Homer1 have been demonstrated to be involved in a number of molecular processes in the glutamatergic PSD. Most prominently, Homer1b/c links mGluR5 to intracellular IP_3 receptors, thereby modulating intracellular Ca^{2+} currents in response to excitatory signaling (Tu et al., 1998; Yuan et al., 2003). Additionally, long-form Homer1b/c mediates kinase activity (Park et al., 2008) and NMDA receptor excitability through a protein complex containing Shank, GKAP and PSD-95 (Tu et al., 1999; Hayashi et al., 2009; Bertaso et al., 2010). Cell surface expression and clustering of type I mGluRs has been shown to be dynamically regulated by both Homer1b/c (Inhibition) and Homer1a (Facilitation) (Roche et al., 1999; Ango et al., 2002). Furthermore, Homer1 has also been demonstrated to affect synapse formation and maturation by modulating PSD complex integrity and Ca^{2+} signaling (Ango et al., 2000; Gasperini et al., 2009; Grabrucker et al., 2011).

Behavioral implications of Homer1

Given the importance of Homer1 on the molecular level, it is not surprising that a manipulation of Homer1 in animal models profoundly impacts behavior. Most strikingly, both short and long Homer1 isoforms are crucially involved in addiction behavior (Szumlinski et al., 2006; Szumlinski et al., 2008). A great number of studies also showed the involvement of Homer1 in memory processes. In Homer1 knockout animals, memory impairments were detected that could be rescued by reinstating functional Homer1b/c (Klugmann et al., 2005; Gerstein et al., 2012). By contrast, overexpression of Homer1a exerts detrimental effects on memory formation in spatial recognition tasks (Celikel et al., 2007), but has been shown to be crucial for functional fear memory formation (Mahan et al., 2012). Another study from Tronson and colleagues implicated an intricate interplay

between Homer1b/c and Homer1a in stress-enhanced fear memory, where select overexpression of Homer1a mimicked the effects of an acute stressor on fear conditioning (Tronson et al., 2010). Given these findings, it is likely that Homer1 mediated pathways play a major role in stress coping mechanisms, both on the molecular and behavioral level. Interestingly, except for the aforementioned publication, no studies have yet reported on the role for Homer1 in the context of acute and chronic stress.

1.5. Modeling mood disorders in rodents

The apparent complexity of a mood disorder such as depression on the physiological, psychological and molecular level poses a significant challenge for animal models that are developed to mimic the situation in humans. Following a scientific rationale, a model organism should be as closely related to the human as possible (i.e. non-human primates), while ethical reasons speak in favor of organisms with less developed nervous systems. As a compromise, small rodents are widely used to model psychiatric diseases. Nonetheless, it remains unknown whether rats or mice can actually develop a depression-like disease, as most core features of this pathology, which are verbally communicated by patients, cannot be measured at all or only as crude approximations in rodents. Willner and colleagues have therefore devised a framework of criteria, which should be fulfilled by a potential animal model (Willner, 1984) (Figure 1.3). The most obvious criterion is face validity, which describes the similarity of disease symptoms in humans and rodents. In the case of depression, these include for example impaired cognitive function, HPA axis dysregulation or anhedonic behavior (Willner and Mitchell, 2002; Müller and Holsboer, 2006; Kalueff and Murphy, 2007). Another important criterion is the construct validity of the animal model, which aims to mimic the underlying factors that are present in the disease, such as environmental or genetic risk factors. The third validity criterion assesses the predictive power of an animal model, meaning that treatments, which have been shown to be effective in humans should also effectively reverse the disease-related parameters in the given animal model (Broekkamp, 1997). To fulfill these criteria, different manipulations can be applied to the animals. Popular approaches include genetic manipulations that, over the last two decades, developed from initial total knockouts of single genes to short hairpin RNA mediated knockdown of specific target genes or optogenetically mediated silencing or activation of distinct cell populations in specific brain regions (Glaser et al., 2005; Fenno et al., 2011). Another important line of research focuses on the application of environmental factors, such as enriched environment (Freund et al., 2013) or stress situations (Schmidt, 2011). By combining the aforementioned factors, animal models aim to mimic the complex gene by environment interactions that are present in the human situation.

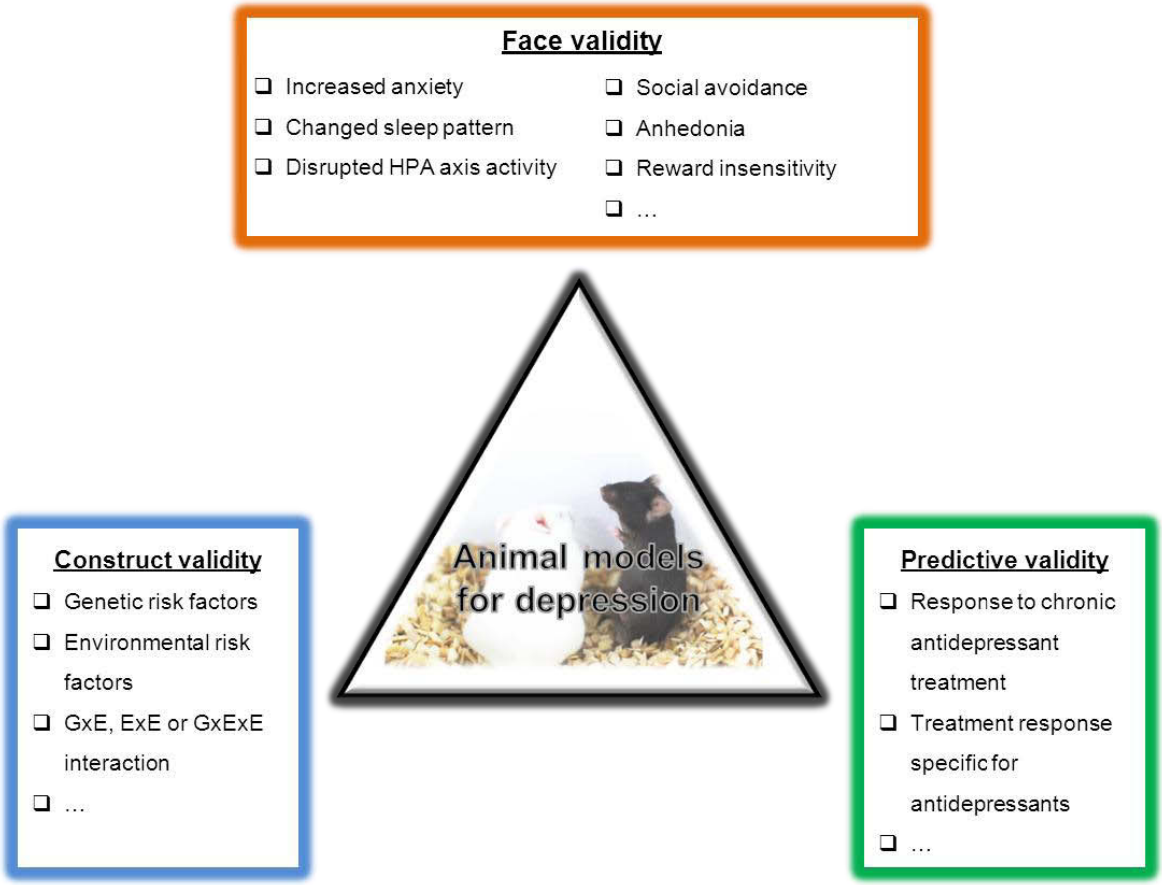

Figure 1.3: Illustration of the three main validity criteria of animal models for depression and some examples of important aspects. GxE, gene by environment; ExE, environment by environment; GxExE, gene by environment by environment

Chronic stress models

Animal models that employ acute and chronic stressors have been extensively studied on their ability to match the aforementioned validity criteria. Chronic stress can be exerted in various ways, with chronic unpredictable mild stress (CUMS) and chronic social defeat stress (CSDS) being amongst the most popular paradigms (Willner, 1997; Krishnan and Nestler, 2008). Unpredictability of the stressor is an important feature of these models, as attenuation and habituation is quickly established in recurring stress episodes (Harris et al., 2004; Girotti et al., 2006). In CUMS models, this is achieved by alternating the time and length of the exposure to a highly variable set of different stressors, such as cold, water, loud noises, as well as restraint or inversion of the light-dark circle (Hollis et al., 2012). Models that apply CSDS can rely on the naturally occurring variance of social defeat intensity to attain unpredictability. Additionally, the number and length of defeat sessions has been shown to critically influence the lasting effects of CSDS. Experimental

protocols can vary from subthreshold defeat sessions, which prime the animals for a vulnerable phenotype (Chaudhury et al., 2013), to extended chronic defeat designs that include 10 to 28 daily defeat bouts (Nestler and Hyman, 2010; Wagner et al., 2011; Chen et al., 2012). Such chronic applications produce long-lasting changes on the behavioral level, such as social avoidance, anhedonia and increased anxiety (Berton et al., 2006; Krishnan and Nestler, 2008). These findings have been complemented with extensive research on the molecular level, where various brain regions, such as the nucleus accumbens, the ventral tegmental area or the hippocampus, have been shown to be crucially involved in the mediation of the behavioral effects (Cao et al., 2010; LaPlant et al., 2010; Lagace et al., 2010). Other popular stress paradigms target different plastic phases of development such as early life (Schmidt et al., 2011b) or adolescence (Schmidt et al., 2007), which also invoke lasting changes on the behavioral and molecular level that ultimately cause the development of phenotypes that are associated with aspects of depression. Taken together, animal models of chronic stress have been demonstrated to be valuable tools to investigate stress-induced pathophysiology and are widely used in preclinical research to elucidate the molecular mechanism of mood disorders.

1.6. Aim of the thesis

The current thesis aims to unravel the role of the glutamate system, with a special focus on Homer1, in stress-induced psychopathology. To achieve this, we first introduce and validate a chronic social stress model, which provides good face, construct and predictive validity (Chapter 1). Next, we investigate the transcriptional regulation of Homer1 in response to chronic (Chapter 2) and acute stress (Chapter 3). In addition, we elucidate the behavioral relevance of Homer1 transcription changes with respect to spatial memory (Chapter 3) and reward associated learning (Chapter 4). Finally, we investigate the behavioral and neuroendocrine effects of chronic stress with regards to genetic and pharmacological intervention targeting the mGluR5/Homer1 pathway (Chapter 5). Taken together, this thesis will provide comprehensive evidence for the involvement of Homer1 in stress-related mood disorders and will serve as a basis for future work aiming to develop novel treatment strategies based on interventions in glutamate signaling pathways.

2. Research articles

Chapter 1: *Differences in FKBP51 regulation following chronic social defeat stress correlate with individual stress sensitivity: Influence of paroxetine treatment*

Neuropsychopharmacology 2012 Dec; 37(13):2797-808

Chapter 2: *Hippocampal Homer1 is regulated by chronic social defeat stress*

Manuscript in preparation

Chapter 3: *Homer1 mediates acute stress-induced cognitive deficits in the dorsal hippocampus*

The Journal of Neuroscience, 2013 Feb 27; 33(9):3857–3864

Chapter 4: *Hippocampal Homer1 levels influence motivational behavior in an operant conditioning task*

Manuscript submitted

Chapter 5: *Homer1/mGluR5 signaling moderates vulnerability to chronic social stress*

Manuscript in preparation

2.1. Differences in FKBP51 regulation following chronic social defeat stress correlate with individual stress sensitivity: Influence of paroxetine treatment

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Abstract

Various clinical studies have identified FK506-binding protein 51 (FKBP51) as a target gene involved in the development of psychiatric disorders such as depression. Furthermore, FKBP51 has been shown to affect glucocorticoid receptor signaling by sensitivity modulation and it is implicated in stress reactivity as well as in molecular mechanisms of stress vulnerability and resilience. We investigated the physiological, behavioral, and neuroendocrine parameters in an established chronic stress model both directly after stress and after a recovery period of 3 weeks and also studied the efficacy of paroxetine in this model. We then examined FKBP51 mRNA levels in the dorsal and ventral part of the hippocampus and correlated the expression to behavioral and endocrine parameters. We show robust chronic stress effects in physiological, behavioral, and neuroendocrine parameters, which were only slightly affected by paroxetine treatment. On the contrary, paroxetine led to a disruption of the neuroendocrine system. FKBP51 expression was significantly increased directly after the stress period and correlated with behavioral and neuroendocrine parameters. Taken together, we were able to further elucidate the role of FKBP51 in the mechanisms of stress resilience and vulnerability, especially with respect to behavioral and neuroendocrine parameters. These findings strongly support the concept of FKBP51 as a marker for glucocorticoid receptor sensitivity and its involvement in the development of psychiatric disorders.

Introduction

Chronic social stress is widely regarded as a risk factor for the development of psychiatric pathologies such as depression and anxiety disorders (McEwen, 2004; Chrousos, 2009). Social stress and economic pressure are most common in western societies and largely increase the risk of psychopathologies (Tennant, 2001). Animal models of social stress, including chronic social defeat stress (CSDS), are widely used to model this situation in rodents and are accepted models for key clinical symptoms of depression (Savignac et al., 2011).

Current treatment strategies for depressed patients focus mostly on the increase of monoamines in the synaptic cleft, for example, via the use of selective serotonin reuptake inhibitors (SSRIs). However, these approaches suffer from the late onset of therapeutic effects, relatively poor response, and high relapse rates (Rush et al., 2006). Treatment efficacy can be increased by combinations of different drugs, but convincing success rates are yet to be reached (Thase, 2006). These data highlight the need to continue the search for novel targets in depression research that may lead to more potent yet well-tolerated drugs for the treatment of affective disorders (Berton and Nestler, 2006).

A malfunction of the hypothalamic-pituitary-adrenal (HPA) axis has been strongly implied in the development of mood disorders (de Kloet et al., 2005). Chronic HPA axis activation may lead to a disruption of the feedback process, which results in an overshooting stress response and promotes the risk for developing psychiatric diseases. The glucocorticoid receptor (GR) plays a crucial role in these feedback circuits and therefore in the termination of the stress response (Ulrich-Lai and Herman, 2009). In line with these findings, many depressed patients show altered GR signaling (Pariante and Miller, 2001).

A chaperone-receptor heterocomplex consisting of heat shock protein 90 and, among others, FK506-binding protein 51 (FKBP51) regulates GR signaling by modulating the activation and trafficking of the receptor as well as its gene transcription properties (Pratt et al., 2006). It has been shown that FKBP51 alters ligand binding sensitivity of the GR, reducing nuclear translocation of the GR-complex, and therefore modulating HPA axis feedback sensitivity (Wochnik et al., 2005; Binder, 2009). In a study conducted by Binder et al. (2004), significant associations between FKBP51 polymorphisms and depressive episodes as well as antidepressant responses were shown. In recent years, a growing body of evidence suggests an important role of genetic variants of FKBP51 in stress

susceptibility and occurrence of major depression (Ising et al., 2008; Zimmermann et al., 2011). Other studies could show the involvement of FKBP51 in suicide events (Roy et al., 2012) and post-traumatic stress disorder (Sarapas et al., 2011).

Further support for an involvement of FKBP51 in stress system regulation comes from animal models. FKBP51 mRNA was upregulated in stress-related brain regions such as the hippocampus in response to acute stressors or a glucocorticoid challenge (Scharf et al., 2011). Additionally, FKBP51 knockout mice were reported to show increased active stress coping behavior in the forced swim test (Touma et al., 2011) and a resilient phenotype in response to CSDS (Hartmann et al., 2012), suggesting a prominent role of FKBP51 in stress coping behavior.

In our study, we aimed to investigate the interaction between FKBP51 and antidepressant treatment in modulating depression-related parameters in male mice. We therefore applied an established chronic stress model and studied its direct and long-term effects on physiology and behavior, neuroendocrine parameters, as well as GR-sensitivity related mRNA and protein levels. Additionally, we investigated the interactions of a commonly prescribed antidepressant, paroxetine, with chronic stress and FKBP51 regulation, hypothesizing that FKBP51 regulation may support stress resilience.

Materials and Methods

Animals and animal housing

For all experiments, male C57Bl/6N mice (Charles River Laboratories, Maastricht, The Netherlands) were used. At the beginning of the experiment, the age of all animals was 12 weeks. The mice were held under standard conditions (12L:12D light cycle, lights on at 08:00 AM, temperature 23 ± 2 °C), were single housed, and were acclimated to the room for 2 weeks before the beginning of the experiments. Food (Altromin 1314, Altromin GmbH, Germany) and tap water were available *ad libitum*. Male CD1 mice (16 - 18 weeks of age) served as resident mice, which were held under the conditions described above. They were allowed to habituate to the social defeat cage for 2 weeks before the experiment. All experiments were carried out in the animal facilities of the Max Planck Institute of Psychiatry in Munich, Germany. The experiments were carried out in accordance with the European Communities' Council Directive 86/609/EEC. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

Experimental design

Experiment 1

In the first experiment, the direct effects of CSDS on various parameters were investigated. A total of 48 mice were randomly split into 2 × 2 groups (Control vehicle (n = 13), control paroxetine (n = 13), chronic stress vehicle (n = 11), and chronic stress paroxetine (n = 11)) and subjected to the chronic stress procedure described below. The paroxetine treatment commenced at the first day of the stress procedure and lasted until the day of killing (Figure 1A). All behavioral tests were performed during the third week of the stress procedure.

Experiment 2

In the second experiment, the same parameters that were investigated in experiment 1 were studied after a 3-week period of recovery following the chronic stress exposure (Figure 3A). A total of 64 mice were divided into two groups (Control and chronic stress)

and they underwent the chronic stress paradigm described below. After cessation of the stressor, both groups were subdivided into vehicle-treated and paroxetine-treated animals (n = 16 each). The treatment phase lasted for 3 weeks and all animals independent of condition were handled twice per week. All behavioral tests of experiment 2 took place during the last week of the paroxetine treatment.

Chronic stress procedure and physiological parameters

The CSDS paradigm lasted for 21 days and was conducted as described previously (Wagner et al., 2011). Briefly, the experimental mice were introduced into the home cage (45 cm × 25 cm) of a dominant resident mouse and defeated shortly after. When the defeat was achieved, the animals were separated by a wire mesh, preventing physical but allowing sensory contact for 24 h. Each day, stressed animals were defeated by another unfamiliar, dominant resident mouse in order to exclude a repeated encounter throughout the experiment. The daily defeat was performed between 11:00 AM and 04:00 PM; varying starting times reduced the predictability of the stressor and therefore minimized a potential habituation effect. Experimental mice were always defeated by resident males during the entire stress period. Control mice were housed in their home cages during the course of experiment. Both stress and control animals were handled daily during the stress procedure; body weight was assessed at the beginning of the experiment as well as before killing. In experiment 2, body weight for all mice was assessed at the beginning of the experiment, after the cessation of the stress period, and on the day of killing. Animals that underwent the stress procedure were subsequently single housed in standard cages.

Paroxetine treatment

Paroxetine was obtained from GlaxoSmithKline (Munich, Germany) as a solution and was diluted in tap water to a final concentration of 0.16 mg/ml. With average water consumption of 5 ml/mouse/day, the daily dose of paroxetine was ≈ 20 mg/kg body weight. Fluid intake was monitored daily and the variation of fluid intake was found to be < 10% over the course of the experiment. The chosen dosage has been reported to be effective in chronic stress models (Schmidt et al., 2007) and we confirmed this in a control sample, where paroxetine levels in basal blood plasma were measured (Data not shown).

In experiment 1, a simultaneous condition × treatment setup was chosen to provide insights into paroxetine × stress interactions. In experiment 2, paroxetine was administered after the CSDS period to further elucidate the possibly improved recovery from stress.

Behavioral analysis

The behavioral tests were carried out between 08:30 AM and 12:30 PM in the same room in which the mice were housed. The testing order was as follows: Open-field (OF), social avoidance (SA), elevated plus-maze (EPM), female urine sniffing test (FUST), forced swim test (FST), and acute stress response. All tests were analyzed using an automated video-tracking system (Anymaze 4.20, Stoelting, Wood Dale, IL). A detailed description of the testing procedures can be found in the supplementary information. All animals underwent the same testing battery in the same order of tests. To minimize possible carryover effects of the different behavioral tests, the sequence of tests was arranged from the least stressful to the most stressful (McIlwain et al., 2001).

Sampling procedure

All animals were killed by decapitation following quick anesthesia by isoflurane at the end of the experiment. Basal trunk blood samples were processed as described above. Brains were removed, snap-frozen in isopentane at -40 °C, and stored at -80 °C for *in situ* hybridization. Adrenal and thymus glands were removed, dissected from fat, and weighed.

In situ *hybridization*

Frozen brains were sectioned at -20 °C in a cryostat microtome at 18 µm, thaw mounted on Super Frost Plus slides, dried and stored at -80 °C. *In situ* hybridization using ^{35}S UTP labeled ribonucleotide probes (FKBP51, Metallothionein-1) was performed as described previously (Schmidt et al., 2007). A detailed protocol can be found in the supplemental materials section.

Western blot

An additional cohort of animals (Control vehicle vs. CSDS vehicle, n = 8 each) underwent the same CSDS paradigm as in experiment 1 except for the behavioral tests. At 24 h after the last defeat session, animals were deeply anesthetized and quickly decapitated. Hippocampal tissue was extracted and subcellular fractions (Cytosol, nucleus) were purified using a commercially available kit (Calbiochem ProteoExtract, Merck Millipore). Western blots were then performed as previously described (Wang et al., 2011a). A detailed description of the protocol is found in the supplemental material.

Statistical analysis

The data presented are shown as means ± standard error of the mean (SEM), analyzed by the commercially available software SPSS 16.0. Student's *t*-test was employed for comparison of two independent groups. Two factorial (Condition and treatment) ANOVA was employed for all other parameters. Correlations between behavioral parameters and mRNA expression were analyzed with the Pearson product moment test. A nominal level of significance $P < 0.05$ was accepted and adjusted according to Bonferroni correction by all posteriori tests (Univariate F-tests, test of simple effects, or contrasts).

Results

Experiment 1

In the first experiment, we investigated the immediate effects of CSDS and paroxetine by killing the animals 24 h after the last defeat session (Figure 1A).

Physiology

At the beginning of the experiment, no differences in body weight were apparent. Also, there was no effect in body weight gain at the end of the experiment between either groups (Control vehicle: 2.17 ± 0.26 g, control paroxetine: 2.78 ± 0.25 g, CSDS vehicle: 2.07 ± 0.35 g, CSDS paroxetine: 2.43 ± 0.26 g). Adrenal glands size was increased and thymus glands size decreased after CSDS, independent of treatment (Figure 1B, C).

Neuroendocrinology

Three weeks of chronic defeat stress increased circulating corticosterone under basal conditions independent of treatment (Figure 1D). In response to a novel stressor, defeated animals showed a significantly increased response to an acute stressor compared to control animals (Figure 1E). Also, paroxetine treatment resulted in an increased corticosterone response compared to vehicle treatment. At 90 min after onset of the acute stressor, defeated animals showed a significantly diminished ability to recover from the acute stressor (Figure 1F). This effect was largely increased in paroxetine-treated animals.

Behavior

Chronic defeat stress markedly altered the animals' behavior in various tests. Reduced locomotion in the OF and increased anxiety-related behavior in the EPM, depicted by reduced open arm time, were induced by CSDS (Figure 1G and H). Paroxetine did not have any alleviating effect in these tests. In the SA test, a treatment effect was revealed, showing a significant increase in the interaction ratio of paroxetine-treated animals compared with vehicle-treated animals (Figure 1I). A stress-related effect could not be found in this test. Defeated animals showed increased anhedonic behavior, as depicted by reduced sniffing time in the FUST urine trial but not in the water trial (Figure 1J). In the FST, defeated mice that received paroxetine displayed a significantly decreased time in

immobile posture compared with both vehicle-treated stress animals and paroxetine-treated control animals (Figure 1K), indicating a more active stress coping behavior.

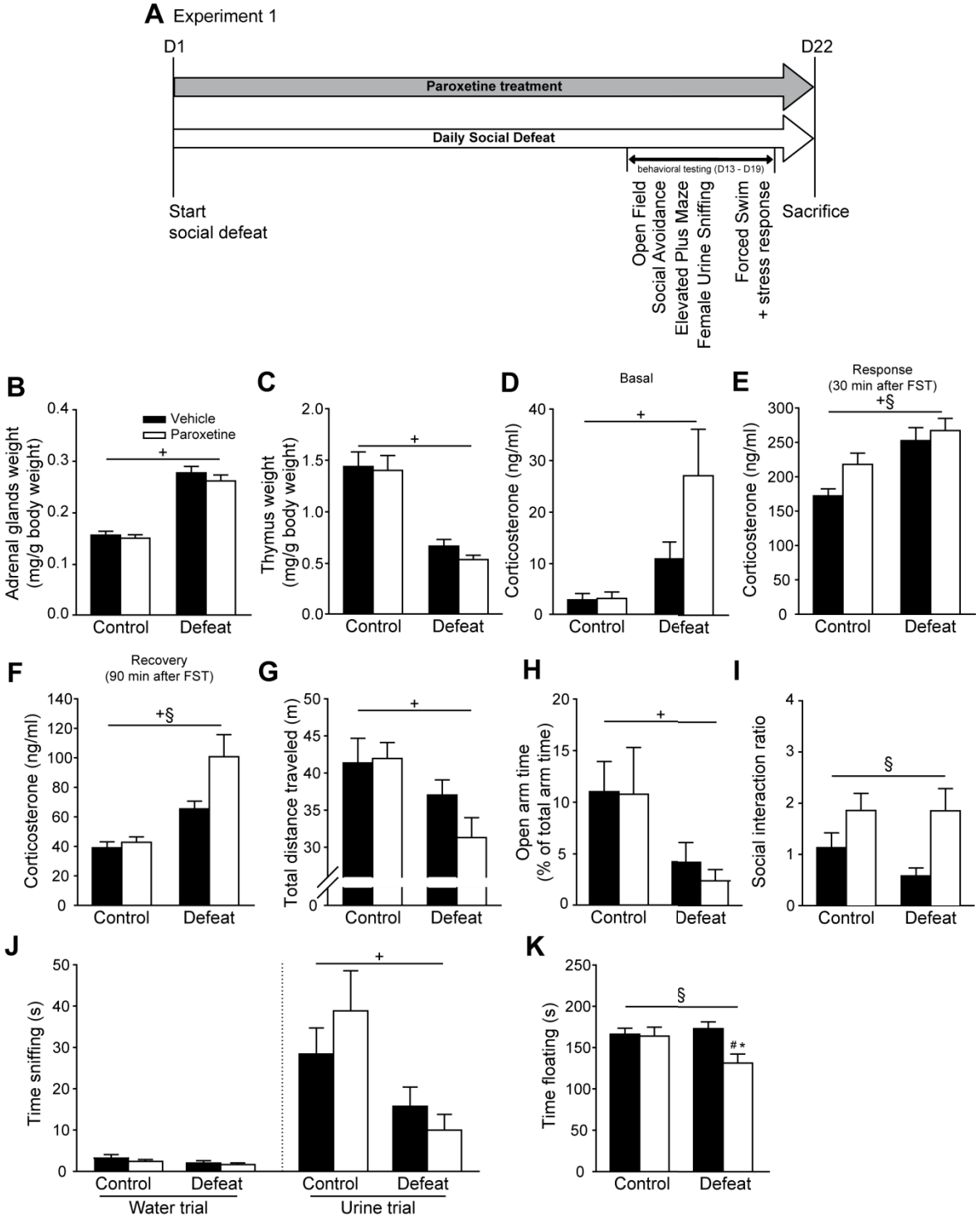

Figure 1: Chronic social defeat stress (CSDS) strongly affects the physiology, behavior and neuroendocrine profile of mice. (A) Time course of experiment 1: Treatment with paroxetine and the chronic stress procedure are performed simultaneously. The behavioral testing is carried out in the last week of the treatment and stress phase. (B,C) ANOVA showed a condition effect in adrenal gland weight ($F_{1,47} = 129.185$, $p < 0.001$) as well as in thymus weight ($F_{1,47} = 53.734$, $p < 0.001$) with chronic stress increasing adrenal gland size and reducing thymus weight, independent of the treatment. (D) Basal

corticosterone levels were increased by exposure to CSDS as shown by an ANOVA condition effect ($F_{1,44}$ = 11.248, p < 0.01). (E) ANOVA revealed both a condition ($F_{1,47}$ = 19.921, p < 0.001) and a treatment effect ($F_{1,47}$ = 5.055, p < 0.05) in circulating corticosterone 30 min after an acute stressor. Paroxetine increased hypothalamic-pituitary-adrenal axis (HPA axis) activity already under control conditions. (F) Corticosterone recovery was disrupted in stressed animals, an effect that appears to be increased in mice treated with paroxetine (ANOVA condition ($F_{1,47}$ = 24.573, p < 0.001), treatment effect ($F_{1,47}$ = 5.082, p < 0.05)). In both the OF (G) and the EPM (H), ANOVA revealed a condition effect, with a reduced locomotion in the OF ($F_{1,47}$ = 7.814, p < 0.01) and a reduced time on the open arms in the EPM ($F_{1,43}$ = 7.534, p < 0.01). (I) Paroxetine increased social interaction in the social avoidance test with no significant effect of CSDS (ANOVA treatment: $F_{1,41}$ = 8.647, p < 0.01). (J) Anhedonic behavior was increased in stressed mice (Urine: ANOVA condition: $F_{1,41}$ = 8.859, p < 0.01; Water: ANOVA condition: $F_{1,41}$ = 2.114, p = 0.154) and was not ameliorated by antidepressant treatment. (K) In the FST, ANOVA reported both a treatment effect ($F_{1,47}$ = 5.229, p < 0.05) as well as a condition × treatment interaction ($F_{1,47}$ = 4.208, p < 0.05). Here, paroxetine only exhibited antidepressant effects in the forced swim test when combined with CSDS. * Significantly different from control condition of the same treatment group, p < 0.05; # Significantly different from vehicle treatment of the same condition group, p < 0.05; + Significant condition effect, p < 0.05; § Significant treatment effect, p < 0.05.

Gene expression analysis

Investigation of FKBP51 mRNA expression revealed a significant increase in the CA1 and the DG of the dorsal hippocampus in defeated animals compared with controls independent of treatment (Figure 2A - C). In the ventral hippocampus, we found FKBP51 mRNA expression to be upregulated in the CA1 and the DG. Animals treated with paroxetine also showed a slight increase in FKBP51 mRNA levels compared with vehicle-treated animals in the CA1. Levels of MT-1 mRNA, a known GR-responsive gene, were not regulated by CSDS or paroxetine treatment in the investigated hippocampal regions CA1 and DG (Supplementary figure S1).

Experiment 2

In the second experiment, we investigated the effects of 3 weeks of recovery from CSDS combined with paroxetine treatment (Figure 3A).

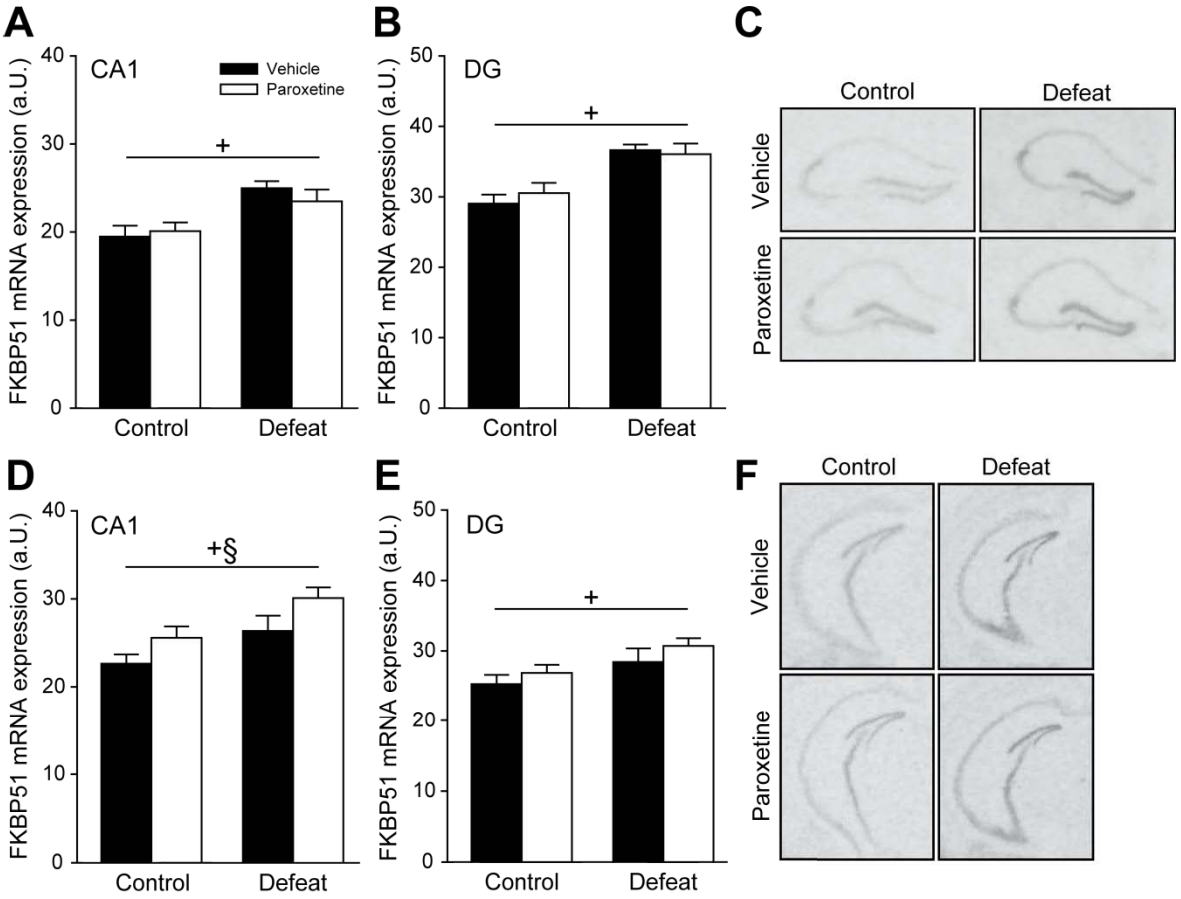

Figure 2: FK506-binding protein 51 (FKBP51) mRNA expression is significantly increased in the hippocampus of stressed animals. (A,B) Both in the CA1 (ANOVA condition: $F_{1,46} = 15.309$, $p < 0.001$) and the dentate gyrus (DG) (ANOVA condition: $F_{1,46} = 24.272$, $p < 0.001$) of the dorsal hippocampus, CSDS increased FKBP51 levels independent of treatment. (C) Representative autoradiographs of FKBP51 mRNA in the dorsal hippocampus. (D,E) Similar to the dorsal hippocampus, FKBP51 expression was increased in the ventral hippocampus of stressed animals (CA1 ANOVA condition: $F_{1,47} = 9,888$, $p < 0.01$; DG ANOVA condition: $F_{1,47} = 6.515$, $p < 0.05$), an effect that is slightly more pronounced in animals treated with paroxetine (CA1 ANOVA treatment: $F_{1,47} = 6.524$, $p < 0.05$). (F) Representative autoradiographs of FKBP51 mRNA in the ventral hippocampus. * Significantly different from control condition of the same treatment group, $p < 0.05$; # Significantly different from vehicle treatment of the same condition group, $p < 0.05$; + Significant condition effect, $p < 0.05$; § Significant treatment effect, $p < 0.05$; Other abbreviations as in figure 1.

Physiology

Although the initial body weight was not different between control and defeated animals, after 3 weeks, chronically stressed animals showed a significantly increased body weight gain ($T_{62} = -3.096$, $p < 0.01$, control: 1.80 ± 0.20 g, CSDS: 2.58 ± 0.24 g). On the day of killing, ANOVA revealed a treatment effect ($F_{1,63} = 19.222$, $p < 0.001$) as well as a condition × treatment interaction ($F_{1,63} = 8.227$, $p < 0.01$), with paroxetine-treated mice showing increased body weight gain and vehicle-treated mice that underwent the stress paradigm being heavier than their control littermates (Control vehicle: 2.42 ± 0.27 g,

control paroxetine: 4.63 ± 0.42 g, CSDS vehicle: 3.88 ± 0.21 g, CSDS paroxetine: 4.34 ± 0.28 g). Stressed animals still showed increased adrenal gland size, with paroxetine-treated animals having a reduced overall adrenal gland weight (Figure 3B). The size of the thymus glands was not significantly altered at the end of the experiment (Figure 3C).

Neuroendocrinology

Basal corticosterone levels were significantly increased directly after the cessation of the stressor on day 22 (Figure 3D). At day 43, paroxetine increased circulating corticosterone levels when mice were previously exposed to the chronic defeat paradigm (Figure 3E). In response to a novel acute stressor, paroxetine also largely increased the corticosterone response (Figure 3F), independent of the condition. At 90 min after the acute stressor, defeated animals recovered worse from the acute challenge, depicted in prolonged increased corticosterone levels (Figure 3G). Also, paroxetine-treated animals showed higher corticosterone levels than their vehicle-treated littermates.

Behavior

In the third week of the treatment phase, stressed animals showed increased locomotion in the OF test, with paroxetine animals being less active than their vehicle-treated littermates (Figure 3H). In the EPM, neither a condition nor a treatment effect could be detected (Figure 3I), whereas a preceding CSDS significantly decreased social interaction in the SA test (Figure 3J). Although mice showed increased interest in the urine-dipped cotton swab compared with the water-dipped swab in the FUST, no condition or treatment effect could be revealed in the urine trial (Figure 3K). In the FST, paroxetine-treated mice floated less when previously exposed to the chronic stress paradigm (Figure 3L).

Fkbp51 gene expression

In both the dorsal and the ventral hippocampus, FKBP51 mRNA expression was not influenced by chronic defeat (Supplementary figure S2).

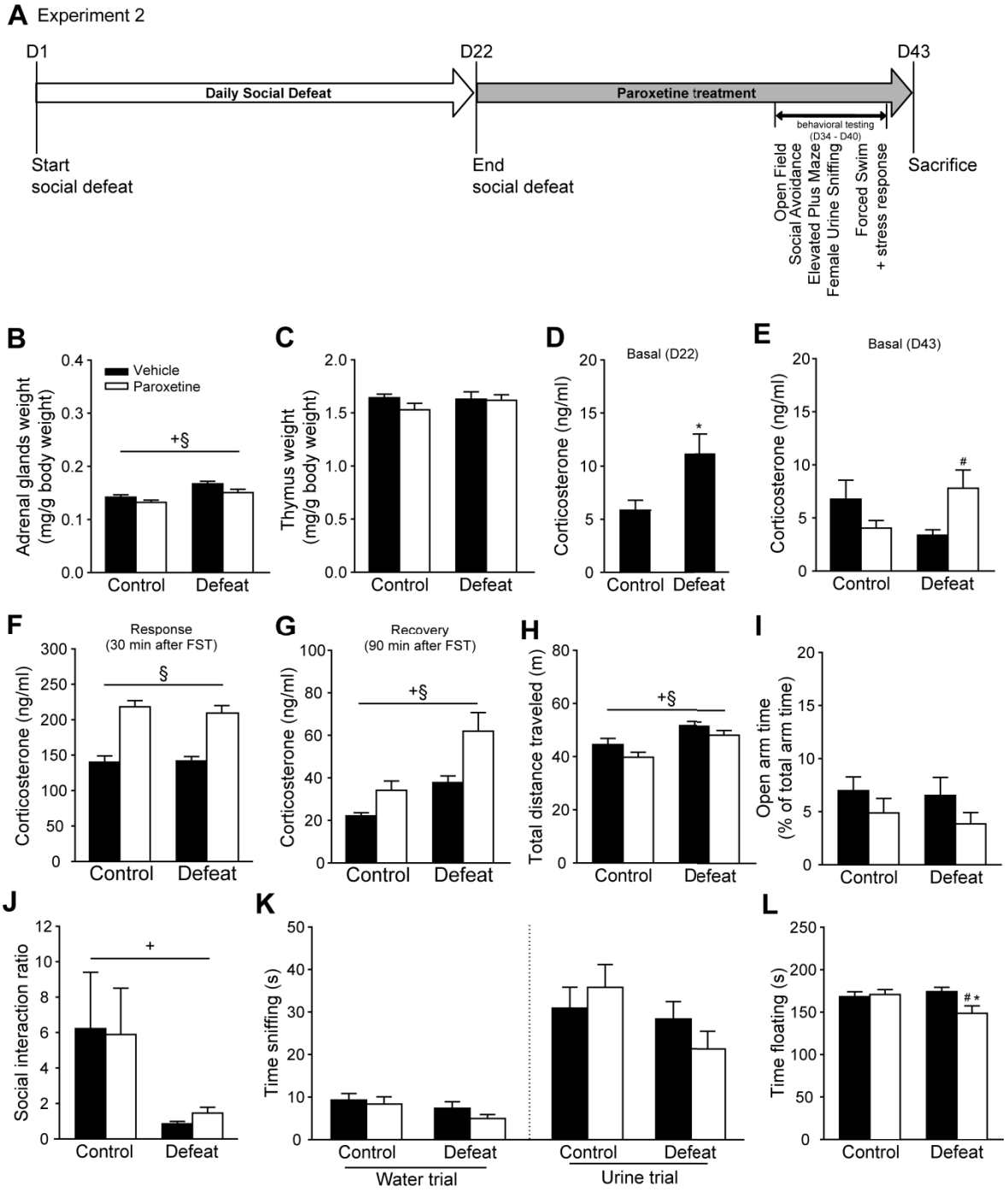

Figure 3: Physiological and neuroendocrine effects of CSDS were still present after a recovery period of 3 weeks but behavioral alterations are mostly restored. (A) Time course of experiment 2: Treatment with paroxetine commences after the stress procedure. The behavioral testing is performed in the last week of the treatment phase. (B) Investigation of the adrenal glands' weight revealed a condition ($F_{1,63}$ = 18.999, p < 0.001) and a treatment effect ($F_{1,63}$ = 7.000, p < 0.01). Adrenal glands were enlarged in stressed animals, but paroxetine diminished the stress effect. (C) Thymus weight was equal throughout all experimental groups. (D,E) Basal corticosterone levels directly after stress were increased (T_{62} = -2.488, p < 0.05) and subsequent paroxetine treatment disrupted HPA axis recovery to normal levels (ANOVA condition × treatment interaction ($F_{1,62}$ = 7.261, p < 0.01)). (F) After challenging the animals with a novel acute stressor, paroxetine treated mice showed an increased corticosterone response independent of condition in the response (ANOVA treatment: $F_{1,63}$ = 69.884, p < 0.001). (G) 2 weeks after cessation, CSDS led to an impaired ability to recover from an acute stressor, an effect that was strongly enhanced by paroxetine (ANOVA condition ($F_{1,63}$ = 17.708, p < 0.001) and treatment effect ($F_{1,63}$ = 12.243, p < 0.001)).

(H) Stressed animals showed a hyperactive phenotype in the open field (ANOVA condition: $F_{1,63} = 17.028$, $p < 0.001$), while paroxetine treatment resulted in less activity (ANOVA treatment: $F_{1,63} = 4.543$, $p < 0.05$). (I,J) While there was no effect on anxiety-related behavior, social interaction was still disrupted in mice that underwent the CSDS paradigm (ANOVA condition: $F_{1,59} = 5.186$, $p < 0.05$). (K) In the female urine sniffing test, no significant anhedonic effect could be found after recovery from the CSDS. (L) As in experiment 1, paroxetine exerted its antidepressant effects only in conjunction with CSDS as shown in reduced floating time in the forced swim test (ANOVA condition × treatment interaction: $F_{1,63} = 4.568$, $p < 0.05$). * Significantly different from control condition of the same treatment group, $p < 0.05$; # Significantly different from vehicle treatment of the same condition group, $p < 0.05$; + Significant condition effect, $p < 0.05$; § Significant treatment effect, $p < 0.05$; Abbreviations as in figure 1.

FKBP51 correlation analyses

Correlation analyses were performed in both experiments and significant effects could be found between FKBP51 expression and behavioral and neuroendocrine parameters in the vehicle-treated stress animals of experiment 1 (Figure 4). FKBP51 mRNA expression in the CA1 region of the dorsal hippocampus correlated both with the time struggling in the FST (Figure 4A) and the total distance traveled in the OF directly after cessation of the CSDS (Figure 4C). In the same experimental subgroup, FKBP51 expression in the CA1 of the dorsal hippocampus also correlated with corticosterone values of the acute stress response test (Figure 4E and G). In the DG, FKBP51 mRNA also correlated with the corticosterone values as well as with the locomotive behavior in the OF (Supplementary figure S2). These effects were not present after 3 weeks of recovery (Figure 4, right panels). In paroxetine-treated animals, no significant correlations could be shown.

GR sensitivity

To test whether varying FKBP51 levels would result in an altered GR sensitivity, we also measured the expression of a known GR target gene, MT-1 (Wang et al., 2004). MT-1 mRNA expression in the CA1 of the hippocampus was correlated to both FKBP51 levels in the same region (Figure 5A) and struggling time in the FST (Figure 5B). Again, in all other experimental subgroups of experiment 1, no significant correlations could be shown. To further investigate GR sensitivity in response to CSDS, we measured the relative protein levels of GR in the cytosolic and nucleic fraction of hippocampal tissue. Here, GR levels were shifted to the nuclear fraction when subjected to CSDS, compared with GR levels in control animals (Figure 5C and D). Overall levels of GR protein were not significantly different from control animals.

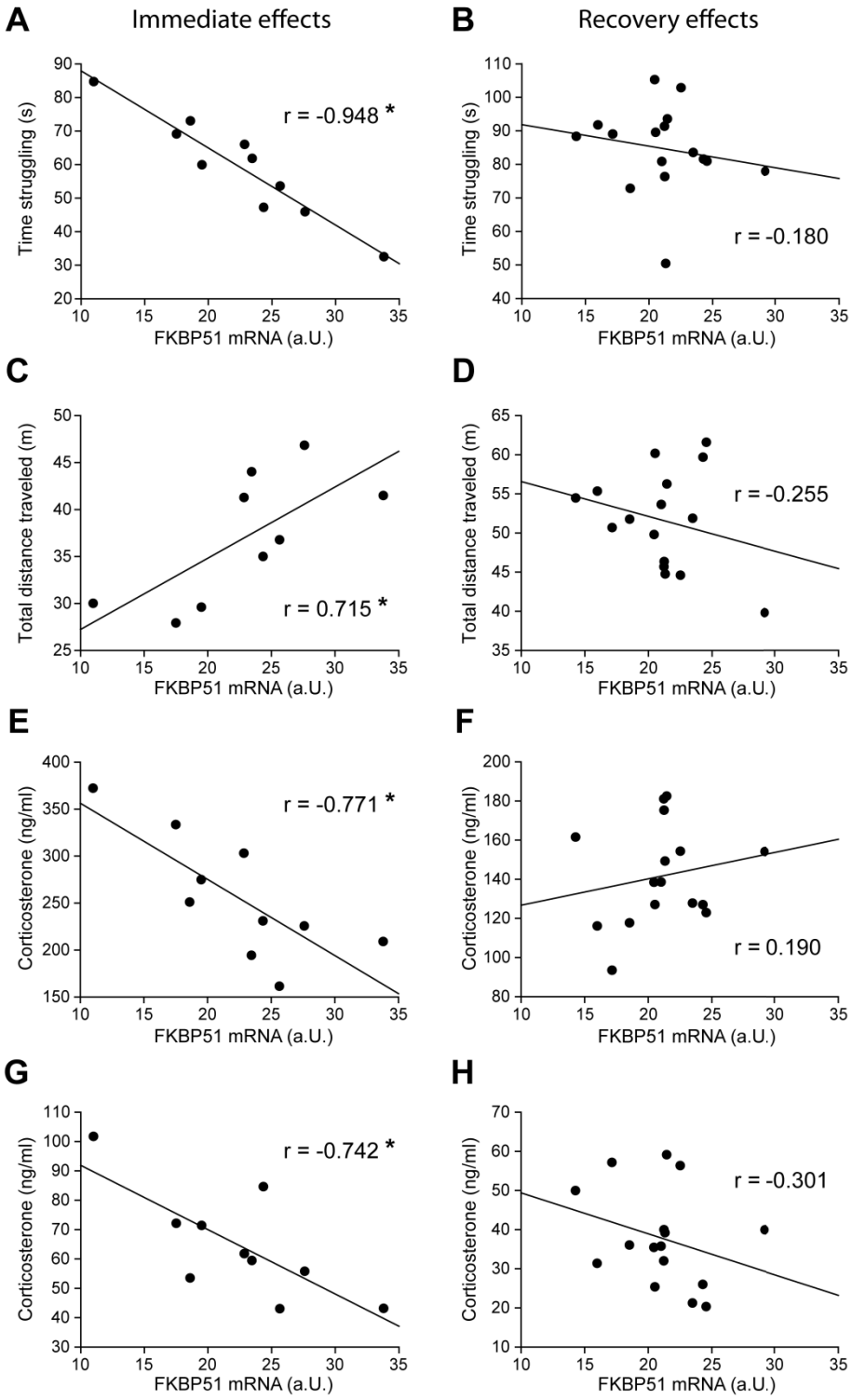

Figure 4: FKBP51 mRNA expression correlates with behavioral and neuroendocrine parameters in stressed animals. (A,B) The stronger the FKBP51 expression levels in the dorsal hippocampus, the lower the time spent with active stress coping in the forced swim test (r = -0.948, p < 0.001). This effect is only visible in a system activated by CSDS, as there is no significant correlation after the recovery period. (C,D) While CSDS reduced locomotion in the open field, an enhanced FKBP51 expression counteracts this behavioral phenotype (r = 0.715, p < 0.05) that is also exclusively visible in an activated system. (E,F,G,H) Circulating corticosterone is directly correlated to the increased FKBP51 levels at both the response to an acute stressor and the recovery from it (Response: r = -0.771, p < 0.01; recovery: r = -0.742, p < 0.05). * Significant correlation, p < 0.05; Abbreviations as in figures 1 & 2.

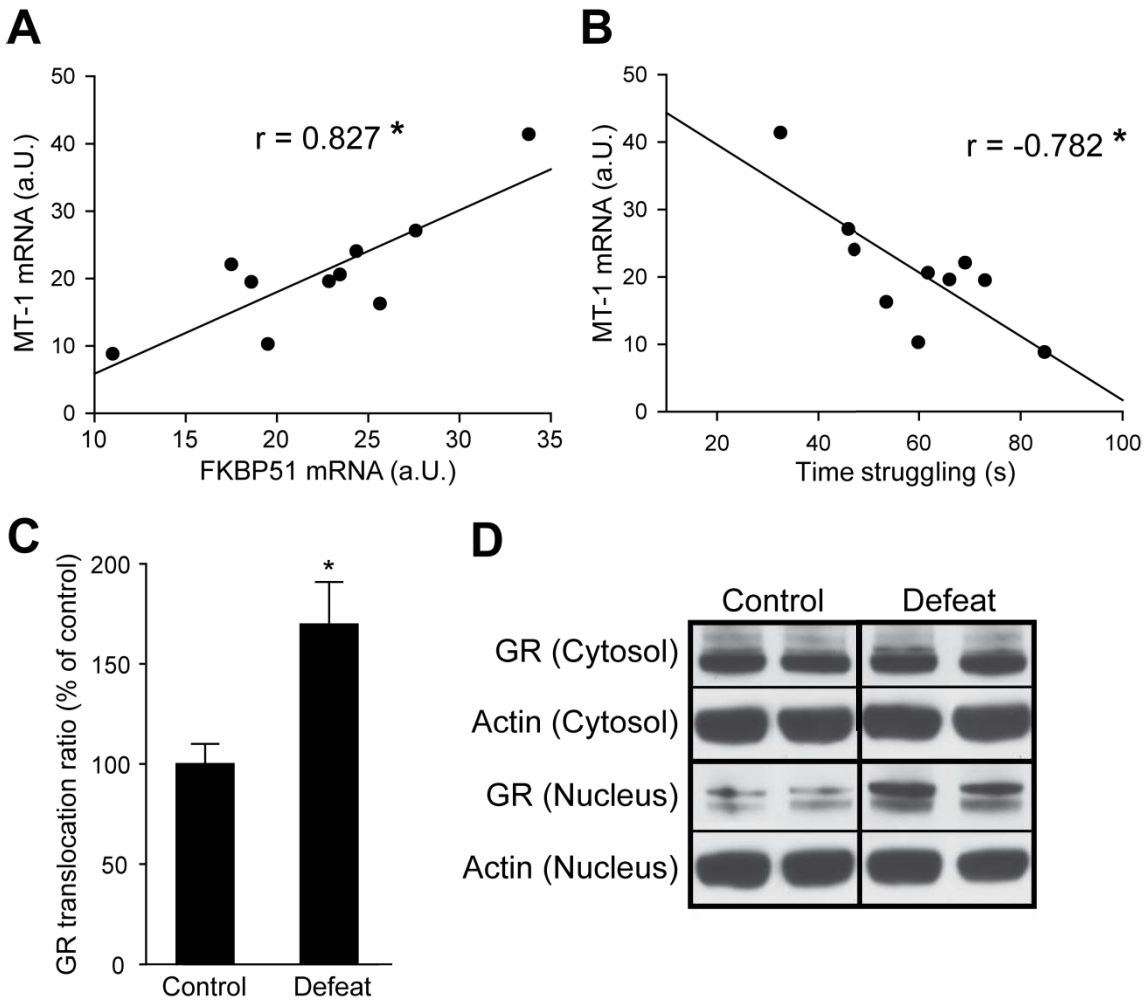

Figure 5: GR sensitivity is increased depending on FKBP51 levels. (A) Metallothionein-1 (MT-1) mRNA levels correlate significantly with FKBP51 levels in the CA1 region of the hippocampus, when animals underwent the CSDS paradigm (r = 0.827, p < 0.01). Since MT-1 is a downstream target of glucocorticoid receptors (GRs), this suggests increased GR sensitivity in response to FKBP51 activation. (B) MT-1 mRNA also correlates to struggling behavior in the FST (r = -0.782, p < 0.01). (C) Animals that underwent the CSDS paradigm, have an increased rate of GR translocation to the nucleus compared to control mice (T_{14} = -3.113, p < 0.01). (D) Protein bands of GR (94 kDa) and Actin (42 kDa) in the cytosolic and nucleic fraction of hippocampal tissue. * Significant from control, p < 0.05. Abbreviations as in figures 1 & 2.

Discussion

In this study, we show an association between FKBP51 and the behavioral and neuroendocrine response to chronic stress. Our CSDS model generated robust changes in physiology, neuroendocrinology, and behavior, both directly after the stress and after a recovery period of 3 weeks. These effects included increased anxiety-related behavior, a disturbed HPA axis function, as well as increased adrenal gland size. Treatment with the commonly used SSRI paroxetine had only small effects in ameliorating the stress-induced phenotype with regard to behavioral changes and deteriorated the neuroendocrine system independent of the time point of the treatment. FKBP51 mRNA expression was increased by CSDS and the level of induction is significantly correlated to both behavioral and neuroendocrine parameters, suggesting an important role of FKBP51 during HPA axis activity and GR sensitivity in a challenging environment. This is further supported by an increased GR translocation to the nucleus in stressed animals as well as FKBP51-correlated expression levels of a downstream target of GR.

The complex immediate phenotype induced by the CSDS model applied in experiment 1 of this study reproduced previous findings to a large extent (Wagner et al., 2011; Wang et al., 2011a; Hartmann et al., 2012). An increase in adrenal gland weight is consistently regarded as a reliable marker for a successful chronic stress paradigm (Schmidt et al., 2007), whereas body weight alterations in mice seem to underlie more intricate mechanisms, including type and intensity of the stressor as well as stress duration and social status of the animals involved (Bartolomucci et al., 2005). However, a tendency to increased body weight after CSDS is in line with previous observations made with this paradigm. Also, HPA axis function was severely disrupted in experiment 1, with an increase in corticosterone release and diminished feedback recovery after an acute stressor (Bartolomucci et al., 2005; Schmidt et al., 2010). We were able to replicate several behavioral phenotypes that have been frequently described, such as disturbed exploratory and social behavior as well as increased anxiety-related and anhedonic behavior (Choleris et al., 2001; Berton et al., 2006; Malkesman et al., 2010; Hartmann et al., 2012).

Regarding the long-lasting effects of our CSDS model, which were investigated in experiment 2, most assessed parameters returned to basal levels. We were not able to show an anxiety-related or anhedonic phenotype, and locomotion was, contrary to the

immediate effects of CSDS, slightly increased. This increased explorative behavior after recovery from CSDS may possibly resemble psychomotor agitation (Gupta, 2009). Additionally, a strong social avoidance was still visible in experiment 2, a finding that is in line with previous reports in which the applied CSDS reliably led to a strong aversion toward social targets (Berton et al., 2006; Tsankova et al., 2006). In these studies, important roles of various brain regions in stress resilience, including the nucleus accumbens and the ventral tegmental area, are highlighted, which are likely to play a role in the recovery mechanisms observed in our study (Krishnan and Nestler, 2008). Additionally, although corticosterone levels did not show differences under both basal and challenging conditions, the recovery from an acute stressor was still impaired, suggesting lasting changes in GR feedback mechanisms, possibly in the paraventricular nucleus of the hypothalamus and the prefrontal cortex (Mizoguchi et al., 2003). Taken together, the strong immediate effects of CSDS on physiology, neuroendocrinology, and behavior can mostly be restored by sufficient recovery time, in this case, 21 days. However, some alterations, such as increased social avoidance and diminished HPA axis feedback, are still present and promote the role of CSDS as a risk factor for the development of psychiatric diseases.

Chronic treatment with the SSRI paroxetine was only partly able to ameliorate the various phenotypes evoked by CSDS. Notably, paroxetine treatment led to elevated HPA axis activity and responsiveness as well as to reduced feedback ability independent of the condition. This is surprising as previous studies reported HPA axis normalization after chronic stress exposure when treated with antidepressants (Reul et al., 1993). Chronic paroxetine treatment had a positive effect on social and anhedonic behavior, but did not influence the anxiety-like phenotype observed in the EPM or reduced locomotion in the OF. Previous studies provide inconsistent results concerning the behavioral effects of SSRIs, with some reporting reduced anxiety (Burghardt et al., 2004) whereas others showing unchanged or even increased anxiety depending on the duration of treatment (Kurt et al., 2000; Norcross et al., 2008). A recent study by Thoeringer et al. (2010) could report anxiolytic action of paroxetine only after acute but not chronic administration. In the current study, paroxetine also led to a significant decrease of floating time in the FST when combined with CSDS and increased social behavior, thereby showing positive chronic treatment effects (Sillaber et al., 2008). We therefore conclude that paroxetine

treatment in mice, although showing therapeutic efficacy in some parameters, was not able to fully restore the CSDS-induced phenotype. In line with our findings, it has recently been suggested that the behavioral effects of CSDS models are largely independent of the serotonergic system (Venzala et al., 2012). Regarding our study, it can be speculated that these effects might rather be driven by HPA axis activation and sensitivity.

In recent years, it has been shown that FKBP51 plays a major role in stress reactivity and GR-mediated feedback processes that are crucial for a functional HPA axis. We further contribute to these understandings by reporting a distinct increase in FKBP51 expression in response to chronic stress. Additionally, the level of FKBP51 induction in the hippocampus is significantly correlated to the neuroendocrine and behavioral phenotype in a complex manner. In FKBP51 KO mice, Touma et al. (2011) reported an increased active stress coping in the FST, which was only present after a strong stressor. In line with these findings, we here show that higher FKBP51 levels in response to a challenge, in this case CSDS, were correlated to a reduction in active stress coping. These findings can be attributed to a higher GR sensitivity in the presence of low FKBP51 levels. Interestingly, higher FKBP51 levels following CSDS also correlated with higher locomotion in a novel environment. Accordingly, FKBP51 KO mice that underwent the same CSDS paradigm showed a strong reduction in locomotion that even exceeded the stress-induced effect seen in wild type animals (Hartmann et al., 2012).

A modulation in GR signaling and sensitivity has been found in both *in vitro* and *in vivo* studies and is suggested to be an important cofactor for the development of depression (Pariante and Miller, 2001). In line with these findings, increased FKBP51 induction correlated with reduced corticosterone response and recovery values. It has been proposed that the magnitude of FKBP51 induction is a marker of GR sensitivity. Indeed, this has recently been shown for FKBP51 mRNA induction in peripheral blood in humans (Menke et al., 2012). In this study, Menke et al. (2012) could show that a dexamethasone challenge is rapidly increasing FKBP51 mRNA levels in peripheral blood, suggesting a prominent role of FKBP51 in the intracellular short feedback loop to immediately reduce GR sensitivity in response to a stressor (Vermeer et al., 2003). Our findings support this hypothesis by showing that FKBP51 mRNA upregulation is connected to neuroendocrine parameters that resemble increased GR sensitivity (Wulsin et al., 2010). We could also show an increased GR translocation to the nucleus in stressed animals compared with

control littermates, which indicates increased GR signaling processes. An increase in expression of the GR-sensitive gene MT-1 has been shown to be induced by GR activity (Wang et al., 2004) and was also directly correlated to FKBP51 mRNA levels in the CA1 region of the hippocampus and to struggling time in the FST. However, during *in vivo* processes it is difficult to disentangle the effects of a strong FKBP51 induction, which would indicate a high GR sensitivity and a consequently high FKBP51 expression that would again decrease GR sensitivity. The dynamics of this ultrashort feedback loop are likely also brain region dependent and may explain why FKBP51 expression can correlate with the endocrine and behavioral phenotype in apparently opposite directions. Also, although FKBP51 and MT-1 mRNA strongly correlate with coping styles in the FST, there was no main effect of CSDS in this test. This may suggest that individuals challenged by CSDS resort to different molecular coping mechanisms than animals under basal conditions.

In paroxetine-treated animals, FKBP51 expression and the parameters mentioned above were not correlated. At first glance, this is surprising as FKBP51 mRNA induction was equally present in both treatment groups, but significant effects of antidepressants on GR activity and synthesis have been described (Pariante et al., 2004; Carvalho and Pariante, 2008). It is therefore likely that extensive paroxetine treatment manipulates the native feedback system to a large extent, which is overruling any regulative effect that FKBP51 may have on GR signaling. This is also reflected in increased plasma corticosterone responses in paroxetine-treated animals, irrespective of the condition (Linthorst and Reul, 2008).

This study also revealed some findings that are not fully in line with previous literature reports. Most prominent, paroxetine treatment was not able to induce more active coping behavior in the FST in control animals: a treatment effect was only detected in mice that previously underwent CSDS. The low efficacy of paroxetine concerning this parameter may be attributed to the application method, the dosage, or the fact that chronic treatment, when compared with an acute treatment with SSRIs, has been reported to elicit reduced behavioral effects (Thoeringer et al., 2010). It has also been suggested that C57Bl/6 mice, in contrast to other strains such as CD1 mice, are not as responsive to SSRI treatment in the FST (Petit-Demouliere et al., 2005). Another possible confounding factor may be the extensive testing battery that all animals underwent

(Blokland et al., 2012). Although the order of the tests was chosen to reduce carryover effects to a minimum (McIlwain et al., 2001), it cannot be excluded that there is a test × condition interaction. However, it has also been shown that a combination of stressors and different behavioral tests do not necessarily lead to confounding interactions (Chourbaji et al., 2008). Furthermore, it has to be pointed out that the measurements of the behavioral and neuroendocrine phenotypes and the mRNA sampling are temporally separated, and hence it cannot be ruled out that the FKBP51 expression levels observed at the time of killing are not the same as at the time of the test. However, it is likely that the inductive effects of CSDS on FKBP51 mRNA levels have reached a steady state by the time the behavioral testing occurs, and thus the levels at the time point of killing can give a meaningful insight into the mechanisms of the individual's stress response.

In summary, we could provide evidence that FKBP51 expression is strongly involved in adaption to chronic stress on both behavioral and neuroendocrine levels. When the stress system is chronically activated due to external challenges, higher FKBP51 levels are closely correlated to a more passive stress coping strategy, possibly because of rapid changes in the short feedback of GR sensitivity. This is indicated by increased GR translocation in stressed animals as well as a correlational increase in a GR-activated downstream target. In conjunction with previous studies, these findings highlight the important role of FKPB51 in the development of stress-associated psychiatric disorders and especially emphasize FKBP51 as a biomarker for GR sensitivity in response to stressful challenges, thus making it a potential target for future treatment options.

Supplemental Material

Behavioral tests performed:

Open-field test

The open-field test was performed to investigate locomotion differences. Testing was carried out in an empty open-field arena (50 cm × 50 cm × 50 cm) made of gray polyvinyl chloride (PVC), which was evenly illuminated with 15 lux. The low illumination of the open field arena was chosen to specifically investigate locomotion behavior and not create an aversive center region that may induce anxiety-related behavior. Testing time was 15 min and main parameter of interest was the total distance traveled.

Social avoidance test

The social avoidance test was performed as described previously (Golden et al., 2011). Briefly, animals were allowed to explore the open field arena for 2.5 min with an empty wire mesh cage placed at one side of the apparatus. In a second stage, the animals were confronted with an unfamiliar CD1 resident mouse in the wire mesh cage for another 2.5 min. The ratio between the time in the interaction zone of the no-target trial and the time in the interaction zone of the target trial serves as a marker for disturbed social behavior associated with depressive disorders. Animals that did not explore the interaction zone at all were excluded from the analysis.

Elevated plus-maze

The elevated plus-maze was conducted to display changes in anxiety-related behavior. The device consisted of a plus-shaped platform with two opposing open arms (30 cm × 5 cm × 0.5 cm) and two opposing enclosed arms (30 cm × 5 cm × 15 cm), made of gray PVC, which were connected by a central area (5 cm × 5 cm). The whole device was elevated 50 cm above the floor. The illumination was 25 lux in the open arms and less than 10 lux in the closed arms. Testing duration was 10 min and mice were placed into the center zone facing one of the enclosed arms at the start of the test. The time spent in the open arms compared to the total arm time as well as the number of open arm entries

were analyzed. Animals that fell off the open arm of the apparatus were excluded from the analysis.

Female urine sniffing test

The female urine sniffing test has been described by Malkesman and colleagues as a measurement of anhedonia in male mice (Malkesman et al., 2010). Briefly, 1 h before the test mice were habituated to a sterile cotton swab inserted into their home cage. In the first stage of the test, mice were exposed to a cotton swab dipped in water for 3 min and sniffing time was scored. After an intertribal interval of 45 min, mice were exposed to a cotton swab dipped in urine from estrous females of the same mouse strain. Again, total sniffing time was scored. The test was performed in a dark environment (<3 lux). Animals that escaped from the apparatus in any trial were excluded from the analysis.

Forced swim test

In the forced swim test, each mouse was put into the a 2 liter glass beaker (Diameter: 13 cm, height: 24 cm) filled with tap water (21 ± 1 °C) to a height of 15 cm, so that the mouse could not touch the bottom with its hind paws or tail. Testing duration was 5 min. Time spent immobile (Floating) and time spent struggling was scored by an experienced observer, blind to treatment or condition of the animals.

Acute stress response

The FST also served as an acute stressor in order to determine the stress response by measuring corticosterone plasma concentrations. After the FST, all mice were towel-dried and placed into their home cage to recover from the acute stressor. Blood samples were taken by tail cut (Fluttert et al., 2000) 30 min (Stress response) and 90 min (Stress recovery) after the onset of the FST. Samples were collected in 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany). All blood samples were kept on ice and later centrifuged at 8000 rpm at 4 °C for 15 min. Plasma was transferred to new, labeled tubes and stored at -20 °C until determination of corticosterone by radioimmunoassay (MP Biomedicals Inc; sensitivity 12.5 ng/ml).

In situ *hybridization*

For *in situ* hybridization, prepared sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. The antisense cRNA riboprobes of FKBP51 (Forward primer: 5'-CTTGGACCACGCTATGGTTT; reverse primer: 5'-GGATTGACTGCCAACACCTT) and Metallothionein-1 (Forward primer: CACGACTTCAACGTCCTGAG; reverse primer: CGGTAGAAAACGGGGGTTTA) were transcribed from a linearized plasmid. Tissue sections were saturated with 100 µl of hybridization buffer containing approximately 3 - 5 × 10^6 cpm ^{35}S labeled riboprobe. Brain sections were coverslipped and incubated overnight at 55 °C. The following day, the sections were rinsed in 4 × SSC (Standard saline citrate), treated with RNAse A (20 mg/l) and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1 × SSC for 1 h at 65 °C and dehydrated through increasing concentrations of ethanol. The slides were exposed to Kodak Biomax MR films (Eastman Kodak Co., Rochester, NY) and developed. Autoradiographs were digitized, and expression was determined by optical densitometry utilizing the freely available NIH ImageJ software. The mean of two measurements of two different brain slices was calculated for each animal. The data were analyzed blindly, always subtracting the background signal of a nearby structure not expressing the gene of interest from the measurements.

Western blot

Samples containing 30 µg of protein were resolved by 10% sodium dodecyl sulphate–polyacrylamide gels, and transferred onto nitrocellulose membranes (Invitrogen). Membranes were labeled with primary antibodies overnight at 4 °C. The primary antibodies used were rabbit anti-GR (1:1000, Santa Cruz Biotechnology), and goat anti-actin (1:2000, Santa Cruz Biotechnology). Following incubation with horseradish peroxidase-conjugated secondary antibodies (1:2000, DAKO) for 3 h, bands were visualized using an enhanced chemiluminescence system (Amersham Biosciences) and quantified by densitometry (Quantity One 4.2, Bio-Rad). GR protein levels were normalized to the corresponding actin levels in the subcellular fraction.

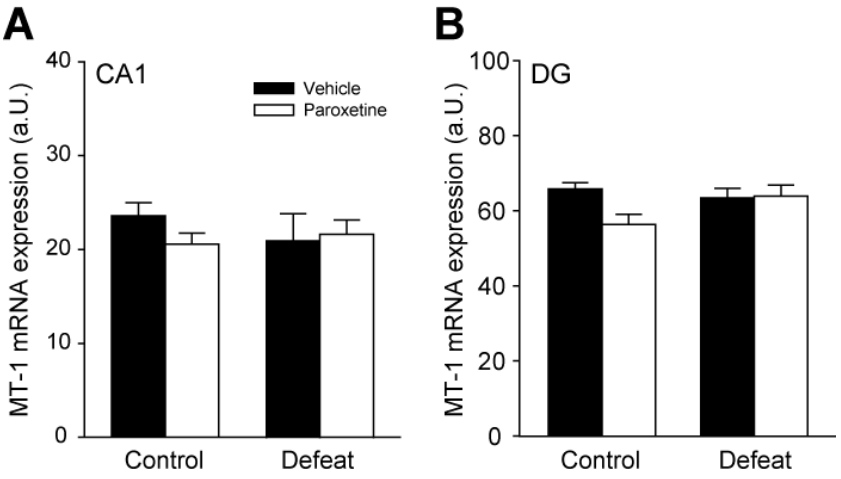

Figure S1: Metallothionein-1 mRNA levels are not influenced by chronic defeat stress or paroxetine treatment in (A) the CA1 region of the dorsal hippocampus and (B) the dentate gyrus (DG) of the dorsal hippocampus

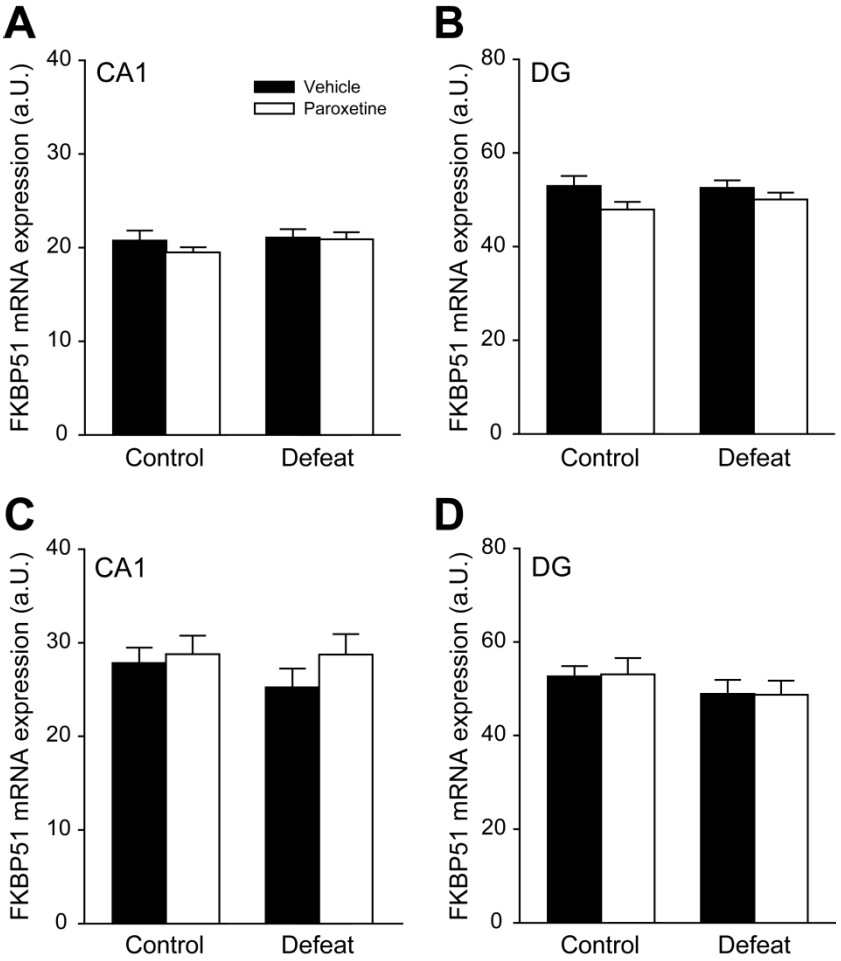

Figure S2: FKBP51 mRNA levels have returned to normal levels three weeks after the chronic defeat stress. (A,B) In the dorsal hippocampus, no effect of condition or treatment could be shown. (C,D) No significant effects of either stress or paroxetine were visible in the ventral hippocampus.

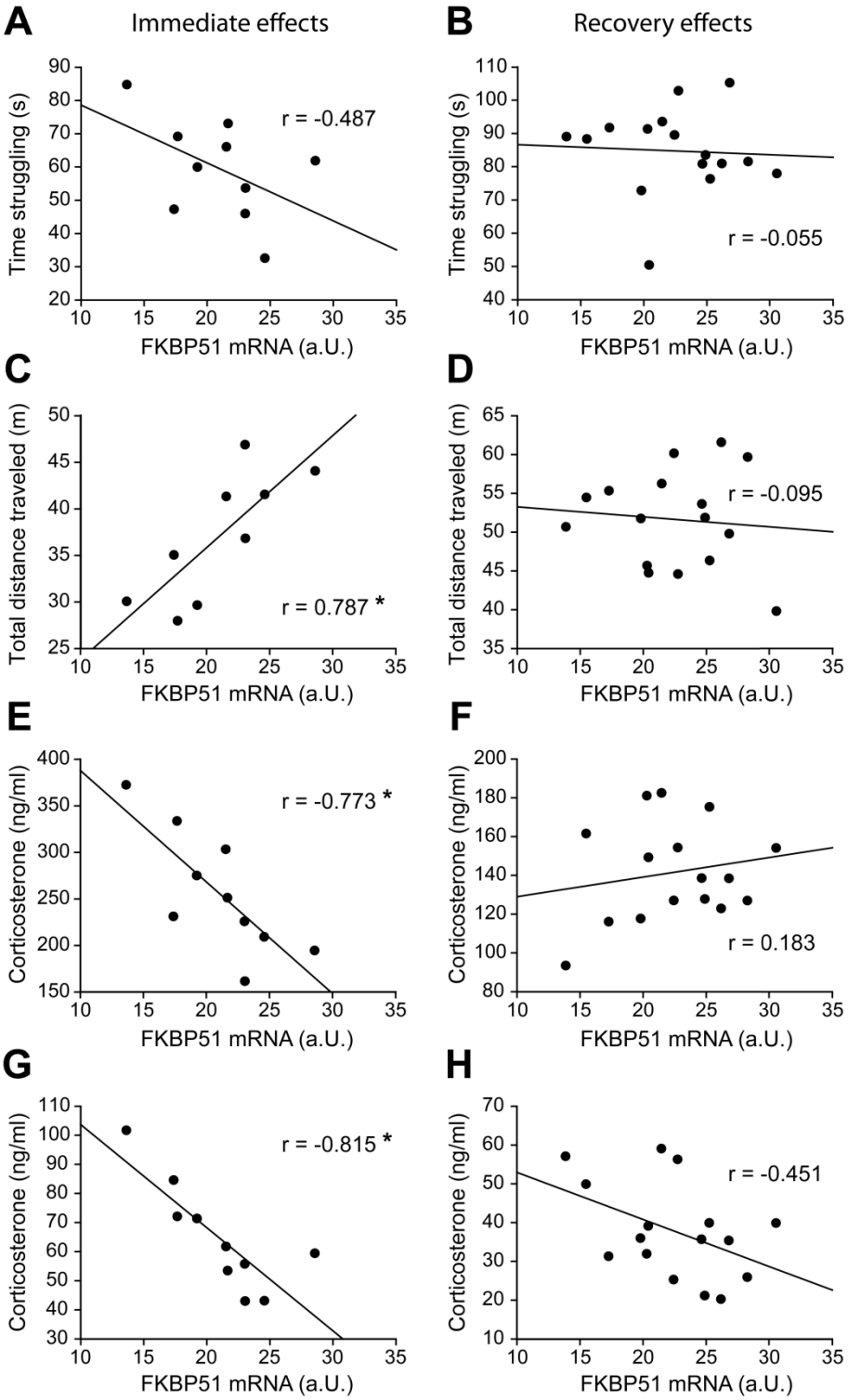

Figure S3: FKBP51 mRNA expression in dentate gyrus (DG) correlates with behavioral and neuroendocrine parameters in stressed animals. (A,B) In the forced swim test, struggling time × FKBP51 expression correlation failed to reach significant levels. (C,D) In the open field, an enhanced FKBP51 expression is strongly correlated to FKBP51 expression levels in the DG directly after stress, but not after a recovery phase of 3 weeks. (E,F,G,H) Circulating corticosterone is also directly correlated to FKBP51 expression levels at both the response to an acute stressor and the recovery from it. Again, after a recovery phase of 3 weeks, no correlation could be found. * Significant correlation, $p < 0.05$;

2.2. Hippocampal Homer1 is regulated by chronic social defeat stress

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Abstract

Chronic stress has prominent detrimental effects on the individual's well-being, which renders it a relevant risk factor for the pathology of psychiatric diseases. The glutamate system has been clearly implicated in mediating the (patho-)physiological effects of chronic stress, yet the involved molecular mechanisms are still poorly understood. In the current study we aimed to discover new candidate genes and biomarkers that are regulated by chronic stress, with a specific focus on the glutamate system. We employed the chronic social defeat stress (CSDS) model in mice and screened for altered mRNA expression in the hippocampus. Candidate genes identified by microarray analysis were technically validated by quantitative reverse transcriptase PCR and subsequently confirmed by *in situ* hybridization in an independent sample. After elimination of all non-validated candidates, we could identify Homer1, a postsynaptic scaffolding protein in glutamate neurons, as stress-regulated gene in the CA1 and CA3 region of the hippocampus. Additionally, a more in-depth analysis of the protein turnover of Homer1 by applying a $^{14}N/^{15}N$ labeled diet during CSDS revealed that approximately 30% of hippocampal Homer1 protein is metabolized and renewed in 7 days with no effect of CSDS on the turnover rate. Taken together, our results suggest Homer1 as a novel candidate gene, which may be involved in mediating detrimental effects of chronic stress in glutamate signaling pathways, thereby ultimately contributing to the emergence of psychiatric disorders.

Introduction

Mood disorders such as major depression are a considerable burden for modern societies and are predicted to become the second leading cause of disability worldwide by 2020 (Murray and Lopez, 1996). One major risk factor that is significantly contributing to the development of psychiatric disorders is chronic stress (Lupien et al., 2009). A prolonged overactivation of the stress systems, most prominently the hypothalamic-pituitary-adrenal (HPA) axis, results in an increased allostatic load that may ultimately lead to detrimental consequences to the individual's health (McEwen, 2004; de Kloet et al., 2005). These effects are accompanied by molecular changes in the brain that range from altered neurogenesis (Petrik et al., 2012) to dysfunctional neurotransmission (Joëls and Baram, 2009), yet the exact molecular mechanisms that turn chronic stress into a risk factor for psychopathology are still poorly understood.

Animal models of chronic stress have proven to be powerful tools to investigate the molecular underpinnings and behavioral consequences of stress-related disorders (Cryan and Holmes, 2005; Savignac et al., 2011). While monoaminergic neurotransmitter systems were studied in great detail (Keeney et al., 2006; Linthorst and Reul, 2008; Krishnan and Nestler, 2008), it was not until recently that the glutamate system has received growing attention in its role in psychopathology (Popoli et al., 2012; Sanacora et al., 2012).

Glutamate is the major excitatory neurotransmitter in the nervous system (Orrego and Villanueva, 1993) and has been linked with both cognitive (Citri and Malenka, 2007; Neves et al., 2008) and emotional processes (Phillips et al., 2003a; Phillips et al., 2003b). Postsynaptic signaling of glutamate is mediated via ionotropic (NMDA, AMPA, kainate) and metabotropic (mGluR1 - mGluR8) receptors. Various scaffold proteins in the postsynaptic density (PSD) bind different channel subunits to change receptor signaling properties by altering surface expression (Bhattacharyya et al., 2009), synthesis, degradation (Park et al., 2004) or recycling (Wang et al., 2008) of receptors. Additionally, mGluRs have been shown to modulate ionotropic signaling by scaffold interactions in the PSD with proteins as Shank and Homer1 (Tu et al., 1999; Hayashi et al., 2009; Bertaso et al., 2010). The latter is constitutively expressed in its long isoform, Homer1b/c, which consists of a proline rich binding site for mGluR5 and a coiled coil structure that allows for multimerization and subsequent downstream signaling (Brakeman et al., 1997; Tu et al., 1998). The shorter splice variant Homer1a, an immediate early gene that is not able to

multimerize, is induced by synaptic activity and can act as a dominant negative to Homer1b/c (Xiao et al., 1998). Recent studies suggested a crucial role of Homer1 in cognition (Ronesi and Huber, 2008; Tronson et al., 2010), and its involvement in the pathology of psychiatric disorders (Szumlinski et al., 2006; Rietschel et al., 2010), therefore providing further evidence for the importance of glutamate signaling in psychopathology.

In the current study, we aimed to identify and validate novel target genes that are modulated by chronic stress exposure, with a main focus on neurotransmission of the glutamatergic system to further dissociate the molecular components of stress coping mechanisms. We therefore utilized an established chronic social defeat stress paradigm and screened hippocampal mRNA transcripts via microarray. Potential candidate genes were then validated using quantitative reverse transcriptase PCR and *in situ* hybridization. Finally, we investigated the protein turnover rate of Homer1, a validated candidate gene for the development of psychiatric disorders.

Materials and Methods

Animals

For all experiments, male C57Bl/6N mice (Charles River Laboratories, Maastricht, the Netherlands) were used if not noted otherwise. At the beginning of the experiment, the age of all animals was 12 weeks. The mice were held under standard conditions (12L:12D light cycle, lights on at 08:00 AM, temperature 23 ± 2 °C), were single housed and acclimated to the room for two weeks before the beginning of the experiments. Food (Altromin 1314, Altromin GmbH, Germany) and tap water were available *ad libitum* unless otherwise specified. Male CD1 mice (16 - 18 weeks of age) served as resident mice, which were held under the conditions described above. They were allowed to habituate to the social defeat cage for two weeks prior to the experiment. All experiments were carried out in the animal facilities of the Max Planck Institute of Psychiatry in Munich, Germany. The experiments were carried out in accordance with the European Communities' Council Directive 86/609/EEC. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany.

Experimental design

Experiment 1

In the first experiment, we subjected 16 mice (n = 8/group) to the chronic social defeat stress (CSDS) paradigm described below. After the stress period, animals were sacrificed and hippocampal RNA was extracted for microarray and quantitative reverse transcription (q)-PCR analysis.

Experiment 2

To independently validate the results obtained from experiment 1, we performed *in situ* hybridization in brain slides of an independent batch of animals that were exposed to the CSDS paradigm (n = 9 - 11/group). The physiological and behavioral parameters of these animals have been reported before (Wang et al., 2011a).

Experiment 3

In the third experiment, we investigated the protein turnover of candidate genes by applying $^{14}N/^{15}N$ labeling as described below. A total of 48 mice were randomly split into 2x2 groups (Control standard diet (n = 12), control $^{14}N/^{15}N$ diet (n = 12), CSDS standard diet (n = 12), CSDS $^{14}N/^{15}N$ diet (n = 12)) and subjected to the chronic stress procedure described below. The animals received ^{14}N labeled diet based on Ralstonia eutropha (U-14N-SILAM-Mouse, Silantes GmbH, Munich, Germany) 1 week prior to the start of the CSDS for a total of 21 days. In the last week of the defeat, these animals switched to ^{15}N labeled diet (U-15N-SILAM-Mouse, Silantes GmbH, Munich, Germany) with the same composition as the corresponding ^{14}N-labeled diet for a total of 7 days until sacrifice. A detailed schematic of the experimental time course is depicted in figure 1. Hippocampal tissue samples were extracted for turnover analysis.

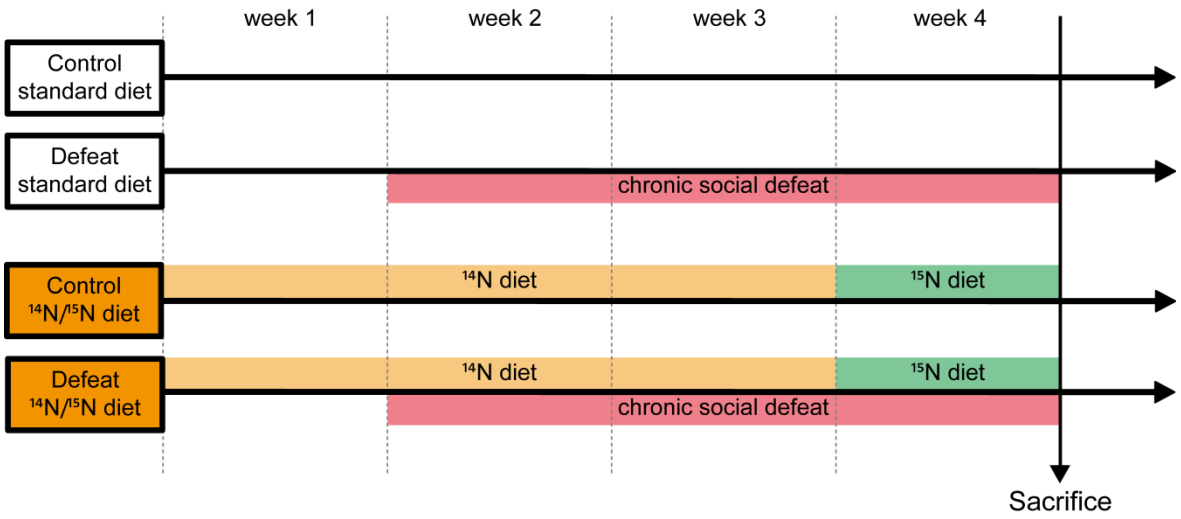

Figure 1: Experimental time course. Experiments 1 and 2 only contained standard diet groups, while in experiment 3 all four depicted groups were included. Here, the ^{14}N diet was administrated 1 week prior to the start of the chronic defeat paradigm. In the last week of the stress, the diet was switched to ^{15}N labeled food, while animals that received standard diet were kept under the same feeding condition over the course of the whole experiment.

Social defeat stress procedure

The CSDS paradigm lasted for 21 days and was conducted as described previously (Wagner et al., 2011). Briefly, the experimental mice were introduced into the home cage (45 cm × 25 cm) of a dominant resident mouse and defeated shortly after. When the defeat was achieved, the animals were separated by a wire mesh, preventing physical but allowing sensory contact for 24 h. Each day, stressed animals were defeated by another

unfamiliar, dominant resident mouse, in order to exclude a repeated encounter throughout the experiment. The daily defeat was performed between 11:00 AM and 04:00 PM; varying starting times reduced the predictability of the stressor and therefore minimized a potential habituation effect. Experimental mice were always defeated by resident males during the entire stress period. Control mice were housed in their home cages during the course of experiment. Both stress and control animals were handled daily during the stress procedure; body weight and fur state were assessed at the beginning of the experiment as well as before the sacrifice. The evaluation of the fur state was carried out as described previously (Mineur et al., 2003). Briefly, furs were rated on a scale from one to four by an experienced investigator, where 1 represents a perfect, clean fur, while 4 stands for a dishevelled, scruffy fur, often including wounds and scurf. Ratings of 2 and 3 represent intermediate fur states, respectively.

Sampling procedure

All animals were sacrificed by decapitation following quick anesthesia by isoflurane at the end of the experiment. Basal trunk blood samples were collected in 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany). All blood samples were kept on ice and later centrifuged at 8000 rpm at 4 °C for 15 min. Plasma was transferred to new, labeled tubes and stored at -20 °C until determination of corticosterone by radioimmunoassay (MP Biomedicals Inc; sensitivity 12.5 ng/ml). Brains were removed and processed as described below. Adrenal glands were removed, dissected from fat and weighed.

RNA processing

RNA from the whole hippocampi (Experiment 1) was isolated using the TRIZOL reagent (Invitrogen) as described previously (Schmidt et al., 2010). The quality of the RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The concentration and purity of total RNA was also assessed by 260 nm UV absorption and by 260/280 ratios, respectively (Nanophotometer, Implen, Munich, Germany). All samples had a RNA integrity number greater than or equal to 7 (7.0 - 8.9, mean 8.0 ± SD 0.4).

Processing of microarrays

RNA samples were processed for microarray as described previously (Schmidt et al., 2010; Menke et al., 2012). Briefly, sample labeling was performed using the Illumina TotalPrep RNA Amplification Kit (Ambion, TX, USA, catalog number AMIL1791). Biotin-labeled cRNA (1.5 µg) was hybridized to Illumina mouse BeadChips (n = 6 per chip, equally distributed between control and defeat condition) (Illumina, San Diego, CA, USA). Gene expression was analyzed using the Illumina BeadStudio software (Version 1.5.1.3).

Quantitative reverse transcriptase PCR

RNA samples from experiment 1 were transcribed into cDNA applying a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's protocol. qPCR of 100ng cDNA per sample was performed using the Quantifast SYBR Green PCR Kit (Quiagen) and the Lightcycler 2.0 (Roche) according to the standard protocols given in the manufacturer's manuals. The primers used for analysis of the target genes are depicted in Table 1. All samples were normalized to the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Symbol	Forward Primer	Reverse Primer
Rimbp2	GCAGGCTCAGGTTGAAGCTA	ACACGGCATTTCTCTTCCAG
Nell2	GGGAATAATGCAGGATGTGC	AAGTCGTTGCATGTTGGACA
Homer1	TGGACTGGGATTCTCCTCTG	TCCATCTTCTCCTGCGACTT
Ipmk	CGGCATGAGGGTTTATCATC	TTCCTTCAGGGTCTCTTTCG
mGluR1	GGAGGTCTGGTTCGATGAGA	TCATAGCGATTAGCCTCTGTGT
Rap1gap	GAAAAGATGCAGGGAAGCAG	CCCAAAACCTCATGGACACT
Slc6a1	GGGGTCCCTGATTGCTCT	AGTTGATGCAGCAAACGATG
Acvr1b	CTGGGAGATTGCACGAAGAT	CGGAGGGCACTAAGTCGTAA
Dlgap1	GTCACCACCGAGGATAGGAA	CCATTTTCTCCGGCTCTTC
Mrvi1	CTCAGCTCTGGGGCTTCTC	GACTGAGGGCCACTGCATA
Nnat	TCATCATCGGCTGGTACATC	CTGTGTCCCTGGAGGATTTC
Slc6a13	CTCCCCTGTCATCGAGTTCT	AGGAGGCACAGGACCAGTT

Table 1: Primers used for quantitative reverse transcriptase PCR. The left column indicates the transcript abbreviation commonly used in public databases. The middle and right column show the sequences of the forward and reverse primers in 5´ to 3´direction.

In situ *hybridization*

Frozen brains from experiment 2 were sectioned at -20 °C in a cryostat microtome at 18 µm, thaw mounted on Super Frost Plus slides, dried and stored at -80 °C. *In situ* hybridization using a ^{35}S UTP labeled ribonucleotide probe for Homer1b/c (Forward primer: AACACTGGGAGGCTGAGCTA; Reverse primer: TACTGCGGAAAGCCTCTTGT; transcript size: 531 nucleotides) and Homer1a (Forward primer: TGGTTGCTCAAGTTGACTGAA; Reverse primer: CCAGTAATGCCACGGTACG; transcript size: 400 nucleotides) was performed as described previously (Schmidt et al., 2007). Briefly, prepared sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. The antisense cRNA riboprobes of Homer1b/c and Homer1a were transcribed from a linearized plasmid. Tissue sections were saturated with 100 µl of hybridization buffer containing approximately $3 - 5 \times 10^6$ cpm ^{35}S labeled riboprobe. Brain sections were coverslipped and incubated overnight at 55 °C. The following day, the sections were rinsed in 4 × SSC (Standard saline citrate), treated with RNAse A (20 mg/l) and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1 × SSC for 1 h at 65 °C and dehydrated through increasing concentrations of ethanol. The slides were exposed to Kodak Biomax MR films (Eastman Kodak Co., Rochester, NY) and developed. Autoradiographs were digitized, and expression was determined by optical densitometry utilizing the freely available NIH ImageJ software. The mean of two measurements of two different brain slices was calculated for each animal. The data were analyzed blindly, always subtracting the background signal of a nearby structure not expressing the gene of interest from the measurements.

Quantitative in vivo *protein turnover analysis*

In vivo protein turnover was analyzed as described previously (Zhang et al., 2011). Briefly, after adaptation to the bacterial diet (Silantes GmbH, Munich, Germany) for 7 days, mice received ^{14}N bacterial diet for three weeks and were then switched to ^{15}N bacterial diet for 7 days in order to ^{15}N label newly synthesized protein. Synaptosomal protein fractions of hippocampal tissue were enriched as described previously (Filiou et al., 2010; Filiou et al., 2011) and 100 µg of this protein fraction was resolved by SDS gel electrophoresis. In

gel digestion, peptide extraction, liquid chromatography–mass spectrometry (LC-MS)/mass spectrometry (MS) analysis and peptide and protein identification was performed as described previously (Webhofer et al., 2013). MS data acquisition was performed in Selected Ion Monitoring mode for increased S/N levels and improved data quality of selected peptides of interest. The labeled peptide fraction, a measure of protein turnover, was calculated using the *Protunyzer* software (Zhang et al., 2011).

Statistical analysis

The data presented are shown as means ± SEM, analyzed by the commercially available software SPSS 18.0. Two-tailed student's t-test was employed for comparison of two independent groups (Control and CSDS). Two-factorial (Condition and diet) ANOVA was employed in experiment 3 when appropriate. For fur state analysis, the Wilcoxon signed-rank-test was applied for within group comparison, the Mann-Whitney-U-test was utilized for between-group analysis. A nominal level of significance $P < 0.05$ was accepted and adjusted according to Bonferroni correction by all posteriori tests (Univariate F-tests, test of simple effects or contrasts).

Results

Experiment 1

In the first experiment, we aimed to identify genes that are regulated by our CSDS procedure. Three weeks of chronic stress did not significantly change basal circulating corticosterone levels at the timepoint of the sacrifice (Figure 2A). However, we could identify a significant increase in adrenal gland weight in mice that underwent CSDS compared to control animals (T_{14} = -7.031, $p < 0.001$) (Figure 2B). A difference in body weight gain over the course of the experiment was not apparent (Figure 2C). The fur state was not significantly different at the start of the experiment, but mice of the CSDS group showed decreased fur quality over time (Z = -2.449, $p < 0.05$) (Figure 2D).

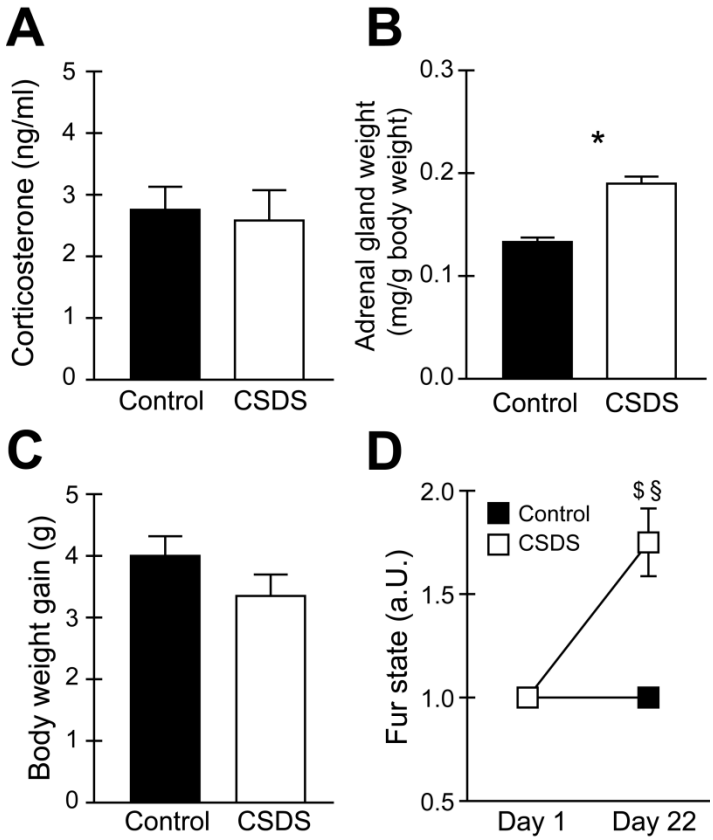

Figure 2: Neuroendocrine and physiological parameters from experiment 1. (A) Chronic social defeat stress (CSDS) did not alter basal corticosterone levels in the blood. (B) Adrenal glands were increased in animals that underwent CSDS compared to their control littermates, indicating enhanced hypothalamic-pituitary-adrenal (HPA) axis activation. (C) No effect of CSDS on the animals body weight gain was apparent at the time point of sacrifice. (D) At the beginning of the experiment, the fur state of all animals was in excellent condition. This was significantly changed by CSDS, as animals that were stressed showed an increased fur state index at day 22 of the experiment, compared to control animals (Condition effect) as well as compared to experimental day 1 (Time effect). * Significant from control; $ Significant from day 1 (Within-subjects time effect); § Significant from control of same time point (Between-subjects condition effect); $p < 0.05$.

Symbol	Accession	Gene name	Fold change	t-value	p-value
Rimbp2	NM_001081388.1	RIMS-binding protein 2	1.15	-2.643	**0.019**
Nell2	NM_016743.2	Protein kinase C-binding protein NELL2	1.15	-3.058	**0.009**
Homer1	NM_152134.2	Homer protein homolog 1	1.07	-3.244	**0.006**
Ipmk	NM_027184.1	Inositol polyphosphate multikinase	0.90	2.589	**0.021**
mGluR1	NM_016976.3	Metabotropic glutamate receptor 1	0.90	2.628	**0.020**
Rap1gap	NM_001081155.2	Rap1 GTPase-activating protein 1	0.90	2.976	**0.010**
Slc6a1	NM_178703.4	Sodium- and chloride-dependent GABA transporter 1	0.90	2.704	**0.017**
Acvr1b	NM_007395.3	Activin A receptor, type 1B	0.89	2.250	**0.041**
Dlgap1	NM_027712.3	Discs, large (Drosophila) homolog-associated protein 1	0.88	3.377	**0.005**
Mrvi1	NM_194464.2	Protein MRVI1	0.85	2.275	**0.039**
Nnat	NM_010923.2	Neuronatin	0.82	3.171	**0.007**
Slc6a13	NM_144512.2	Sodium- and chloride-dependent GABA transporter 2	0.81	2.184	**0.046**

Table 2: Selected candidate transcripts from the microarray. First and second column indicate the transcript abbreviation as well as the accession code used to identify the transcript in the ncbi database (http://www.ncbi.nlm.nih.gov). The third column describes the full name of the transcribed protein as given in the aforementioned database. In the next columns, the fold change of CSDS animals compared to control animals is listed, followed by the statistical t-value for the comparison as well as the respective p-value. Abbreviations as in figure 2.

At the day of sacrifice, stressed animals also showed a significantly increased fur state index compared to control animals ($Z = -3.000$, $p < 0.01$).

Analysis of hippocampal mRNA levels on the Illumina microarray revealed a total of 898 regulated genes ($p < 0.05$), however no gene withstood correction for multiple testing ($q > 0.05$). In a next step, we excluded all genes that showed a fold ratio of 0.90 to 1.05 ($n = 388$) as well as all signals that did not significantly exceed the microarray background range ($n = 210$). We furthermore eliminated all duplicate ($n = 28$) and not yet clearly

identified sequences such as predicted gene products (n = 157). From the remaining 115 sequences, we picked all genes that were reported to be associated with traits that are linked to psychiatric pathology such as neurogenesis, neurotransmission, and intracellular signaling for further analysis and validation.

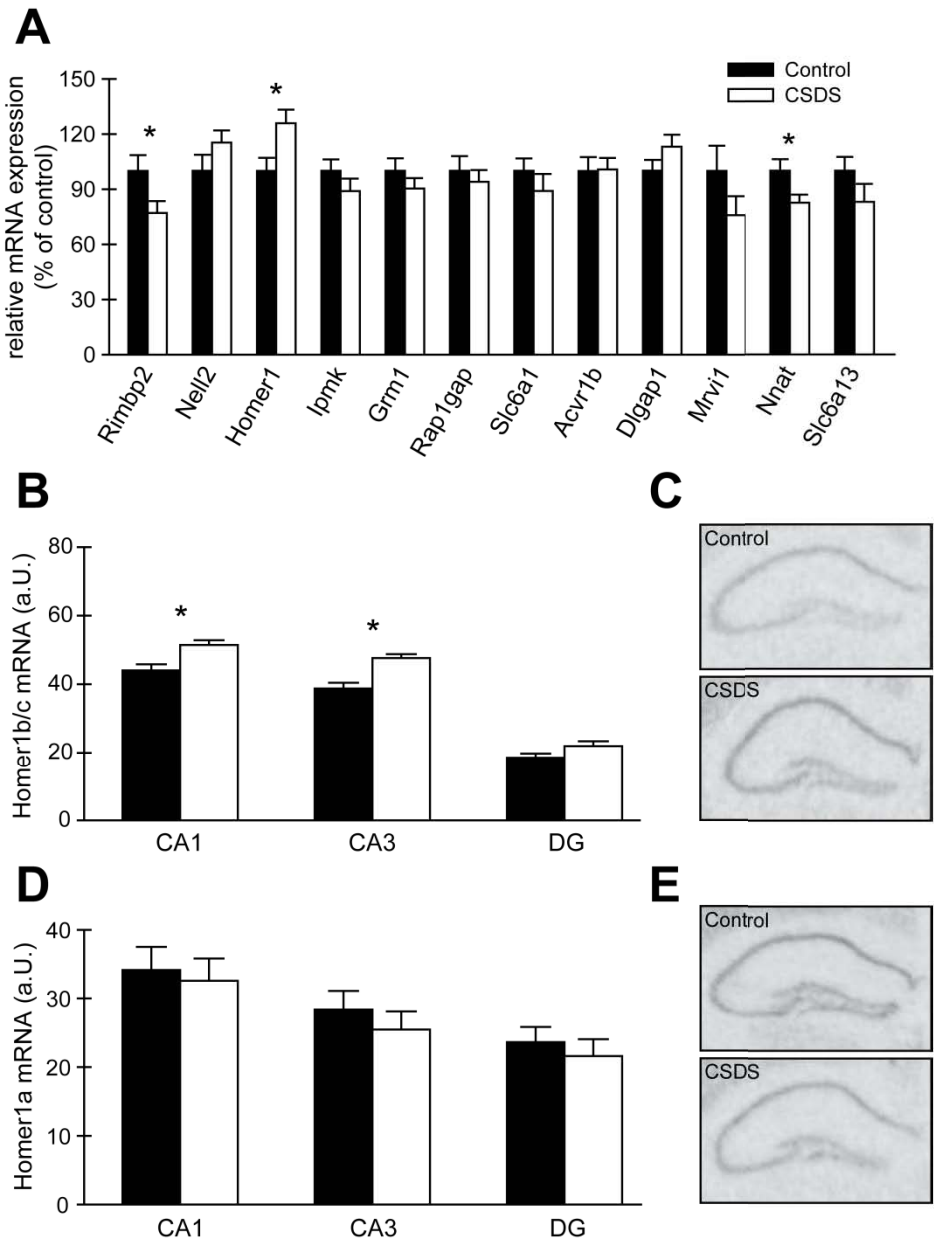

Figure 3: Validation of candidate genes from the microarray. (A) From 12 candidates, only the mRNA transcripts of 3 genes were significantly regulated in the hippocampus when analyzed by quantitative reverse transcriptase PCR, with Rimbp2 showing a regulation in the opposite direction compared to the microarray. Homer1 was significantly up- and Nnat downregulated, which is in line with the microarray results. (B) Validation of the constitutively expressed Homer1b/c in the dorsal hippocampus by *in situ* hybridization revealed an upregulation in the CA1 and CA3, but not in the dentate gyrus (DG). (C) Representative autoradiograph of Homer1b/c mRNA in the dorsal hippocampus. (D) Homer1a was not upregulated in response to chronic stress. (E) Representative autoradiograph of Homer1a mRNA in the dorsal hippocampus. * Significant from control; $p < 0.05$.

A total of 14 Illumina chip sequences were then double-checked with the basic local alignment tool (http://blast.ncbi.nlm.nih.gov), which eliminated 2 more candidates that were not specific for the specified gene. The 12 remaining candidates are depicted in Table 2.

To validate the chosen candidates from the microarray results, we performed qPCR as a technical control replicate using the RNA samples obtained from experiment 1. All 12 candidates were detected and normalized against GAPDH. From all candidates, only Rimbp2 ($T_{14} = 2.260$, $p < 0.05$), Homer1 ($T_{14} = -2.519$, $p < 0.05$), and Nnat ($T_{14} = 2.260$, $p < 0.05$) were significantly regulated in the qPCR analysis (Figure 3A). However, Rimbp2 was negatively regulated in the qPCR analysis, while the microarray results showed an upregulation.

Experiment 2

To independently replicate the mRNA differences seen in the hippocampus of stressed animals, as well as gain subregion-specific information about these alterations, we performed *in situ* hybridization with riboprobes against the two isoforms of Homer1, Homer1a and Homer1b/c, in a different set of animals (Wang et al., 2011a). Here we could detect significant differences in Homer1b/C mRNA levels in the CA1 ($T_{18} = -3.275$, $p < 0.01$) and CA3 ($T_{18} = -4.556$, $p < 0.001$) but not the dentate gyrus (DG) ($T_{18} = -1.791$, $p = 0.090$) region of the dorsal hippocampus (Figure 3B). On the other hand, Homer1a was not significantly regulated in any of the investigated regions of the hippocampus (Figure 3C).

Experiment 3

In a follow-up experiment, we investigated the turn-over rate of the protein Homer1b/c by measuring differences in $^{14}N/^{15}N$ labeled protein samples. To validate the efficiency of the CSDS paradigm, basal circulating corticosterone levels were measured at the day of sacrifice. Here, two-factorial ANOVA revealed a significant effect of the $^{14}N/^{15}N$ diet compared to standard diet ($F_{1,43} = 4.077$, $p < 0.05$), indicating that the bacterial diet increases basal corticosterone levels independently of the stressor (Figure 4A). Regarding the physiological effects of the CSDS paradigm on the animals, we detected a significant increase in adrenal gland weight due to CSDS ($F_{1,43} = 27.505$, $p < 0.001$) but no changes in

body weight gain (Figure 4B, C). Also, the bacterial diet did not significantly alter both investigated parameters. The fur state did not differ between the groups at the beginning of the experiment (Figure 4D). Over the course of the CSDS procedure, stressed animals showed a significant reduction in fur quality compared to both the beginning of the experiment (Standard diet: $Z = -3.500$, $p < 0.001$; $^{14}N/^{15}N$ diet: $Z = -3.176$, $p < 0.001$) and to their respective control group (Standard diet: $Z = -4.804$, $p < 0.001$; $^{14}N/^{15}N$ diet: $Z = -4.550$, $p < 0.001$). The diet did not influence fur state quality. In a subset of these experimental mice ($n = 3$/group, $^{14}N/^{15}N$ diet), we investigated the turnover rate of Homer1b/c in the synaptosomal fraction of the hippocampus during the last week of the CSDS paradigm, but did not find a significant change (Figure 4D).

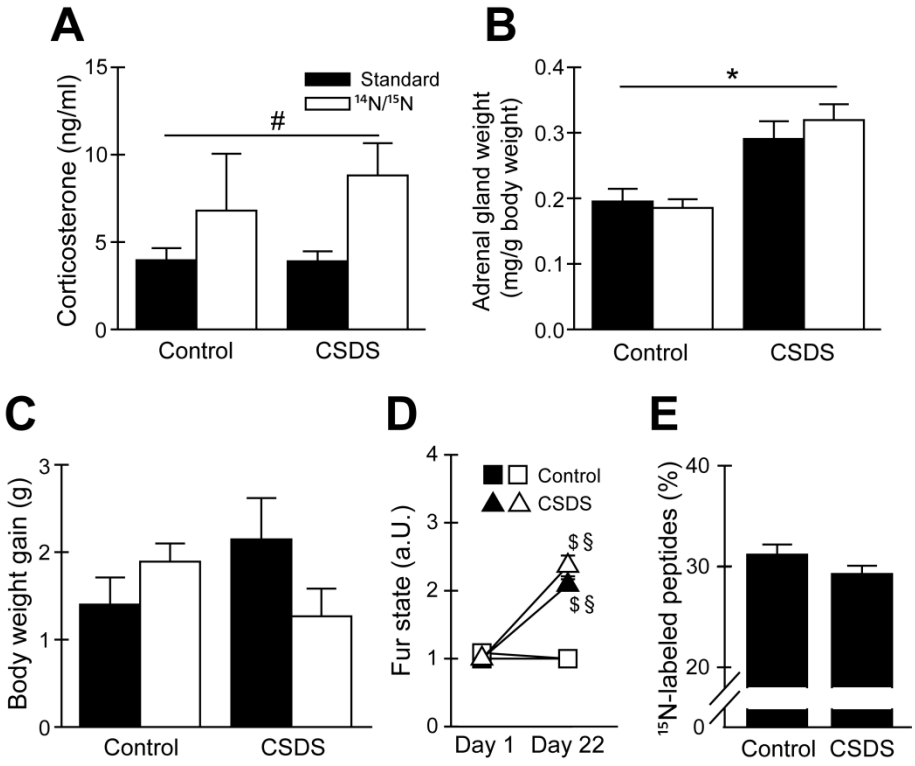

Figure 4: Results from experiment 3. (A) While CSDS did not affect basal corticosterone levels, the $^{14}N/^{15}N$ diet significantly increased circulating levels in the blood. (B) Adrenal glands were enlarged in the CSDS group with no effect of the diet. (C) Also, neither CSDS nor the applied diet significantly influenced the animals' body weight gain over the course of the experiment. (D) The fur state index was increased after the CSDS procedure in both diet groups, both with respect to the initial fur state index at day 1 of the respective subgroup (Time effect) and to the respective control group at day 22 (Condition effect). The diet itself did not affect the quality of the fur. (E) Protein turnover was not altered by CSDS, since both groups showed the same amount of ^{15}N labeled Homer1 protein in the synaptic fraction of the hippocampus. * Significant condition effect; # Significant diet effect; $ Significant from day 1 (Within-subjects time effect); § Significant from control of same time point (Between-subjects condition effect); $p < 0.05$. Abbreviations as in figure 2.

Discussion

In the present study, we aimed to identify novel target genes that are involved in neurotransmission and are regulated by chronic stress exposure. We therefore analyzed alterations in hippocampal RNA levels following an established chronic stress paradigm by microarray. We then aimed to validate potential candidates by both qPCR and *in situ* hybridization in independent samples. Following validation, we identified Homer1 as a potential gene that is regulated by chronic stress in the hippocampus. To further investigate the nature of this regulation, we also investigated the turnover rate of Homer1 protein in animals that were exposed to stress but did not find a difference. In summary, we were able to identify and validate a novel candidate gene in the glutamate system that may be involved in stress-induced molecular and behavioral alterations, which may ultimately lead to psychopathology.

Chronic stress has considerable consequences for the animals' physiology, which is displayed in increased basal circulating corticosterone and a concomitant increase in size of the adrenal glands (Keeney et al., 2006; Schmidt et al., 2007; Hartmann et al., 2012). These changes indicate a chronic overactivation of the HPA axis, which has been shown to be one of the main causes for the development of stress-related disorders (de Kloet et al., 2005). In the current study however, basal corticosterone levels were not significantly changed. It is important to note that basal blood samples were collected at one timepoint, which does not allow correcting for the ultradian rhythm that underlies corticosterone secretion (Lightman et al., 2008; Lightman and Conway-Campbell, 2010). This may explain why the measured corticosterone levels were not different between stressed and control animals. Yet, we were able to detect a robust increase in adrenal gland size in all experiments, which is a reliable indicator of HPA axis hyperactivity over the course of the chronic stress period, thereby indicating a successful chronic stress induction (Karst and Joëls, 2003; Schmidt et al., 2007). This is further supported by a reduction in fur state quality, which has previously been reported as a result of chronic stress exposure (Denmark et al., 2010; Wagner et al., 2011). While changes in body weight are frequently observed in the context of stress (Bartolomucci et al., 2009), we did not find a significant change in body weight gain in stressed animals. Indeed, results from previous studies applying this CSDS paradigm are inconsistent with respect to body weight data, reporting both increased body weight gain (Wagner et al., 2011; Wang et al.,

2011a; Hartmann et al., 2012) or no change (Wagner et al., 2012). The underlying molecular mechanisms of body weight alterations in response to chronic stress are still poorly understood, therefore further studies are warranted regarding this issue, but are not the main focus of the present study.

Next to physiological effects, chronic stress is reported to have major effects on the central nervous system, that range from macroscopic changes like hippocampal atrophy (Lupien et al., 1998) and neurogenesis (Schoenfeld and Gould, 2012) to alterations on subsynaptic levels such as the composition of the PSD (Donohue et al., 2006; Cohen et al., 2011). Structural and molecular changes are often accompanied by regulations on the mRNA level of various genes, which in turn translate into altered protein levels. We therefore performed a microarray analysis to identify genes that are regulated by our CSDS paradigm. Of initially 23273 sequences that were tested on the microarray, we chose 12 significantly regulated genes that have previously been implicated in neurotransmission or neurogenesis, but were only successful in validating two of them (Homer1, Nnat) in a technical replication, with an upregulation of Homer1 also being replicated in an independent sample.

Interestingly, in a study by Berton and colleagues, Homer1 was also reported to be upregulated by chronic defeat stress in the nucleus accumbens (Berton et al., 2006). Furthermore, microarray data from another study in our lab indicated Homer1 to be differentially regulated between stress resilient and vulnerable animals (Schmidt et al., 2010), which was also technically replicated by *in situ* hybridization (Personal communication). This is in line with our findings, showing the constitutively expressed isoform Homer1b/c to be regulated in the hippocampus of stressed mice in an independent experiment. Homer1b/c and its membrane interaction partner has recently been suggested to be critically involved in various neurological disorders, such as fragile X syndrome (Michalon et al., 2012), schizophrenia (Szumlinski et al., 2005; Jaaro-Peled et al., 2010; Spellmann et al., 2011) and depression (Rietschel et al., 2010). In the context of learning impairments, a phenotype typically observed in psychiatric disorders, Homer1b/c has also been shown to be involved (Szumlinski et al., 2004; Gerstein et al., 2012). To our knowledge, this is also the first study to report a protein turnover rate of Homer1b/c. While CSDS did not affect the turnover rate, we could show that after 7 days of treatment

with ^{15}N diet, around 30% of the present Homer1b/c protein was labeled with the heavy isotope.

To further specify the observed regulatory effects, we also investigated the short isoform Homer1a, which was not significantly regulated after three weeks of stress. Previous studies reported the immediate early gene Homer1a to be induced by e.g. a maximal electroconvulsive seizure, which is also a severe acute stressor (Brakeman et al., 1997; Hu et al., 2010). Acute induction of Homer1a by stress in turn exerts major effects on memory formation, also involving interactions with Homer1b/c, strengthening the evidence for an important role of this signaling pathway in learning behavior (Inoue et al., 2009; Tronson et al., 2010). Yet, no studies have been published that investigate the involvement of Homer1a in the context of chronic stress.

There are some limitations to this study that make the interpretation of the results more difficult. First, all gene regulations found on the microarray are only significant after a single t-test and do not withstand correction for multiple testing. However, it is unlikely that CSDS does not alter hippocampal gene expression, since this paradigm and other similar paradigms have been shown to strongly affect mice in various aspects (Berton et al., 2006; Tsankova et al., 2006; Wagner et al., 2011; Wang et al., 2011a; Hartmann et al., 2012; Wagner et al., 2012). Compared to e.g. tumor biology, effect sizes due to chronic stress are of lower magnitude, which may in turn cause sensitivity problems with current microarray technology (Shippy et al., 2006). This is in line with previous observations, also failing to show significant differences follow chronic stress after correction multiple testing (Datson et al., 2012). Novel strategies and techniques, such as next generation sequencing, should therefore be considered for future studies applying screening approaches (Shendure and Ji, 2008; Mehta et al., 2010; Shendure and Lieberman Aiden, 2012). Inferior microarray resolution may also be the cause for the poor replication rate obtained by qPCR that eliminated most of the chosen candidates. An increase in group size (i.e. from 8 to 12/group) may also resolve some of the problems mentioned above since this would greatly reduce the variance within the experimental groups. With regard to the upregulation of Homer1, further studies will need to clarify whether the increased mRNA levels also translate into elevated protein levels, which may in turn lead to alterations in the synaptic composition of glutamate neurons.

Summarizing, our results implicate Homer1 as a potential novel target for development and treatment of stress-related psychiatric disorders. This is evidenced by a distinct elevation of Homer1 mRNA levels in the hippocampus, which can subsequently alter mGluR5-mediated signaling pathways that ultimately lead to changes in glutamatergic neurotransmission. Given the evidence presented in this study, we suggest performing future studies that further dissect the molecular and behavioral consequences of modulating Homer1 in the context of stress and psychopathology.

2.3. Homer1 mediates acute stress-induced cognitive deficits in the dorsal hippocampus

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Abstract

In recent years, the glutamatergic system has been implicated in the development and treatment of psychiatric disorders. Glutamate signaling is processed by different receptors, including metabotropic glutamate receptors (mGluRs), which in turn interact with the scaffolding protein Homer1 to modulate downstream Ca^{2+} signaling. Stress is a major risk factor for the incidence of psychiatric diseases, yet acute stress episodes may have diverging effects on individuals. Cognitive impairments have often been shown to occur after episodes of stress, however the specific role of mGluR5/Homer1 signaling in the interaction of stress and cognition has not yet been elucidated. In this study we show that a single episode of social defeat stress is sufficient to specifically induce cognitive impairments in mice 8 h after the stressor without affecting the animals' locomotion or anxiety levels. We also demonstrate that Homer1b/c levels as well as mGluR5/Homer1b/c interactions in the dorsal hippocampus are reduced up to 8 h after stress. Blockade of mGluR5 during the occurrence of social stress was able to rescue the cognitive impairments. In addition, a specific overexpression of Homer1b/c in the dorsal hippocampus also reversed the behavioral phenotype, indicating that both mGluR5 and Homer1b/c play a crucial role in the mediation of the stress effects. In summary, we could demonstrate that stress induces a cognitive deficit that is likely mediated by mGluR5/Homer1 signaling in the hippocampus. These findings help to reveal the underlying effects of cognitive impairments in patients suffering from stress-related psychiatric disorders.

Introduction

Stressful life events are commonly accepted as risk factors for the development of psychiatric disorders (de Kloet et al., 2005). Social and work stress in particular are prevalent in western societies and psychopathologies emanating from such stressors result in high economic losses (Tennant, 2001). Stress paradigms in animal models produce a variety of behavioral, physiological and neuroendocrine changes that are related to clinical symptoms of psychiatric disorders (Nestler and Hyman, 2010). One major indication of these disorders is cognitive impairment, and numerous studies have investigated the interactions of stress and cognitive dysfunction at different stages in life in both animal models and humans (Lupien et al., 2009). Early life stress, chronic stress and chronically elevated circulating glucocorticoids (GCs) have been shown to induce cognitive impairments (Landfield et al., 1981; Borcel et al., 2008; Wang et al., 2011a; Wang et al., 2011b). In contrast, acute effects of stress and GCs critically depend on the timing and magnitude of the stressor or GC exposure and the stage of memory formation, consolidation or retrieval (Sandi, 2011). In this context, glutamatergic pathways and their role in memory formation have received growing attention in the last years, with a particular focus on glutamate receptor signaling (Popoli et al., 2012).

Homer1 is a scaffolding protein located in the postsynaptic density (PSD) and interacts with various binding partners, most prominently group I metabotropic glutamate receptors (mGluRs) and inositol trisphosphate receptors (Brakeman et al., 1997; Tu et al., 1998). The best described Homer1 isoforms are on the one hand Homer1b/c, consisting of a conserved amino-terminal target-binding domain and a coiled-coil structure that allows for multimerization (Xiao et al., 1998). On the other hand, Homer1a, a short form which is missing the coiled-coil structure, has been shown to act as a dominant negative for long Homer1 isoforms at the mGluR and IP_3 binding sites. The constitutively expressed Homer1b/c has been shown to mediate ligand-dependent signaling of mGluRs that may lead to downstream translational activation of protein kinase pathways (Ronesi and Huber, 2008; Ronesi et al., 2012) but is also involved in cell surface expression of mGluRs (Ango et al., 2002). In contrast, Homer1a induces ligand-independent activation of mGluRs, thus interfering with intracellular Ca^{2+} release (Ango et al., 2001). In humans, a role of Homer1 has been suggested in the emergence of major depressive disorders

(Rietschel et al., 2010), and was also found to be implicated in memory formation and cognition (Szumlinski et al., 2005; Lominac et al., 2005).

In the current study, we hypothesized that mGluR5/Homer1 signaling plays a crucial role in mediating effects of stressful life events. To test this, we first devised an experimental setup that specifically produces a learning impairment in response to an acute stress exposure. Next, we investigated both mRNA and protein levels of Homer1 in response to the stressor. Additionally, we tested whether pharmacological modulation of the glucocorticoid receptor (GR) or mGluR5/Homer1 signaling or Homer1 overexpression can attenuate the stress-induced cognitive deficits.

Materials and Methods

Animals

For all experiments, male C57Bl/6N mice (Charles River Laboratories, Maastricht, the Netherlands) at the age of 12 weeks were used. The mice were held under standard conditions (12L:12D light cycle, lights on at 08:00 AM, temperature 23 ± 2 °C) and were single housed and acclimated to the room for 2 weeks before the beginning of the experiments. Food (Altromin 1324, Altromin GmbH, Germany) and tap water were available *ad libitum*. Male CD1 mice (16 - 18 weeks of age) served as resident mice, which were held under the conditions described above. They were allowed to habituate to the social defeat cage for two weeks prior to the experiment. All experiments were carried out in the animal facilities of the Max Planck Institute of Psychiatry in Munich, Germany. The experiments were carried out in accordance with the European Communities' Council Directive 2010/63/EU. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany.

Experimental design

For all experiments, a separate batch of animals was used (n = 8 - 12 mice per group). The defeat procedure was performed between 08:00 AM and 12:00 PM. Sampling and testing was performed after the stated amount of time. For *in situ* hybridization and western blot, brains were removed, frozen in isopentane at −40 °C, and stored at −80 °C until further processing. For immunohistochemistry, animals were deeply anesthetized with ketamine/rompun and perfused intracardially with 4% paraformaldehyde. Brains were removed, postfixed overnight in 4% paraformaldehyde following overnight incubation in 30% sucrose solution at 4 °C, and then stored at −80 °C. Blood samples were kept on ice and later centrifuged at 8000 rpm at 4 °C for 15 min. Plasma was transferred to new, labeled tubes and stored at -20 °C until determination of corticosterone by radioimmunoassay (MP Biomedicals Inc; sensitivity 12.5 ng/ml).

Social defeat stress procedure

Animals were exposed to an aggressive CD1 resident mouse with short attack latency for 5 min, and then returned to their homecage until testing or sacrifice. During the defeat procedure, fighting was not interfered with unless a severe injury occurred. In this case, the defeat was stopped and the experimental animal was excluded from further analysis. Control animals were allowed to explore an empty novel cage similar to the resident cage for 5 min as well.

In situ *hybridization and immunohistochemistry*

For *in situ* hybridization, frozen brains were coronally sectioned in a cryostat microtome at 18 µm and kept at −80 °C. *In situ* hybridization using a ^{35}S UTP-labeled ribonucleotide probe for Homer1b/c (Forward primer: AACACTGGGAGGCTGAGCTA; Reverse primer: TACTGCGGAAAGCCTCTTGT) and Homer1a (Forward primer: TGGTTGCTCAAGTTGACTGAA; Reverse primer: CCAGTAATGCCACGGTACG) was performed as described previously (Schmidt et al., 2007). For fluorescence immunohistochemistry, serial coronal sections were cut at 30 µm thickness. Double-labeling immunofluorescence (Rabbit anti-Homer1, 1:1000, Synaptic Systems; goat anti-GFP, 1:500, Abcam) was performed on free-floating sections (n = 3 per mouse) as described previously (Wang et al., 2011a).

Coimmunoprecipitation

For coimmunoprecipitation (CoIP), a separate batch of experimental animals was killed 8 h after the defeat procedure and the dorsal hippocampus was dissected. Membrane fractions were isolated using the Calbiochem ProteoExtract Kit (EMD Biosciences) as described previously (Wagner et al., 2012). The protein concentration was determined, and 1.2 mg of lysate was incubated with 2.5 µg mGluR5 antibody (Millipore) overnight at 4 °C. Twenty microliters of BSA-blocked Protein G Dynabeads (Invitrogen, catalog no. 100-03D) were added to the lysate-antibody mix followed by 3 h incubation at 4 °C. The beads were washed three times with PBS, and protein-antibody complexes were eluted with 100 µg/ml mGluR5-peptide solution (Millipore) in CoIP buffer for 30 min at 4 °C. Fifteen micrograms of the cell lysates and 10 µl of the immunoprecipitates were further processed by western blot analysis.

Western blot

Purification of synaptosomal fraction of hippocampal tissue (Filiou et al., 2010) and western blot analysis (Wang et al., 2011a) was performed as described before. Antibodies used were rabbit anti-Homer1 (1:1000, Synaptic Systems), rabbit-anti mGluR5 (1:1000, Millipore) and goat anti-actin (1:2000, Santa Cruz Biotechnology) for primary as well as horseradish peroxidase-conjugated secondary antibodies (1:2000, DAKO).

Behavioral testing

All behavioral tests were recorded using a video-tracking system (Anymaze 4.20; Stoelting, Wood Dale, IL, USA). The following behavioral tests were performed: Spatial object recognition (sOR), Y-Maze, elevated plus maze (EPM) and female urine sniffing test (FUST). The testing procedures were performed as described below.

Spatial object recognition

The spatial object recognition task was performed in an open field apparatus (50 × 50 × 50 cm) under low illumination (15 lux) as described previously (Schmidt et al., 2011a). Prominent spatial cues were provided. Mice were habituated to the testing environment for 10 min on two consecutive days before testing. During the acquisition trials, mice were presented with two identical aluminum cubes (5 × 5 × 5 cm) and allowed to freely explore the objects two times for 10 min separated by a 15 min intertrial interval (ITI). During the 5 min retrieval trial, 30 min following the last acquisition trial, mice were presented with a nondisplaced object and a relocated one. The percentage of time exploring the displaced and the non-displaced objects was calculated, with a higher preference for the novel object being rated as intact spatial recognition memory. Animals that did not explore any of the two objects during the retrieval phase were excluded from the analysis.

Y-Maze

The Y-maze was performed as described previously (Schmidt et al., 2011a). Briefly, the apparatus is made of gray polyvinyl chloride (PVC) and consisted of three evenly illuminated arms (30 × 10 × 15 cm, 15 lux) with an angle of 120° between each arm. The apparatus was surrounded by various spatial cues. To ensure that the mice had sufficient

spatial cues available without having to stretch up and look outside of the maze, we also introduced intramaze cues (Triangles, bars, and plus signs) that served the same purpose as the external cues. The Y-maze test comprises two trials separated by an ITI to assess spatial recognition memory. During the first trial (Acquisition phase), the mouse was allowed to explore only two of the three arms for 10 min while the third arm was blocked. After 30 min ITI, the second trial (Retrieval phase) was conducted during which all three arms were accessible for 5 min. The percentage of distance traveled in the novel arm compared with the known arms was scored with a significantly higher percentage than chance level (33.3%) rated as successful spatial memory.

Elevated plus maze

The EPM was conducted to display changes in anxiety-related behavior. The device consisted of a plus-shaped platform with two opposing open arms (30 cm × 5 cm × 0.5 cm) and two opposing enclosed arms (30 cm × 5 cm × 15 cm), made of gray PVC, which were connected by a central area (5 cm × 5 cm). The whole device was elevated 50 cm above the floor. The illumination was 20 lux in the open arms and < 10 lux in the closed arms. Testing duration was 5 min and mice were placed into the center zone facing one of the enclosed arms at the start of the test. The time spent in the open arms compared to the total arm time was analyzed.

Female urine sniffing test

The FUST was performed as described previously (Malkesman et al., 2010; Wagner et al., 2012). Briefly, 1 h before the test mice were habituated to a sterile cotton swab inserted into their home cage. In the first stage of the test, mice were exposed to a cotton swab dipped in water for 3 min and sniffing time was scored. After an ITI of 45 min, mice were exposed to a cotton swab dipped in urine from estrous females of the same mouse strain. Again, total sniffing time was scored. The test was performed in a dark environment (< 3 lux). Animals that escaped from the apparatus in any trial were excluded from the analysis.

Drugs

The selective GR agonist dexamethasone (DEX) (Ratiopharm) or vehicle (0.9% NaCl) was administered subcutaneously (10 mg/kg body weight). Animals were then placed back in their homecage until sacrifice or behavioral testing.

For blockade of GR signaling, the specific GR antagonist RU486 (Sigma) was administered 5 min before the defeat procedure. Animals received a subcutaneous injection of either RU486 (100 mg/kg body weight) or vehicle (Polyethylene glycol).

For blockade of mGluR5 signaling, the specific mGluR5 antagonist MTEP (Tocris Bioscience) was solubilized in 0.9% NaCl (Anderson et al., 2003; Busse et al., 2004). Animals received a subcutaneous injection of either vehicle or MTEP (15 mg/kg body weight) 5 min before the defeat procedure.

Drug dosage was chosen according to previous reports (Anderson et al., 2003; Schmidt et al., 2011a).

Viral overexpression of Homer1

Viral overexpression was performed as described previously (Schmidt et al., 2011a). We used an adeno-associated bicistronic AAV1/2 vector (GeneDetect) containing the CAG-Homer1-IRES-EGFP-WPRE-BGH-polyA expression cassette (Containing coding sequence of Homer1 NCBI CCDS ID CCDS36745). For the control group, we used the same vector construct expressing only EGFP. Virus production, amplification, and purification were performed by GeneDetect. Mice were anesthetized with isoflurane, and 0.5 µl of either AAV-Homer1 or AAV-EGFP (Titres: 1.2×10^{12} genomic particles/ml) were bilaterally injected in the dorsal hippocampus at 0.06 µl/min by glass capillaries with tip resistance of 2 - 4 MΩ in a stereotactic apparatus. The following coordinates were used: 1.9 mm posterior to bregma, 1.3 mm lateral from midline, and 1.3/1.8 mm below the surface of the skull, targeting the CA1 and dentate gyrus (DG) region of the dorsal hippocampus. After surgery, mice were treated for 5 days with Metacam via drinking water. Behavioral testing started 4 weeks after virus injection. Successful overexpression of Homer1 was verified by immunofluorescence. Animals that were not infected bilaterally in both the CA1 and DG region were excluded from the analysis (n = 4). Quantification of the overexpression was achieved by *in situ* hybridization using the riboprobe described above.

Statistical analysis

The data presented are shown as means ± SEM and were analyzed by the commercially available software SPSS 16.0. Student's t-test was employed for comparison of means. Two-way analysis of variance was used to investigate effects of locomotion and object interaction in experiments including two conditions (Control/defeat and vehicle/drug treatment). A nominal level of significance $P < 0.05$ was accepted.

Results

A single social defeat induces specific spatial memory impairments after an intermediate period of recovery

We subjected mice to a single defeat session and investigated the corticosterone response as a measure of hypothalamic-pituitary-adrenal axis activity after four distinct time points (Figure 1A). Circulating corticosterone was significantly increased 1 h ($T_{9.614}$ = -8.623, $p < 0.001$), 4 h ($T_{9.898}$ = -3.273, $p < 0.01$), and 8 h (T_{18} = 3.753, $p < 0.01$), but not 24 h after the defeat session. As the defeat stress showed a significant neuroendocrine impact 8 h after the stress, we tested whether stressed animals show a behavioral phenotype. Indeed, 8 h after a defeat session, experimental mice were not able to distinguish a displaced object in the sOR test (Control: T_{22} = 7.721, $p < 0.001$; defeat: T_{22} = -0.234, $p = 0.817$) (Figure 1B). We also replicated this cognitive impairment in the Y-maze test, where stressed animals did not discern between a novel and known arms (Figure 1C). To investigate for potential biases, we also investigated the total distance traveled in the apparatus in both tests, but did not find a difference (sOR: control: 8.70 ± 1.26 m; defeat: 7.21 ± 1.19 m, $p = 0.398$; Y-maze: control: 12.01 ± 0.85 m; defeat: 12.75 ± 2.04 m, $p = 0.724$). Additionally, the total interaction time with both objects in the sOR test did not differ between control and defeated animals (Control: 3.79 ± 0.61 s; defeat: 3.19 ± 0.75 s, $p = 0.542$). These memory deficits were not visible in either test when animals were tested 24 h after the defeat session (Control: displaced object: 78.61% ± 2.62; nondisplaced object: 21.39% ± 2.62; T_{18} = 15.419, $p < 0.001$; defeat: displaced object: 76.72% ± 3.42; nondisplaced object: 23.27% ± 3.42; T_{18} = 11.032, $p < 0.001$). To further narrow down the behavioral phenotype, we also performed an EPM test (Figure 1D) as well as a FUST (Figure 1E) 8 h after the defeat but did not find any difference between control and defeated mice (EPM, time on the open arm: T_{22} = 0.830, $p = 0.415$; FUST, urine sniffing time: T_{22} = 0.284, $p < 0.779$), indicating that there is no underlying locomotion phenotype, anxiety phenotype, or anhedonic phenotype in these animals.

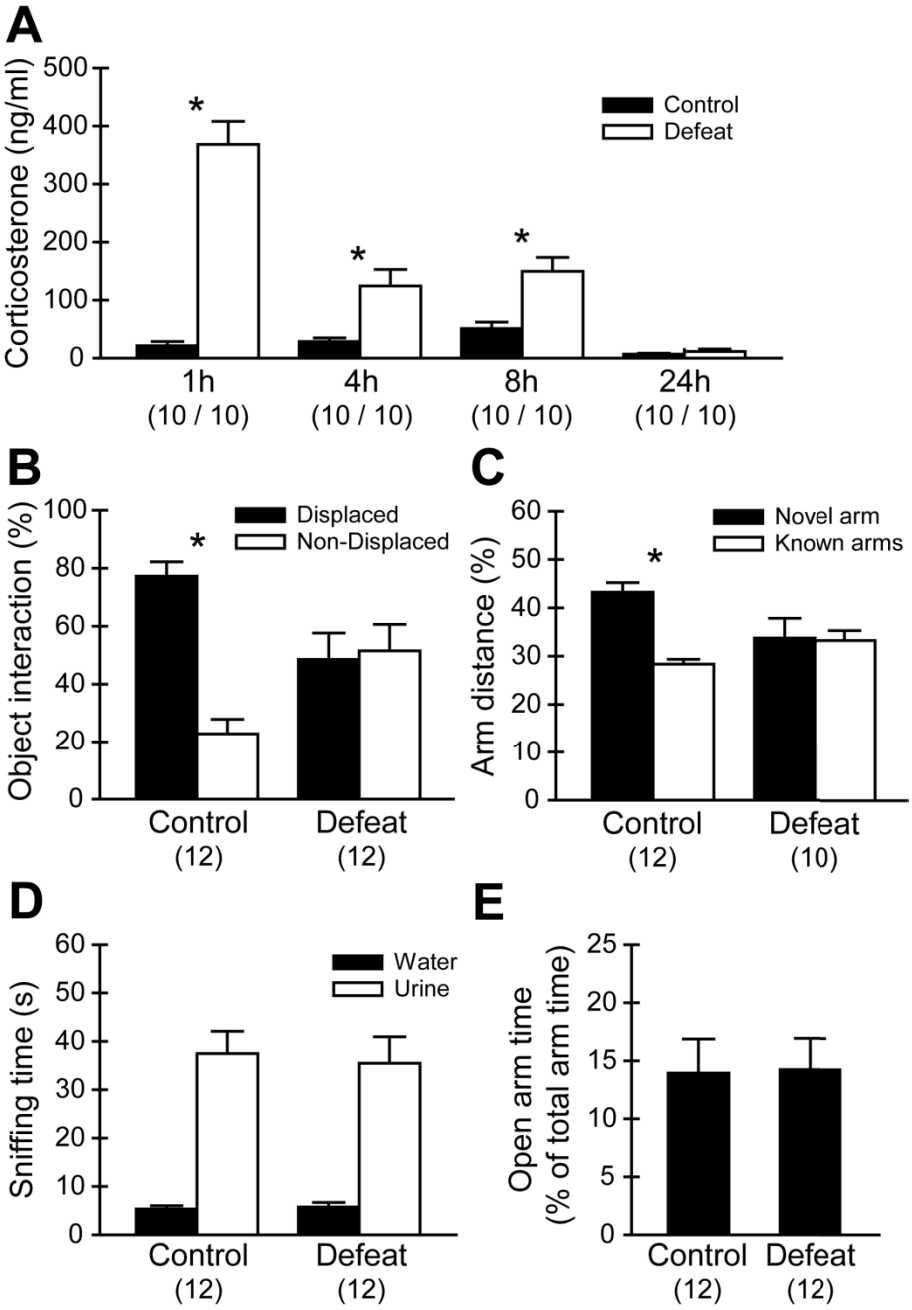

Figure 1: Neuroendocrine and behavioral effects of a single defeat session. (A) Defeated mice show significantly increased corticosterone levels up to 8 h after onset of the stressor. (B) In the spatial object recognition test, animals did not discriminate between the displaced and the nondisplaced object when stressed 8 h before. (C) In the Y-maze, we found the same memory impairment as when animals were defeated 8 h before. (D) Both control and defeated mice spent equal time sniffing in the female urine sniffing test 8 h after the defeat, indicating that there is no anhedonic behavior induced by a single defeat session. (E) In the elevated plus maze, defeated animals show the same anxiety-related phenotype as their control littermates when tested 8 h after the stress. * $p < 0.05$; data are expressed as mean ± SEM

A single defeat immediately reduces Homer1b/c levels in the dorsal hippocampus and decreases mGluR5/Homer1b/c coupling

Brains from the same animals that were killed to investigate corticosterone levels were prepared for *in situ* hybridization, and Homer1 mRNA levels were investigated in the dorsal and ventral hippocampi. We detected a reduction in Homer1b/c mRNA 4 h (T_{18} = 3.734, $p < 0.01$) and 8 h (T_{18} = 2.317, $p < 0.05$) after the stressor in the CA1 (Figure 2A, B) and DG region of the dorsal hippocampus (DG: 4 h: control 22.20 ± 1.39, defeat 16.98 ± 1.03, T_{18} = 3.019, $p < 0.01$; 8 h: control 17.47 ± 0.55, defeat 15.47 ± 0.72, T_{17} = 2.214, $p < 0.05$).

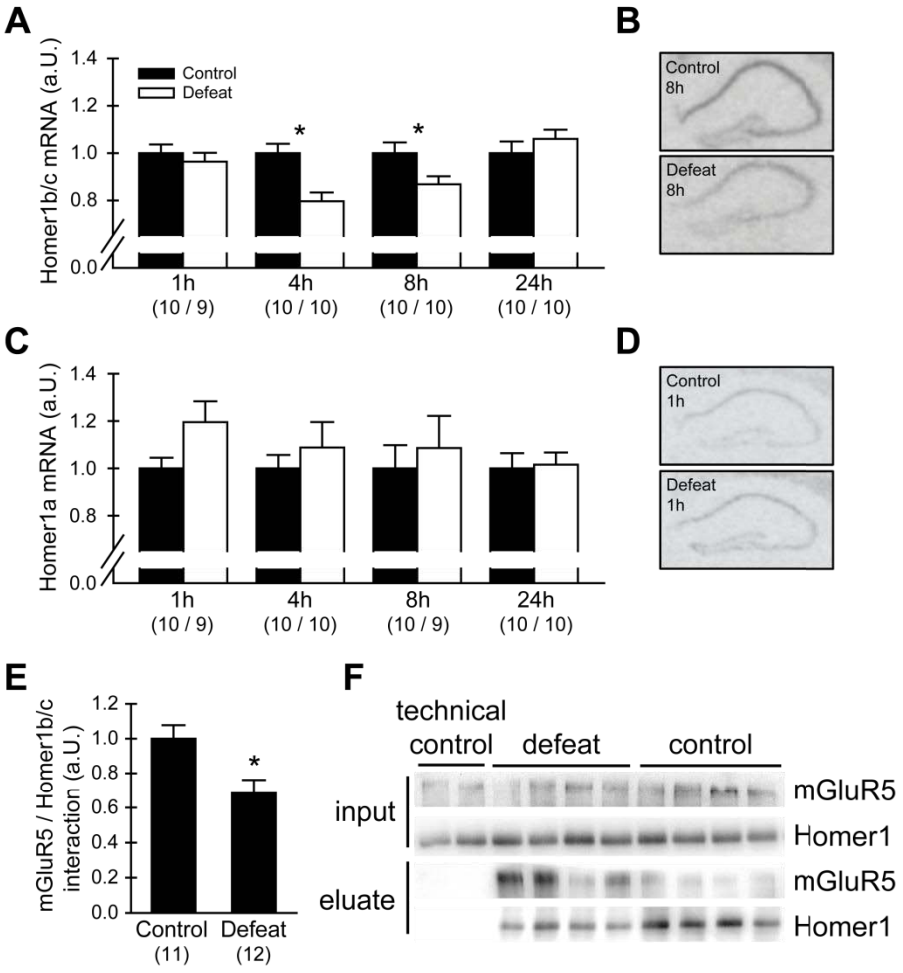

Figure 2: Homer1 mRNA level alterations in response to stress. (A) After 4 and 8 h, Homer1b/c mRNA levels in the CA1 region are reduced. This reduction normalizes after 24 h. (B) Representative autoradiographs of Homer1b/c mRNA levels in the hippocampus. (C) Homer1a levels are not significantly altered in response to a single social defeat. One hour after onset of the stressor, an increase in Homer1a mRNA failed to reach significance (p = 0.077). (D) Representative autoradiographs of Homer1a mRNA levels in the hippocampus. (E) Interactions of mGluR5 and Homer1b/c are decreased 8 h after defeat stress. (F) Representative western blot of the mGluR5/Homer1 immunoprecipitation. For the technical control, a pooled lysate was incubated without primary antibodies. * p < 0.05, data are expressed as mean ± SEM.

After 24 h, no significant effect was visible. Also, we did not find a change in mRNA levels in the ventral hippocampus, indicating a high specificity of this regulation. Additionally, we investigated short-form Homer1a mRNA levels in the CA1 region of the dorsal hippocampus after the same time points but did not see a significant regulation (Figure 2C, D). In a separate cohort of animals that were defeated and killed 8 h after the stressor, we extracted the highly enriched synaptosomal fraction of dorsal hippocampus tissue and measured Homer1b/c levels by western blot. Here, we found a significant decrease of Homer1 protein levels in the synapse when animals were defeated previously ($T_{13} = 2.436$, $p < 0.05$). Another cohort of animals was defeated, killed 8 h after the stressor, and dorsohippocampal membrane fractions were processed for CoIP. While total protein levels remained unchanged in response to stress, coupling of mGluR5/Homer1b/c was significantly reduced ($T_{21} = 2.867$, $p < 0.01$) (Figure 2E, F).

GR signaling is not sufficient to elicit memory impairments, while blockade of mGluR5 signaling reverses stress effects

To investigate whether activation of the GR in the hippocampus is already sufficient to induce changes in Homer1b/c levels that subsequently lead to memory impairments, we injected a batch of experimental animals with the GR agonist DEX to mimic the corticosterone response to a severe stressor. Next, we analyzed the expression profile of Homer1b/c in response to the DEX injection but did not find a regulation in any of the investigated time points (Figure 3A). Furthermore, a DEX injection was not able to induce spatial memory deficits 8 h after the administration (Figure 3B). In the Y-maze test, both vehicle- and DEX-injected animals were able to discriminate between the new and the known arms (Vehicle: $T_{20} = -10.266$, $p < 0.001$; DEX: $T_{20} = -5.178$, $p < 0.001$). Total locomotion was not affected by DEX treatment. We then investigated whether GR or mGluR5/Homer1 signaling during the sensation of the defeat stress is necessary to induce the memory deficits that are occurring 8 h after the stress. We injected either RU486, a specific GR antagonist, or the specific mGluR5 inverse agonist before the defeat and tested the learning behavior in a subsequent sOR test 8 h after the onset of the stressor. Blockade of GR signaling by RU486 was not able to prevent the stress-induced memory impairments in the sOR task (Figure 3C). Here, both unstressed control groups discriminated between the two objects (Vehicle: $T_{16} = 8.747$, $p < 0.001$;

RU486: $T_{16} = 2.796$, $p < 0.05$), while the defeated animals did not learn the novel position of the object regardless of treatment (Vehicle: $T_{14} = -0.067$, $p = 0.948$; RU486: $T_{14} = -0.699$, $p = 0.495$). Total locomotion and total interaction time with both objects did not differ between the experimental groups.

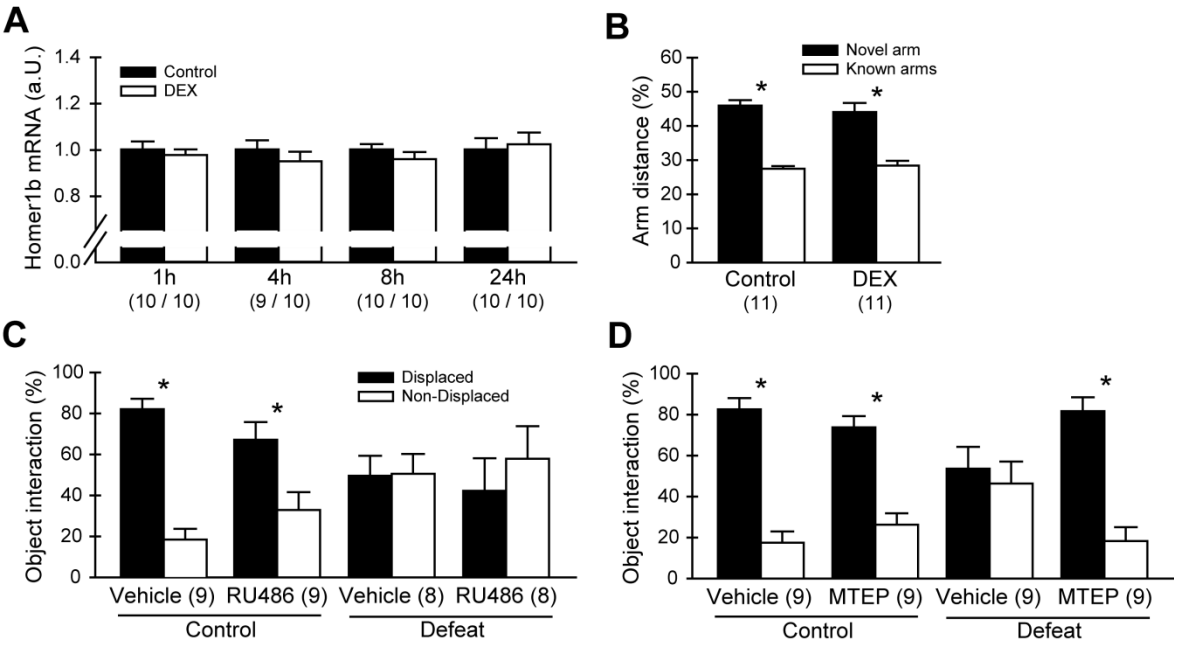

Figure 3: Manipulation of mGluR5/Homer1 but not glucocorticoid receptor signaling rescues cognitive impairments. (A) A single injection of DEX (Dexamethasone) that mimics a corticosterone response to a stressor was not able to induce changes in Homer1b/c mRNA levels. (B) Eight hours after a single injection of DEX, experimental mice are not impaired in the Y-maze memory performance task. (C) Mice that received a RU486 injection before the stressor are still affected by the defeat stress and cannot discriminate between the displaced and the nondisplaced object. (D) Mice that received a MTEP injection before the stressor show functional learning behavior independent of the condition. * $p < 0.05$, data are expressed as mean ± SEM.

When animals were administered with MTEP treatment before the defeat, their learning behavior normalized in a subsequent test 8 h after the onset of the stressor (Figure 3D). In the sOR, both control groups showed normal learning behavior with a significant preference for the displaced object (Vehicle: $T_{16} = 8.367$, $p < 0.001$; MTEP: $T_{16} = 6.065$, $p < 0.001$). In the defeated group, only the MTEP-treated animals showed a learning phenotype (Vehicle: $T_{16} = 0.479$, $p = 0.638$; MTEP: $T_{16} = 6.600$, $p < 0.001$). We also replicated this pharmacologically induced rescue of the behavioral phenotype in the Y-maze test (Control vehicle: novel arm 40.90% ± 3.95, known arms 29.55% ± 1.97, $T_{22} = 2.570$, $p < 0.05$; control MTEP: novel arm 39.75% ± 2.44, known arms 30.12% ± 1.22,

T_{22} = 3.519, p < 0.01; defeat vehicle: novel arm 37.59% ± 4.39, known arms 31.21% ± 2.19, T_{22} = 1.298, p = 0.208; defeat MTEP: novel arm 42.86% ± 4.51, known arms 28.57% ± 2.26, T_{22} = 2.832, p < 0.01). In both experiments we did not find significant differences in locomotion or total object interaction time between any of the groups.

Overexpression of Homer1b/c rescues spatial memory impairments induced by defeat stress

We examined whether we could rescue the stress-induced cognitive decline, which correlated with reduced Homer1 levels in the dorsal hippocampus, by overexpressing Homer1b/c in the dorsal hippocampus by AAV injection. We achieved a stable overexpression in the CA1 and DG regions of the dorsal hippocampus that was quantified by *in situ* hybridization (Figure 4A, B). Viral infection spread (Figure 4C) and verification of the infection sites were additionally controlled by immunofluorescence (Figure 4D). Animals were tested in the sOR test, and while defeated animals infected with a control virus showed cognitive impairments (T_{18} = 0.802, p = 0.433), animals that were overexpressing Homer1b/c were able to discriminate the objects' locations (T_{18} = -4.215, p < 0.001) (Figure 4E). Both control groups were able to discriminate between the displaced and nondisplaced objects (Empty: T_{18} = -5.381, p < 0.001; Homer1 overexpression: T_{18} = -5.411, p < 0.001). Again, no locomotion or total object interaction differences were present when comparing the experimental groups.

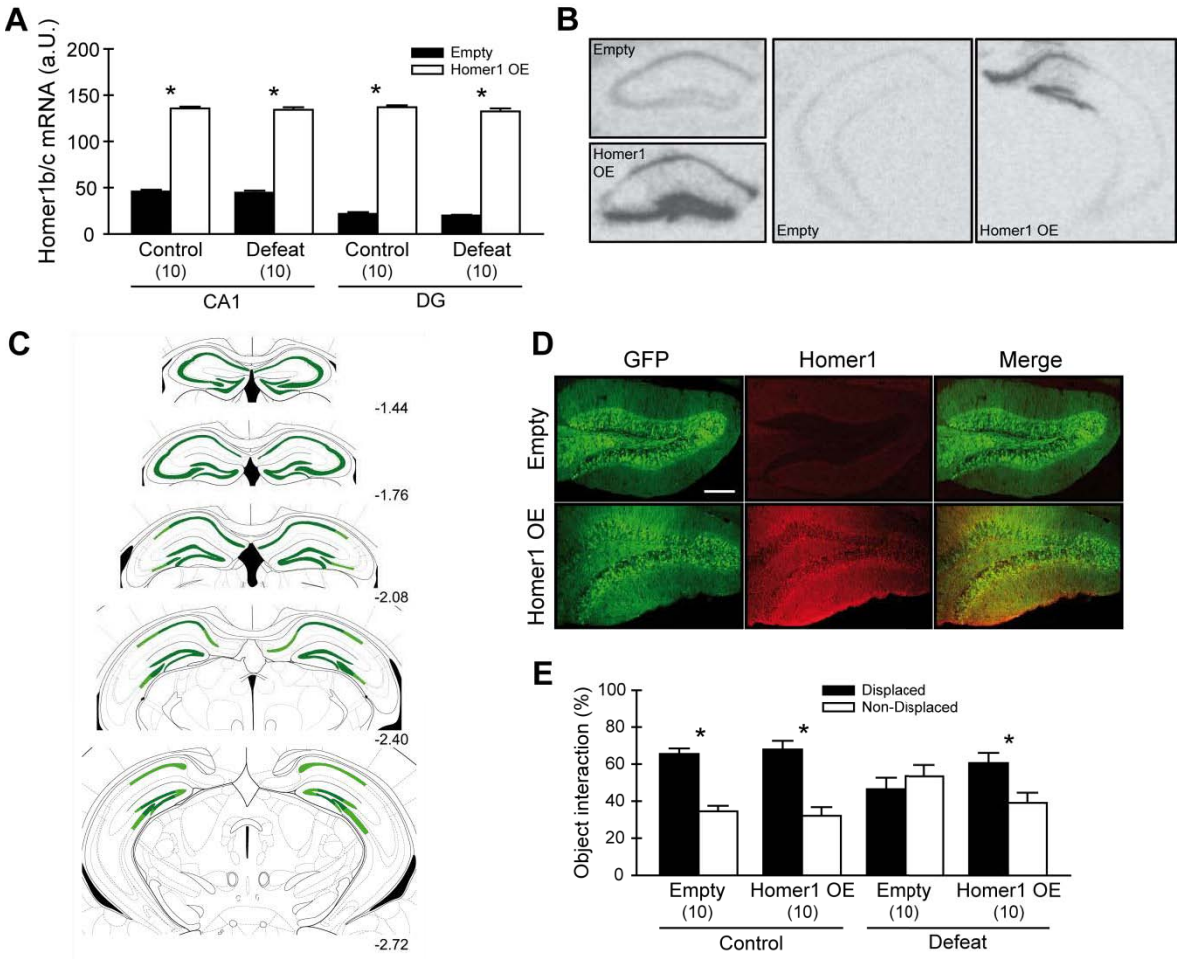

Figure 4: Overexpression of Homer1b/c in the dorsal hippocampus rescues memory impairments induced by defeat stress. (A) Homer1b/c mRNA levels in the dorsal hippocampus. Infection with the viral construct induced a robust increase. (B) Representative autoradiographs of Homer1b/c mRNA levels in the dorsal and ventral hippocampus of control and Homer1 OE animals. (C) Schematic representation of the extent of viral infection in the hippocampus from -1.44 to -2.72 mm posterior to bregma (Dark green: Strongest overexpression; light green: weaker overexpression). (D) Visualization of Homer1b/c expression in the hippocampal DG region 5 weeks after injection of control (Top panels) or Homer1b/c-expressing virus (Bottom panels) (Scale, 200 µm). (E) Overexpression of Homer1b/c in the dorsal hippocampus prevents learning deficits induced by acute defeat stress. * $p < 0.05$, data are expressed as mean ± SEM.

Discussion

In our study, we show that a single social defeat leads to temporally specific cognitive impairment after a recovery of 8 h. In parallel, hippocampal Homer1b/c levels mRNA and protein levels, as well as mGluR5/Homer1b/c interaction, are decreased in response to the stressor. To further investigate the relationship between the behavioral and molecular phenotype, we first blocked mGluR5 signaling by applying MTEP, a specific inverse agonist of mGluR5, before the defeat stress and could thereby rescue the stress-induced phenotype. Furthermore, a specific Homer1b/c overexpression in the dorsal hippocampus by viral transfection was also able to reverse the stress-induced effects on cognition. These results give substantial evidence that postsynaptic signaling via mGluR5 and Homer1b/c is strongly involved in mediating cognitive deficits induced by acute stress.

The impact of stress on cognition and memory formation is well described but strongly dependent on the chosen experimental setup (Schwabe et al., 2010). An acute stress session has been reported to interfere with the induction of long-term potentiation (LTP), but it facilitates long-term depression (LTD) (Joëls and Krugers, 2007). In animal models, acute stressors disrupt the performance in memory tasks when the test is immediately after the sensation of stress (Howland and Cazakoff, 2010), most likely due to elevated GCs during memory encoding (Sandi, 2011). An acute stress session can also induce long-term effects such as an increase in anxiety-related behavior that develops 7 days after the stress session (Kinn Rød et al., 2012). In this study, we were able to induce a specific cognitive deficit by social defeat after an intermediate time of recovery (8 h) without interfering with other behavioral traits such as anxiety or locomotion. A possible confounding factor in this behavioral setup is neophobia, which can be induced by stress as well (Bats et al., 2001). However, all parameters that we measured for control, such as the locomotion in the behavioral tests as well the interaction times with the objects to observe, do not indicate a possible corruption of the data. Furthermore, both the FUST and the EPM test did not show significant effects in anxiety-related behavior and interaction with novel stimuli.

In our molecular analysis, we show that Homer1b/c levels are reduced in response to stress in a time window that coincides with the observed memory deficits. Various studies have identified Homer1 to be involved in memory processes in the prefrontal cortex

(Lominac et al., 2005; Jaubert et al., 2007). Furthermore, Homer1 has recently been associated with stress-induced behavioral changes in the context of fear conditioning (Tronson et al., 2010). There, the intrinsic activity of mGluR5 and its subsequent signaling via Homer1 multimers in the hippocampus have been shown to mediate the enhancing effects of stress on contextual fear. We now confirm that acute stress has a profound effect on metabotropic glutamate signaling pathways in the hippocampus. This is demonstrated by a significant reduction in both Homer1b/c mRNA levels as well as in decreased Homer1b/c protein levels in the PSD and Homer1/mGluR5 interactions 8 h after the defeat stress.

We did not observe a significant induction of Homer1a, an immediate early gene triggered by synaptic activity (Bottai et al., 2002), which is in contrast to previous studies that report increased Homer1a mRNA in response to various stimuli (Sala et al., 2003; Hu et al., 2010; Mahan et al., 2012). Possibly, the induction of Homer1a due to the defeat stress is already diminishing at the 1 h time point, and early time points of investigation might have shown a significant increase in Homer1a mRNA levels. Previous studies have already reported a significant role of Homer1 in memory processes, such as memory impairments of Homer1 knockout mice (Jaubert et al., 2007) that could be rescued by Homer1c overexpression in the hippocampus of these animals (Gerstein et al., 2012). Homer1a, in turn, has been shown to be essentially involved in the processing of fear (Brouillette et al., 2007) and working memory (Celikel et al., 2007). Our findings are contributing further to understanding the role of Homer1 in memory formation, suggesting that Homer1b/c signaling is immediately altered by stress and subsequently changes hippocampal memory processing.

Interestingly, the observed behavioral and molecular effects do not seem to be dependent on GR signaling. This is on the one hand shown by the absence of memory impairments as well as changes in Homer1b/c mRNA levels when the synthetic GR agonist DEX is administered to the animals instead of exposing them to a defeat stress. On the other hand, antagonizing GR by RU486 before stress was not able to recover the memory deficits. Corticosterone has frequently been shown to both positively and negatively alter memory formation, depending on the timing of corticosterone release and learning (Sandi, 2011; Popoli et al., 2012). A very recent study elucidated on the role of GR signaling in the dorsal and ventral hippocampi in memory retrieval, showing that stress

impairs hippocampus-dependent memory up to 105 min after the stressor (Dorey et al., 2012). The present results further complement these findings, suggesting that it is not GR signaling but mGluR5/Homer1-mediated processes that impair memory formation in a more intermediate time window.

Inverse agonists of mGluR5 such as MPEP and MTEP have received growing attention for their potential in the treatment of neurologic disorders and were extensively tested in various behavioral parameters (Simonyi et al., 2010). With regard to learning paradigms, an immediate treatment before the test predominantly led to cognitive impairments (Steckler et al., 2005). When MTEP was applied before the stressor, we could show that stress-induced learning deficits were abolished. Anderson et al. (2003) could convincingly show that MTEP is rapidly metabolized in mice, suggesting that 8 h postadministration, mGluR5 blockade by residual MTEP is unlikely and therefore does not interfere with the behavioral tests performed. Given the anxiolytic and antidepressant-like properties that have been reported for mGluR5 antagonists (Palucha and Pilc, 2007), one can speculate that blockade of the mGluR5 during and directly after the sensation of stress severely alters the perception and processing of the stressor and therefore its long-term consequences. The concept that environmental stimuli can trigger changes in the ability of synapses to express plasticity via intra-cellular signaling cascades has been coined “metaplasticity” (Abraham, 2008). It has previously been shown that prior activation of group I mGluRs can induce metaplasticity, thereby altering the ability of neurons in the CA1 or DG region of the hippocampus to express LTP or LTD (Rush et al., 2002; Gisabella et al., 2003). Our current data provide further evidence for this concept, showing that stress perception and subsequent molecular signaling pathways, in this case driven by mGluR5/Homer1 interactions, are critical for the future capacity of an individual to retain novel spatial information. This is particularly intriguing, since acute stress has been convincingly shown to enhance fear memory via mGluR5 signaling, with Homer1a having been shown to be necessary for the augmenting effects of stress on fear memory (Inoue et al., 2009; Tronson et al., 2010). Previous evidence also suggests that stress impairs spatial memory, which is in line with the results presented here (Sandi, 2011). The reduction of Homer1b/c in response to acute defeat stress, accompanied by increased dissociation from mGluR5, indicates that this pathway exerts detrimental effects on

spatial memory performance, possibly shifting the hippocampal memory formation system towards a more sensitive fear response in an intermediate time frame.

Taken together, our study introduces the mGluR5/Homer1b/c signaling pathway as a major mediator of stress-induced spatial memory deficits. We also provide evidence that mGluR5 signaling plays a critical role during the perception of stress and most likely induces metaplasticity changes that lead to an altered Homer1b/c signaling. These findings underline the importance of glutamatergic pathways in the development of psychopathologies that are related to stress.

2.4. Hippocampal Homer1 levels influence motivational behavior in an operant conditioning task

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Manuscript submitted

Abstract

Loss of motivation and learning impairments are commonly accepted core symptoms of psychiatric disorders such as depression and schizophrenia. Reward-motivated learning is dependent on the hippocampal formation but the molecular mechanisms that lead to functional incentive motivation in this brain region are still largely unknown. Recent evidence implicates neurotransmission via metabotropic glutamate receptors and Homer1, their interaction partner in the postsynaptic density, in drug addiction and motivational learning. As previous reports mainly focused on the prefrontal cortex and the nucleus accumbens, we now investigated the role of hippocampal Homer1 in operant reward learning in the present study. We therefore tested either Homer1 knockout mice or mice that overexpress Homer1 in the hippocampus in an operant conditioning paradigm. Our results show that deletion of Homer1 leads to a diverging phenotype that either displays apparent inability to perform the task or outstanding hyperactivity in both learning and motivational sessions. On the other hand, overexpression of hippocampal Homer1 led to reduced motivation in a progressive ratio task. This phenotype is possibly based on a decreased learning performance that was apparent during the training stage of the testing paradigm. Our results highlight the importance of Homer1-mediated signaling in the hippocampus in motivation-based learning tasks and encourage further investigations regarding the specific molecular underpinnings of the phenotype observed in this study that may ultimately lead to improved treatment options for patients suffering from psychiatric disorders.

Introduction

Memory deficits and motivational impairments are frequently reported to be associated with the emergence of psychiatric pathologies such as depression (Burt et al., 1995; Fairhall et al., 2010) and schizophrenia (Lieberman et al., 2001). Motivational behavior has mainly been associated with amygdaloid structures (Lang and Bradley, 2010), as well as the medial prefrontal cortex (Murray and Wise, 2010) and the nucleus accumbens (Ishikawa et al., 2008). On the other hand, compelling evidence implicates the hippocampus as a major structure of memory disturbances (Squire, 1992; Morgado-Bernal, 2011) and reward-motivated learning (Adcock et al., 2006; Delgado and Dickerson, 2012). As the hippocampal formation is structurally and functionally connected with the amygdala, the prefrontal cortex, and the nucleus accumbens, it can serve as an integrating structure for motivational and memory processes.

In this structural framework, glutamatergic neurotransmission has been shown to be centrally involved in memory formation (Popoli et al., 2012) and reward-seeking behavior, including drug addiction (Vanderschuren and Kalivas, 2000; Baker et al., 2003; McFarland et al., 2003). Specifically group I metabotropic glutamate receptors (mGluRs) have been shown to interact with scaffolding proteins from the Homer family, which are expressed in the postsynaptic density of glutamatergic neurons. Homer1 has been demonstrated to link group I mGluRs to downstream targets such as inositol triphosphate receptors (Brakeman et al., 1997; Tu et al., 1998), TRP cation channels (Yuan et al., 2003), and ryanodine receptors (Feng et al., 2002). Constitutively expressed Homer1b/c multimers have been shown to mediate ligand-dependent signaling (Ronesi and Huber, 2008), while the shorter splice variant Homer1a, an immediate early gene (IEG) that is induced by neuronal activation (Xiao et al., 1998), can induce ligand-independent signaling and is thought to act as a dominant negative to the constitutively expressed isoform (Ango et al., 2001).

A number of clinical and preclinical reports have implicated Homer1 in the pathophysiology of depression (Rietschel et al., 2010), schizophrenia (Norton et al., 2003; Szumlinski et al., 2005) and addiction (Szumlinski et al., 2004; Uys and LaLumiere, 2008). In rodent studies, the Homer1/mGluR5 signaling pathway has previously been shown to be involved in memory formation and cognition in the prefrontal cortex (Lominac et al., 2005) and the hippocampus (Jaubert et al., 2007; Gerstein et al., 2012). Furthermore,

mGluR5/Homer1 interactions have been shown to mediate stress-induced alterations in memory formation of both fear conditioning (Tronson et al., 2010) and spatial information (Wagner et al., 2013) in mice. However, the role of hippocampal Homer1/mGluR5 in operant reward learning and motivation, which are central aspects for mood disorders, is still largely unclear.

In the current study, we therefore aimed to further elucidate the role of hippocampal Homer1 in operant reward learning by testing Homer1 knockout mice as well as mice that overexpress the constitutively expressed Homer1b/c isoform in the hippocampus in an operant conditioning paradigm. We hypothesized that a deletion of Homer1 leads to a reduction of incentive motivation, while overexpression of Homer1b/c in the hippocampus should improve memory formation and thereby may help to improve the performance in the operant conditioning task.

Materials and Methods

Animals

Conventional Homer1KO mice were bred from heterozygous breeding pairs on a C57BL/6N background in the animal facilities of the Max Planck Institute of Psychiatry in Munich, Germany. Generation and genotyping of Homer1KO mice was reported previously (Yuan et al., 2003). Homer1 knockout resulted in complete loss of protein expression and was verified by PCR. For the Homer1b/c overexpression experiment, male C57BL/6N mice (Charles River Laboratories, Maastricht, the Netherlands) at the age of 10 weeks were used. All mice were held under standard conditions (12L:12D light cycle, lights on at 08:00 AM, temperature 23 ± 2 °C) and were single housed and acclimated to the experimental room for 2 weeks before the beginning of the experiments. Tap water was available *ad libitum* during the whole experiment. Food (Altromin 1324, Altromin GmbH, Germany) was available *ad libitum* until start of the food restriction period. All experiments were performed in the animal facilities of the Max Planck Institute of Psychiatry in Munich, Germany. The experiments were carried out in accordance with the European Communities' Council Directive 2010/63/EU. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany.

Experimental design

Experiment 1

Adult male Homer1 knockout (KO) mice or wild type (WT) littermate controls (12 - 14 weeks of age, n = 9 - 10 per group) were tested in the operant conditioning paradigm.

Experiment 2

Adult male C57Bl/6N mice received intra-hippocampal injections of a Homer1b/c overexpression vector (n = 10) or an empty control virus (n = 10) and were tested in the operant conditioning paradigm 4 weeks later.

Operant conditioning

Average daily food intake was measured for 5 days and food was subsequently restricted to 75% of this daily intake to promote incentive motivation in the operant conditioning task (Szumlinski et al., 2005). This mild caloric restriction has been shown to have no negative consequences for the physiological wellbeing of the animals. After one week of food restriction, animals were introduced to the operant conditioning chamber (Bioseb, France) for 5 days. In each 30 min trial, mice received a sucrose reward (Bio-serv, NJ, USA) every 45 s, which was always paired with a 3 s light and sound (5000 Hz) stimulus. Reward delivery and stimuli were operated with commercially available software (Packwin V2.0.01; Panlab, Spain).

The training stage consisted of a fixed ratio/variable ratio (FR/VR) protocol, in which the experimental animals received a reward after a single lever press for the first ten presses (FR1) followed by 1 - 3 lever presses to receive a reward (VR1-3). The 30 min training trial was performed in bouts of 5 consecutive daily trials per week, until 75% of mice in the respective control group (WT or Empty) received at least 10 rewards. In the first experiment, this was the case after 15 training trials. Here, 2 WT and 5 KO animals did not pass the cut-off criterion and were not tested in the progressive ratio task. In the second experiment, 10 training trials were performed. 2 Empty and 4 Homer1b/c OE animals did not pass the cut-off criterion and were excluded from subsequent testing.

Mice that passed the training stage were tested in a progressive ratio (PR) task for 120 min to test the animals for motivation in the previously acquired operant conditioning task. The reward progression for the PR task is outlined in supplemental table 1.

The whole time course of the experiment, including surgery and recovery is shown in supplemental figure 1. All operant conditioning trials were performed between 08:00 AM and 12:00 PM. After experiment 2, all animals were deeply anesthetized with ketamine/rompun and perfused intracardially with 4% paraformaldehyde. Brains were removed, postfixed overnight in 4% paraformaldehyde following overnight incubation in 30% sucrose solution at 4 °C, and then stored at −80 °C until further processing for immunohistochemistry as described below.

Viral overexpression of Homer1

Viral overexpression of Homer1b/c was performed as described previously (Schmidt et al., 2011a). We used an adeno-associated bicistronic AAV1/2 vector (GeneDetect, New Zealand) containing the CAG-Homer1-IRES-EGFP-WPRE-BGH-polyA expression cassette (containing coding sequence of Homer1 NCBI CCDS ID CCDS36745). For the control group, we used the same vector construct expressing only EGFP. Virus production, amplification, and purification were performed by Genedetect. Mice were anesthetized with isoflurane, and 0.5 µl of either AAV-Homer1 or AAV-EGFP (Titres: 1.2×10^{12} genomic particles/ml) were bilaterally injected in the dorsal hippocampus at 0.06 µl/min by glass capillaries with tip resistance of 2 - 4 MΩ in a stereotactic apparatus. The following coordinates were used: 1.9 mm posterior to bregma, 1.3 mm lateral from midline, and 1.3/1.8 mm below the surface of the skull, targeting the CA1 and dentate gyrus (DG) region of the dorsal hippocampus. After surgery, mice were treated for 5 days with Metacam via drinking water. The habituation phase of the operant conditioning paradigm started 4 weeks after virus injection. Quantification and verification of Homer1b/c overexpression were confirmed by *in situ* hybridization and immunofluorescence as described previously (Wagner et al., 2013). Animals that were not infected bilaterally in both the CA1 and DG region were excluded from the analysis (n = 1). One mouse (Empty group) died in the recovery phase after the surgery, before the experiment started.

In Situ *hybridization and immunohistochemistry*

For *in situ* hybridization, frozen brains were coronally sectioned in a cryostat microtome at 18 µm and kept at −80 °C. *In situ* hybridization using a ^{35}S UTP-labeled ribonucleotide probe for Homer1b/c (Forward primer: AACACTGGGAGGCTGAGCTA; Reverse primer: TACTGCGGAAAGCCTCTTGT) was performed as described previously (Schmidt et al., 2007). For fluorescence immunohistochemistry, serial coronal sections were cut at 30 µm thickness. Double-labeling immunofluorescence (Rabbit anti-Homer1, 1:1000, Synaptic Systems; goat anti-GFP, 1:500, Abcam) was performed on free-floating sections (n = 3 per mouse) as described previously (Wang et al., 2011a).

Statistical analysis

The data presented are shown as means ± standard error of the mean, analyzed by the commercially available software SPSS 16.0. Repeated measures ANOVA with time as within-subjects factor and genotype/AAV type as between-subjects factor or Chi Square analysis were used for body weight, habituation and training stage analysis. For the progressive ratio session, data were analyzed with student's *t*-test for normally distributed data. If the data was not normally distributed according to Shapiro-Wilk-test, the nonparametric Mann-Whitney-U (MWU) test was applied. Correlations between lever presses and locomotion were analyzed with the Pearson product-moment test. A nominal level of significance $P < 0.05$ was accepted.

Results

Experiment 1

Homer1KO mice displayed a significantly reduced body weight already at the beginning of the experiment (WT: 27.76 ± 0.62 g, KO: 23.55 ± 0.85 g; $p < 0.001$). Over the course of the operant conditioning paradigm, food restriction resulted in a body weight loss in both groups, independent of the genotype of the animals (Time effect: $F_{2.072} = 11.365$, $p < 0.001$) (Supplemental table 2). Also, the reduced body weight of Homer1KO mice compared to their WT littermates was present during the whole experimental period ($F_{1,10} = 21.312$; $p < 0.001$).

In the habituation phase, repeated measures ANOVA revealed a time effect ($F_{2.591} = 3.190$; $p < 0.05$) but no time × genotype interaction in the number of consumed rewards, indicating that all animals showed increased interest in the sucrose pellets over time (Figure 1A). Yet, it has to be noted that 6 out of 9 Homer1KO mice did not consume any reward in the fifth habituation trial. The same animals did not express interest in the reward in previous habituation trials, while WT mice displayed a normally distributed interest in the reward. This genotype difference becomes significant over several trials when analyzed by a Chi Square test (Trial 1: $p = 0.667$, Trial 2: $p = 0.055$, Trials 3 to 5: $p < 0.05$).

Over the course of the FR/VR training period, WT animals displayed a normal learning behavior with a stable lever press response after 15 training trials (Figure 1B). The mean of Homer1KO mice lever press responses also increased steadily, which is reflected in a significant repeated measures ANOVA main time effect ($F_{2.175} = 5.340$; $p < 0.01$) without significant time × genotype interaction. A more detailed analysis of the Homer1KO dataset revealed that 5 of 9 subjects pressed the lever less than 5 times and did not consume any presented reward in the majority of the training trials. On the other hand, those animals that already showed high interest in the reward during habituation also performed above average in the training stages, thereby largely increasing the variance in the Homer1KO group. After training trial 15, 8 of 10 WT mice passed the cut-off criterion of 10 lever presses, while only 4 of 9 KO animals received more than 10 rewards (Figure 1C). After exclusion of all non-performing mice, Homer1KO animals performed significantly better than their WT littermates (MWU: $Z = -2.378$; $p < 0.05$).

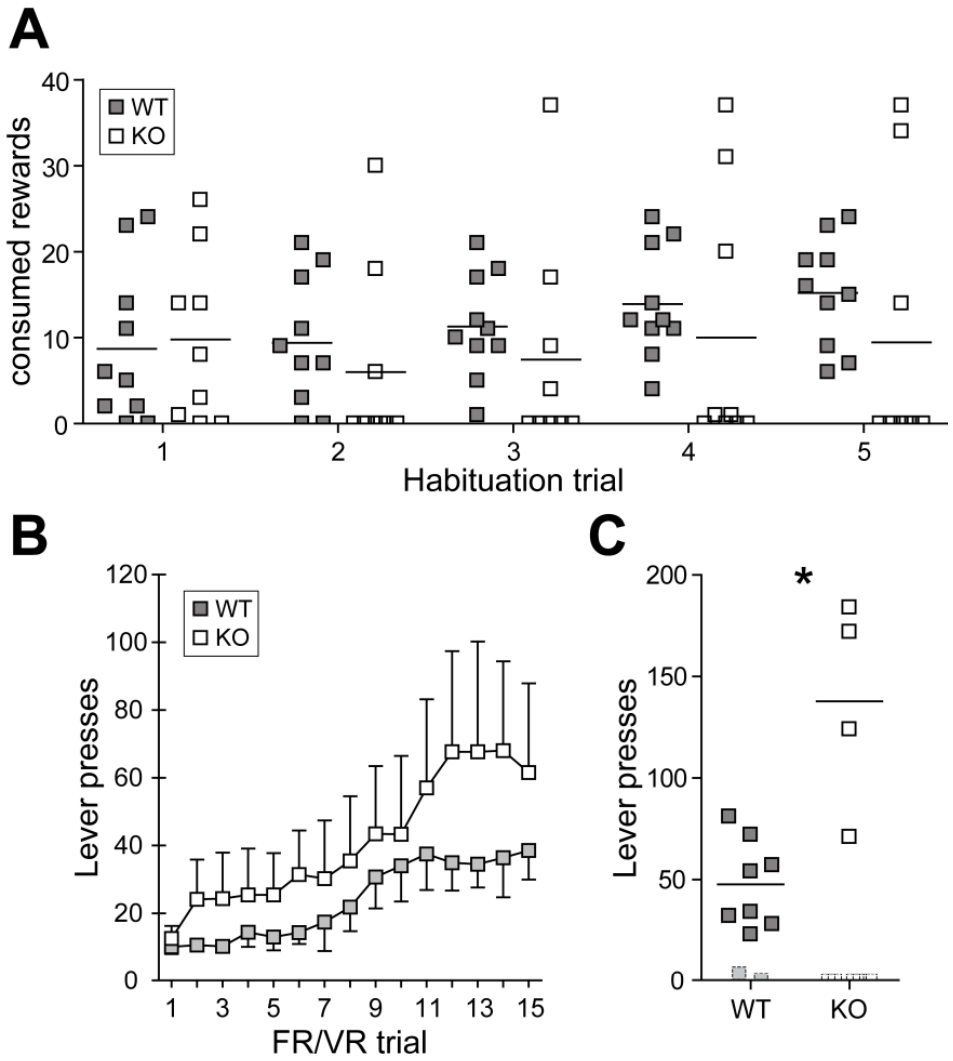


Figure 1: Training performance of Homer1KO mice. (A) In 5 habituation trials, all wild type (WT) mice show growing interest in the presented reward. Most of the Homer1KO mice, however, do not consume the sucrose pellets. (B) The learning curve in the fixed ratio/variable ratio (FR/VR) stage is slightly, but not significantly higher in Homer1KO mice compared to WT animals. This is due to the above-average performance of a subset of Homer1KO mice that already showed a response to the reward in the habituation phase, while the greater part of the Homer1KO animals show a below-average performance, thereby largely increasing the variance of the sample. (C) FR/VR results of training trial 15. Mice that were excluded from subsequent progressive ratio testing are indicated as dotted datapoints and were not taken into account for the analysis of significance in this graph. The performing Homer1KO animals press the lever more often than their WT littermates, indicating higher motivation to receive food rewards. * Significant from WT, $p < 0.05$.

In the PR task, the remaining Homer1KO animals showed a significantly increased lever press activity compared to the WT group (MWU: $Z = -2.378$; $p < 0.05$) (Figure 2A) without significantly affecting total locomotion during the task (Figure 2B). Additionally, locomotion was not correlated to the lever presses (WT: $p = 0.540$; KO: $p = 0.369$). The individual difference of the high-performing animals becomes apparent when taking a

closer look on the lever press profile over the course of the 120 min PR session (Figure 2C). Here, WT animals show a reduced motivation to press the lever once rewards are more difficult to obtain. Homer1KO animals, in contrast, keep active until the end of the testing period, thereby receiving more rewards than their WT littermates.

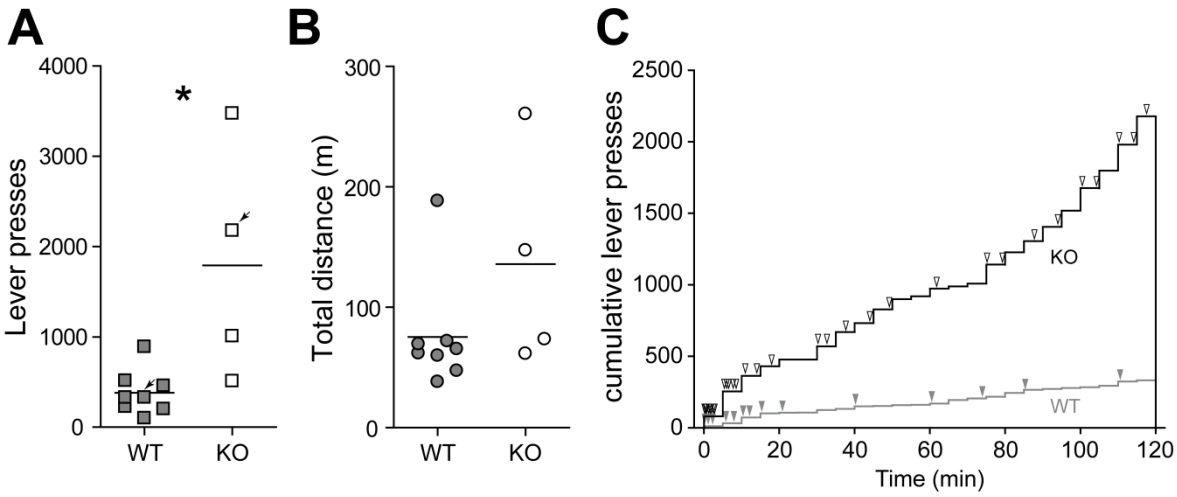


Figure 2: Progressive ratio (PR) performance of Homer1KO mice. (A) Homer1KO mice that passed the pretesting cut-off criterion show a significant increase in motivational behavior displayed by higher lever press numbers. The arrows indicate the datapoints that are plotted in panel c. (B) Locomotion in the PR task. There was no significant effect of total distance traveled between the two groups. (C) Representative cumulative distribution of PR lever presses. Dispensed rewards are marked as triangles. The Homer1KO mouse (Black line) shows high performance over the course of 120 min, thereby receiving constant rewards. In contrast, the wild type mouse (Gray line) shows less operant responses once rewards are obtained more and more slowly. * Significant from WT, $p < 0.05$.

Experiment 2

Successful targeting (Figure 3A) and overexpression of Homer1b/c was validated by immunohistochemistry (Figure 3B) and quantified by means of *in situ* hybridization (Figure 3C). Both in the dorsal (dHC) and the ventral hippocampus (vHC), we detected a significant increase in Homer1b/c mRNA levels in CA1 (dHC: $T_{16} = -22.728$, $p < 0.001$; vHC: $T_{16} = -48.992$, $p < 0.001$) and DG regions (dHC: $T_{16} = -25.885$, $p < 0.001$; vHC: $T_{16} = -101.802$, $p < 0.001$). Viral spread was analogous to our previous study with this viral construct (Wagner et al., 2013).

While overexpression of Homer1b/c did not lead to a significant change in body weight during the experiment, food restriction led to a body weight reduction in both groups over time (Time effect: $F_{1.326} = 21.312$; $p < 0.001$) (Supplemental table 3).

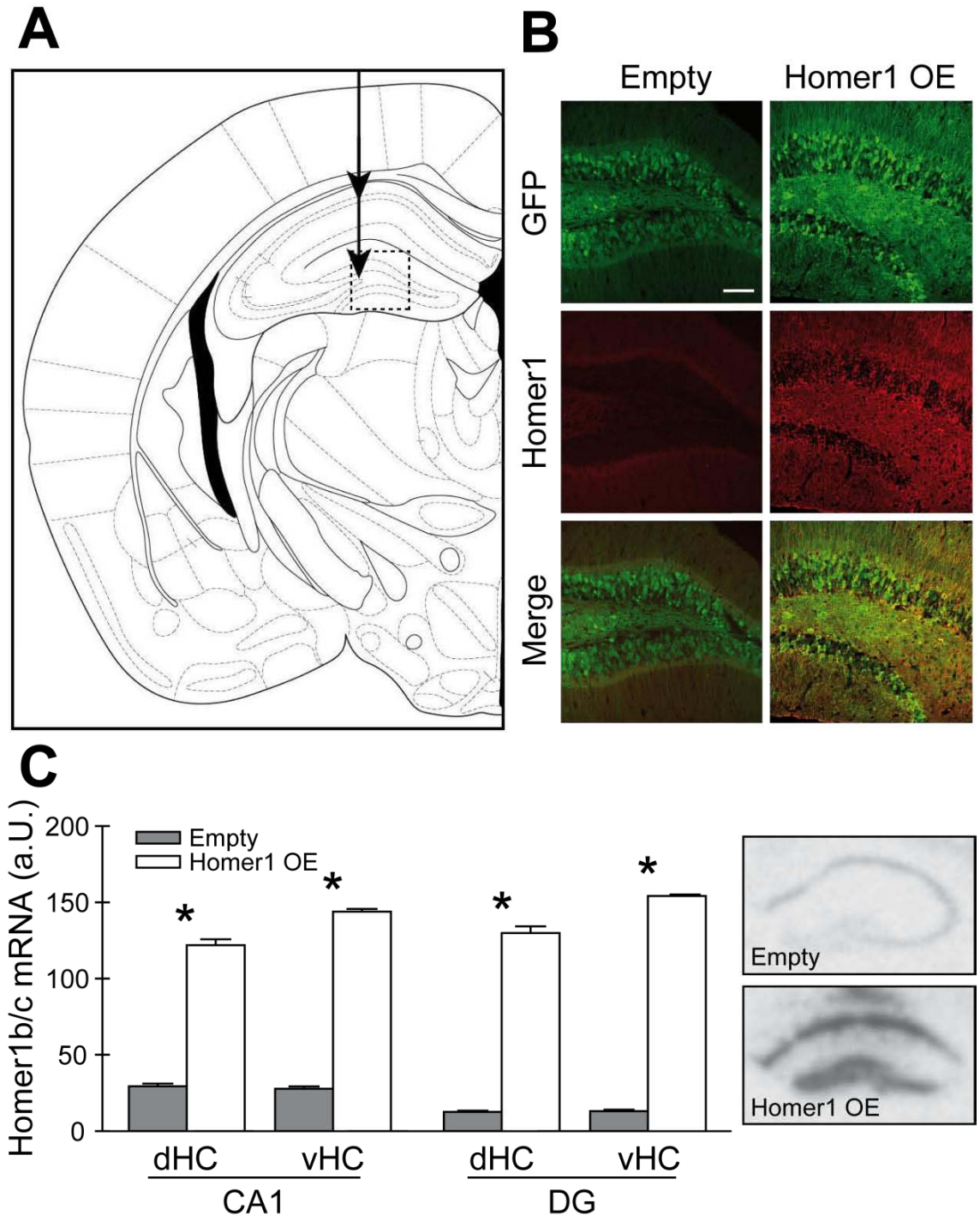


Figure 3: Confirmation and quantification of viral overexpression. (A) Schematic of the injection site of the virus in the CA1 and the dentate gyrus (DG) of the dorsal hippocampus. The dotted square indicates the approximate area of visualization in Panel b. (B) Visualization of Homer1b/c expression in the hippocampal DG region 8 weeks after injection of control (Left panels) or Homer1b/c-expressing virus (Right panels) (Scale: 100 µm). (C) Homer1b/c mRNA levels in the hippocampus. Infection with the viral construct induced a robust increase in both CA1 and DG mRNA levels in the dorsal (dHC) and ventral (vHC) part of the hippocampus. Pictures show representative autoradiographs of Homer1b/c mRNA levels in the dorsal hippocampus of Empty and Homer1 OE animals. * Significant from Empty virus, $p < 0.05$.

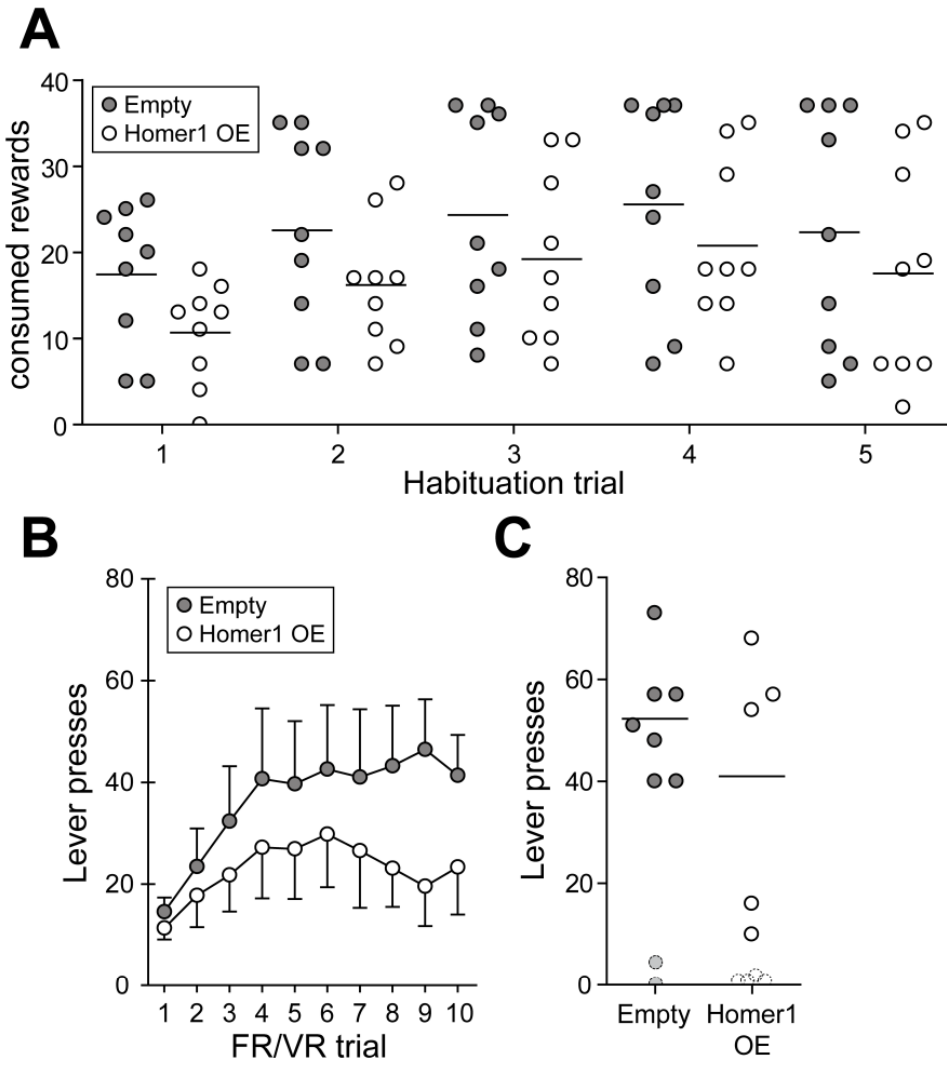


Figure 4: Training performance of Homer1 OE mice. (A) During habituation, both groups quickly recognized and consumed the presented rewards. No differences between the treatments were detected. (B) During the training stage, both Empty and Homer1 OE animals learned to associate lever presses with the reception of a reward. Although Homer1 OE mice appear to show less motivational behavior, repeated measures ANOVA did not reveal a significant time × AAV interaction. (C) Fixed ratio/variable ratio results of training trial 10. Mice that were excluded from subsequent progressive ratio testing are indicated as dotted datapoints and were not taken into account for the analysis of significance in this graph. Yet, no difference in lever press activity was evident between the experimental groups.

Both Empty and Homer1b/c OE animals showed increasing interest in the presented reward over the course of the habituation phase (Time effect: $F_{1.584} = 9.170$; $p < 0.01$) with no time × AAV effect ($F_{1.584} = 0.166$; $p = 0.798$) or Chi Square significance between the AAV types (Figure 4A). Overexpression of Homer1b/c did not have an effect on the consumed reward number. Note that during this stage, a maximum of 37 rewards could be consumed. This limit was reached by three Empty animals in both the 4th and the 5th habituation trial.

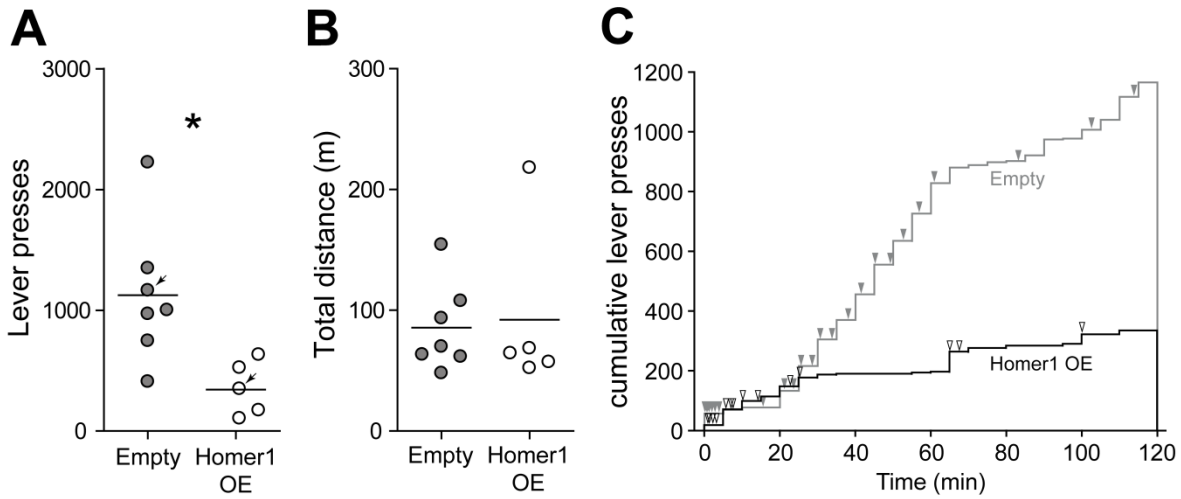


Figure 5: Progressive ratio (PR) performance of Homer1 OE mice. (A) Homer1 OE mice display significantly less lever presses compared to Empty animals in the PR task. The arrows indicate the datapoints that are plotted in panel c. (B) Locomotion in the PR task. Overexpression of Homer1b/c does not lead to a general increase in locomotion. (C) Representative cumulative distribution of PR lever presses. Dispensed rewards are marked as triangles. The mouse infected with Empty virus (Black line) shows high activity up to 60 min into the PR stage, followed by a decreased lever press frequency. This activity decrease appears earlier in the Homer1OE animal (Gray line), which translates into a reduced overall activity over the course of 120 min. * Significant from WT, $p < 0.05$.

During the training trials, both experimental groups showed a time-dependent increase in lever presses to receive rewards (Time effect: $F_{2.977} = 4.087$; $p < 0.05$), without a significant effect of Homer1b/c overexpression or interaction effects (Figure 4B). After training trial 10, 7 out of 9 Empty animals received more than 10 rewards, thereby passing the cut-off criterion. In the Homer1 OE group, only 5 out of 9 animals exceeded the amount of lever presses to pass the criterion. After exclusion of all non-performing mice, no effect of Homer1b/c overexpression was apparent (Figure 4C).

In the PR task, Homer1 OE mice showed a significantly reduced motivation to earn rewards by lever pressing ($T_{10} = 2.699$; $p < 0.05$) compared to animals injected with Empty virus (Figure 5A). An underlying general locomotion effect could not be detected, as displayed in the total distance traveled in the PR task (Figure 5B). Interestingly, Empty animals appeared to be motivated to earn rewards for about 60 min, then subsequently decreasing the number of lever presses, while overexpression of Homer1b/c reduced this time to about 25 min (Figure 5C).

Discussion

In the current study, we provide evidence that hippocampal Homer1 is involved in operant reward learning. In an extensive operant conditioning paradigm, we tested both Homer1KO and Homer1 OE mice with respect to their learning and motivational behavior. In the Homer1KO animals, two distinct subgroups emerged: mice that displayed high motivation and activity and animals that did not perform at all in the operant conditioning task. Overexpression of Homer1b/c in the hippocampus did not affect the basic interest in sucrose rewards, but led to a reduced activity in the PR task. These results extend the current knowledge that Homer1 signaling plays a crucial role in functional incentive motivation specifically in the hippocampus, and further suggests that Homer1 may be a relevant target for the treatment of psychiatric disorders such as depression or schizophrenia.

The complex behavioral phenotype of Homer1KO mice has previously been associated with learning and memory deficits and motivational impairments (Szumlinski et al., 2005). Szumlinski and colleagues could show that Homer1KO animals display less motivation to obtain a sucrose reward. Pronounced hyperactivity indicated by increased locomotion in a novel environment (Szumlinski et al., 2005) and enhanced activity in the rest cycle (Jaubert et al., 2007), has also been reported in these mice, which was confirmed by observations made in our group (Unpublished data). In our study, we observed that most of the Homer1KO animals did not express any interest in the presented reward, while others displayed an abnormally high activity, reflected by excessive retrieval and consummation of the rewards, yet a locomotion effect failed to reach significance. This bimodal distribution is apparent over the course of the various tasks and makes data interpretation difficult, since the results may not necessarily represent learning as much as distinct hyperactivity of a subset of KO animals. Although learning deficits have frequently been reported in the context of Homer1 deletion (Szumlinski et al., 2004; Lominac et al., 2005), it is likely that these impairments play a secondary role to the observed inactive phenotype. Since the animals were not required to learn a task before acquiring a reward in the habituation phase, we suggest that the majority of Homer1KO mice initially showed indifference or even a degree of aversion towards the reward. Subsequently these mice also lacked the motivation to comply with the reward-stimulus paradigm presented in the following weeks. Such findings indicate that deletion of

Homer1 can have opposing effects on individuals in the same task, most likely depending on environmental factors that have yet to be elucidated. The food restriction used here may be one such factor leading to individual differences in the measured behaviors. Indeed, food-sated Homer1KO mice have been reported to show altered performance in reward-seeking tasks when compared to food deprived animals (Szumlinski et al., 2005). In our study, Homer1KO animals presented significantly lower body weight from the onset of the experiment, a phenotype that has not been previously reported (Szumlinski et al., 2004; Jaubert et al., 2007). A reduced absolute weight may alter the severity of the food restriction and therefore confound the motivational alterations in comparison to the WT control group. This is especially intriguing since Homer1 is also deleted in cortical regions, which may interfere with appetite and hunger perception.

To specifically address the question of whether hippocampal Homer1 expression has an impact on operant reward learning, we overexpressed Homer1b/c by viral transfection and exposed these animals to the same operant conditioning paradigm. We did not observe a significant basal difference in the motivation for sucrose reward during the habituation phase. However a large subgroup of Homer1 OE mice did not reach the cut-off criterion in the training stage, suggesting problems with operant conditioning memory processes in these animals. This is in sharp contrast to various studies that link Homer1b/c to improved memory processing (Lominac et al., 2005; Ronesi and Huber, 2008; Gerstein et al., 2012). Nevertheless Homer1b/c overexpression also led to a reduction in activity to obtain a reward during the PR task, which suggests that the previous performance in the FR/VR stages as well as in the habituation may be confounded by underlying motivational deficits. Confounding effects of general activity are less likely, since these animals did not show altered locomotion in the behavioral setup. Additionally, previous phenotyping of animals that overexpress Homer1 in the hippocampus did not reveal basal effects on behavioral core parameters (Wagner et al., 2013). A possible underlying mechanism for these effects may be caused by the imbalance between overexpressed Homer1b/c and the IEG Homer1a, which has been shown to be critically involved in memory formation (Inoue et al., 2009). A recent study has shown that Homer1a is required for fear conditioning and furthermore that fear conditioning induces the upregulation of this gene (Mahan et al., 2012). Hernandez and colleagues reported an increase in Homer1a mRNA levels in rats after an instrumental

learning task, further supporting the importance of this IEG in operant conditioning tasks (Hernandez et al., 2006). The elevated levels of Homer1b/c in the hippocampus of Homer1 OE mice may be causal for the induction of Homer1a not being sufficient to trigger the downstream pathways that in turn stimulate motivational behavior. Interestingly, both KO and OE mice showed problems in reaching the cut-off criterion, suggesting that general modulation or dysbalance of the Homer1 signaling system may be detrimental to learning. However, further studies are needed to provide evidence for hippocampal Homer1a/Homer1b/c interplay that may promote operant conditioning learning and motivational behavior.

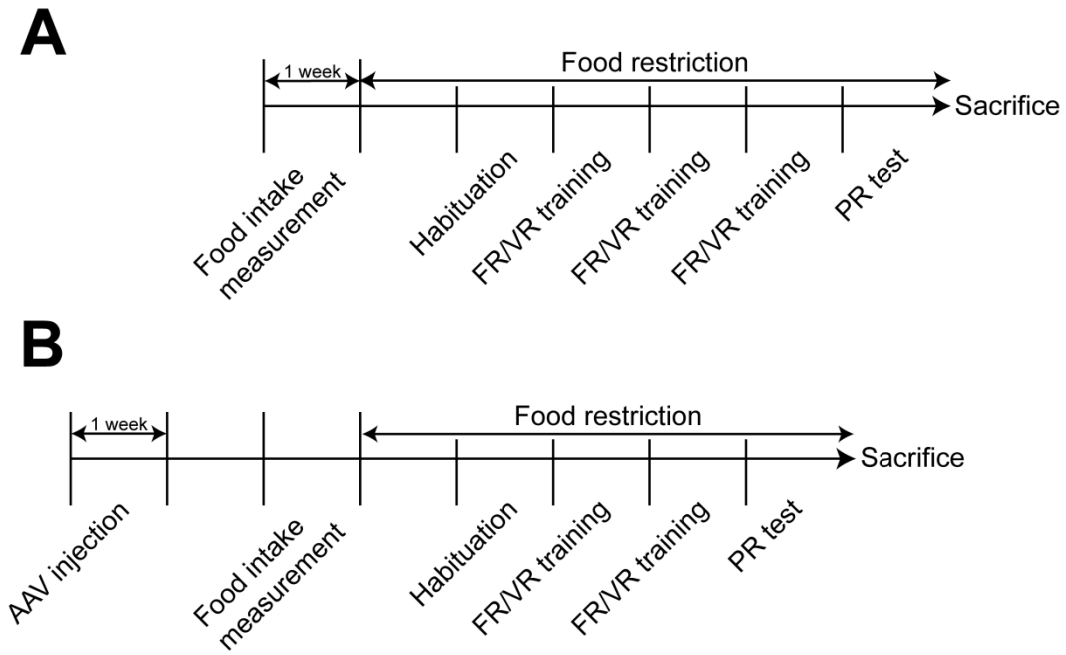
A major limitation of this study emerges from the relatively small number of animals in each experimental group. In particular, the bimodal distribution of the Homer1KO mice complicates the data interpretation. Future studies should consider larger group compositions to further investigate possible underlying mechanisms of this diverging phenotype. Concerning the Homer1 OE animals, follow-up studies need to address the question as to whether loss of reward motivation is indeed linked to the reduced ability to learn the operant conditioning task. Also, the overexpression of Homer 1b/c exclusively was limited to the hippocampus. Conversely, the KO mice suffered from complete loss of all Homer1 subtypes across all brain regions. Furthermore, the performances of the control groups in both experiments differ, yet this may be attributed to the different origin and of the animals used. However, this has to be kept in mind when directly comparing the different phenotypes of both experiments. A more detailed molecular analysis of hippocampal Homer1 interaction partners, especially in Homer1KO mice, may lead to further insight in this respect.

Taken together, we provide first evidence that hippocampal Homer1 is involved in the acquisition of an operant conditioning paradigm, with a profound decrease of motivational behavior in mice that overexpress Homer1b/c in the hippocampus. Additionally we detected hyperactive behavior in a subpopulation of Homer1KO mice that has not been previously described and that could be of specific importance in relation to schizophrenia, suggesting the need to further investigate this mouse model, on both a behavioral and molecular level. The results presented in this study provide further evidence that alterations in signaling pathways, specifically Homer1, may contribute to the emergence of motivational and learning deficits.

Supplemental Material

Reward No.	Lever presses for next reward	Total lever presses	Reward No.	Lever presses for next reward	Total lever presses	Reward No.	Lever presses for next reward	Total lever presses	Reward No.	Lever presses for next reward	Total lever presses
1	1	1	14	49	295	27	132	1486	40	239	3927
2	2	3	15	54	349	28	139	1625	41	248	4175
3	4	7	16	60	409	29	147	1772	42	257	4432
4	7	14	17	66	475	30	155	1927	43	266	4698
5	10	24	18	72	547	31	163	2090	44	275	4973
6	13	37	19	78	625	32	171	2261	45	284	5257
7	17	54	20	84	709	33	179	2440	46	294	5551
8	21	75	21	90	799	34	187	2627	47	304	5855
9	25	100	22	97	896	35	195	2822	48	314	6169
10	29	129	23	104	1000	36	203	3025	49	324	6493
11	34	163	24	111	1111	37	212	3237	50	334	6827
12	39	202	25	118	1229	38	221	3458	51	344	7171
13	44	246	26	125	1354	39	230	3688	52	354	7525

Supplemental table 1: Progressive ratio reward overview. The middle column shows the required amount of lever presses to achieve the next reward. The right column shows the cumulated total lever presses required to receive the respective number of rewards given in the left column.



Supplemental figure 1: Experimental design overview. (A) In experiment 1, Homer1KO mice were trained in the FR/VR protocol for 3 weeks until the PR test session was performed. (B) In experiment 2, Homer1 OE animals were allowed to recover for 3 weeks until food restriction commenced. FR/VR training lasted for 2 weeks. FR/VR: Fixed ratio/Variable ratio; PR: Progressive ratio.

Genotype	Food intake measurement	Food restriction	Habituation	FR/VR training 1	FR/VR training 2	FR/VR training 3	PR test
WT	27.76 ± 0.62	26.42 ± 0.67	25.53 ± 0.55	25.32 ± 0.59	25.74 ± 0.56	25.72 ± 0.56	25.14 ± 0.65
KO	23.55 ± 0.85	21.41 ± 0.92	21.63 ± 0.71	21.02 ± 0.75	21.62 ± 0.71	21.39 ± 0.74	21.06 ± 0.72

Supplemental table 2: body weight progression in experiment 1. Repeated measures ANOVA revealed an effect of time ($F_{5,6}$ = 25.091; p < 0.001) and a significant between subject genotype effect ($F_{1,10}$ = 21.312; p < 0.001). Homer1KO animals were significantly lighter over the course of the experiment than their WT littermates, while both groups were affected by the food restriction (Colored in gray). All values in g.

AAV type	Food intake measurement	Food restriction	Habituation	FR/VR training 1	FR/VR training 2		PR test
Empty	30.46 ± 1.04	27.81 ± 0.95	27.71 ± 0.78	28.44 ± 0.71	26.34 ± 0.60		26.77 ± 0.62
Homer1 OE	31.62 ± 0.80	29.13 ± 0.65	29.23 ± 0.62	29.62 ± 0.45	27.28 ± 0.21		27.06 ± 0.14

Supplemental table 3: body weight progression in experiment 2. Repeated measures ANOVA revealed an effect of time ($F_{5,6}$ = 30.964; p < 0.001) but no effect of the virus on body weight progression. Both groups lost weight in response to the food restriction (Colored in gray). All values in g.

2.5. Homer1/mGluR5 signaling moderates vulnerability to chronic social stress

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Abstract

Psychiatric disorders such as depression have recently been closely linked to changes in glutamate transmission in the central nervous system. Glutamate signaling is processed by different receptors, including metabotropic glutamate receptors (mGluRs) and Homer1 is a scaffold protein that mainly interacts with mGluRs and subsequently modulates Ca^{2+} signaling pathways. Two major isoforms of Homer1 have been described: Homer1b/c, consisting of a conserved amino-terminal target-binding domain and a coiled-coil structure including two leucine zipper motifs, is predominantly expressed in the nervous system. Homer1a, a short form, which is missing the coiled-coil structure, has been shown to act as a dominant negative for long Homer1 isoforms at the mGluR binding site. Our study aims to unravel the role of Homer1 in the mediation of responses to chronic social stress as well as its function in promoting a stress resiliency or vulnerability. We therefore performed a thorough profiling of Homer1KO mice under the effects of chronic defeat stress and found a marked hyperactivity in behavioral parameters as well as a dysregulated hypothalamic-pituitary-adrenal axis activity after acute stressors. Overexpression of Homer1a in the hippocampus, in turn, led to an increased vulnerability to chronic stress, reflected in an increased physiological response to stress as well as enhanced behavioral despair. Chronic administration of the selective, orally bioavailable mGluR5 antagonist CTEP was able to recover behavioral alterations induced by chronic stress, thereby further suggesting a prominent role of the mGluR5/Homer1 pathway in the stress system. In summary, our results present strong evidence for the involvement of the glutamatergic system in the emergence of psychiatric disorders and implicate the mGluR5/Homer1 signaling pathway as a major target for the development of novel antidepressant agents.

Introduction

Individuals are frequently challenged by stressful events and the organism can readily respond to such challenges by activating hormonal pathways such as the hypothalamic-pituitary-adrenal (HPA) axis (Chrousos, 2009). Yet, prolonged activation of these systems by chronic stress leads to chronically elevated cortisol levels which in turn can lead to maladaptive consequences in the organism and may ultimately contribute to the development of psychiatric disorders such as depression (McEwen, 2004; de Kloet et al., 2005). By modeling certain aspects of depressive disorders, animal models of chronic stress exposure have been a valuable tool to understand the molecular underpinnings of stress-induced psychopathology (Cryan and Holmes, 2005; Joëls and Baram, 2009; Savignac et al., 2011) as well as to introduce and validate current and novel treatment strategies for depression (Wagner et al., 2012; Mutlu et al., 2012; Scharf et al., 2013).

Most present treatment options are based on the monoamine hypothesis of depression and aim to increase the amount of monoamines, such as serotonin, in the synaptic cleft (Rush et al., 2006; Prins et al., 2011). Yet, the late onset of therapeutic effects as well as unsatisfactory relapse rates and side effects illustrate the need to develop new drugs that also target different transmitter systems (Thase, 2006).

The main excitatory neurotransmitter is glutamate and glutamate-releasing neurons are present across the brain. Recent studies have provided convincing evidence that dysregulation of glutamate signaling, mainly via its different postsynaptic receptors such as AMPA, NMDA or metabotropic glutamate receptors (mGluRs) contributes to the emergence of psychiatric disorders (Kendell et al., 2005; Sanacora et al., 2012; Mathews et al., 2012). Drugs that modulate glutamate receptor function have been proposed as promising targets for psychiatric drug development (Popoli et al., 2012). Especially positive and negative modulators of mGluR5, such as MTEP, have been implicated as novel agents for the treatment of depression (Palucha et al., 2005; Pilc et al., 2008; Krystal et al., 2010), but the exact molecular mechanisms that mediate these effects are yet to be fully understood.

In this context, Homer1, a postsynaptic scaffolding protein that links mGluR5 to downstream targets such as inositol triphosphate receptors (Tu et al., 1998), but also acts as moderator for NMDA/mGluR5 interactions (Tu et al., 1999; Bertaso et al., 2010), emerged as a potential target protein in psychopathology. Clinical studies provided first

evidence that Homer1 is involved in the development of major depressive disorders (Rietschel et al., 2010), while preclinical studies describe its importance in memory formation (Lominac et al., 2005) and reward-related behaviors (Szumlinski et al., 2004; Jaubert et al., 2007). Furthermore, Homer1a, a splice-variant that is induced by synaptic activity (Brakeman et al., 1997), has been identified as an immediate early gene product, which is crucially involved in behavioral alterations that are related to depression (Celikel et al., 2007; Mahan et al., 2012). However, the impact of Homer1 and its modulatory effects on glutamate signaling via the mGluR5 in chronic stress situations is largely unknown.

In the current study, we therefore aimed to investigate the role of mGluR5/Homer1 in the context of chronic social defeat stress, which has been shown to adequately model certain endophenotypes of depression by us and others (Berton et al., 2006; Nestler and Hyman, 2010; Wang et al., 2011a; Hartmann et al., 2012). We modulated this signaling pathway by using total Homer1 knockout mice, as well as mice that selectively overexpressed Homer1a in the hippocampus and analyzed neuroendocrine, central gene expression, and behavioral alterations. We also investigated the efficacy of CTEP (Lindemann et al., 2011), a novel mGluR5 antagonist, with respect to its anti-depressant properties on HPA axis function and behavior in this model.

Materials and Methods

Animals

For experiment 1, conventional Homer1$^{-/-}$ and wild type littermates were bred from heterozygous breeding pairs on a C57BL/6N background in the animal facilities of the Max Planck Institute of Psychiatry in Munich, Germany. Generation and genotyping of Homer1$^{-/-}$ mice was reported previously (Yuan et al., 2003) and Homer1 knockout was verified by PCR. Male C57BL/6N mice (Charles River Laboratories, Maastricht, the Netherlands) at the age of 10 weeks were used in experiment 2 and experiment 3. All mice were held under standard conditions (12L:12D light cycle, lights on at 08:00 AM, temperature 23 ± 2 °C) and were single housed and acclimated to the experimental room for 2 weeks before the beginning of the experiments. Male CD1 mice (16 - 18 weeks of age) served as resident mice, which were held under the conditions described above. Tap water and food (Altromin 1324, Altromin GmbH, Germany) was available *ad libitum* during the whole experiment. All experiments were performed in the animal facilities of the Max Planck Institute of Psychiatry in Munich, Germany. The experiments were carried out in accordance with the European Communities' Council Directive 2010/63/EU. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany.

Experimental design

Experiment 1

In the first experiment, we aimed to identify the consequences of a deletion of Homer1 in the context of chronic stress (Figure 1A). We therefore exposed 22 Homer1$^{-/-}$ (KO) and 26 wild type (WT) mice (Aged 12 - 15 weeks) to the chronic social defeat stress (CSDS) paradigm as described below. Half of each group (KO: n = 11; WT: n = 13) was randomly assigned to either control or stress condition. During the last 7 days of the CSDS paradigm, all behavioral tests were performed.

Experiment 2

In the second experiment, we used 48 C57BL/6N mice to assess the effects of Homer1a overexpression in the CA1 and dentate gyrus (DG) region of the hippocampus in the context of chronic stress (Figure 1B). We infected 24 mice with an empty viral construct and 24 mice with a viral construct carrying the Homer1a coding sequence as described below. After a recovery period of 3 weeks, the 2 groups were randomly split into control and stress conditions (n = 12/group respectively) and the CSDS paradigm was performed as described below. Behavioral tests were performed as in experiment 1.

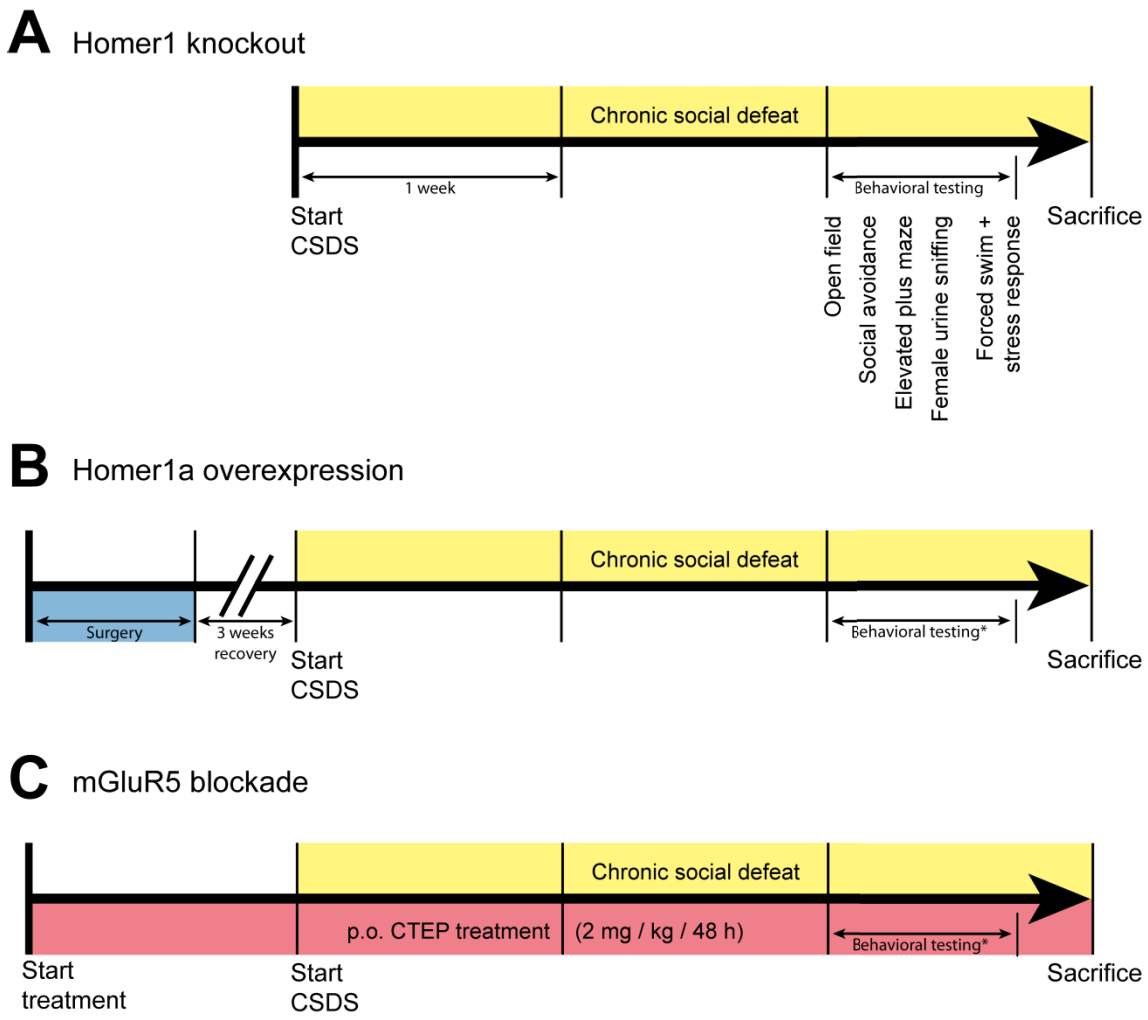


Figure 1: Overview of the experimental time courses. (A) Homer1KO (KO) or Wild Type (WT) mice from heterozygous breeding pairs were randomly distributed in control and chronic social defeat stress (CSDS) groups. CSDS lasted for 21 days, behavioral tests were performed in the third week of the experiment. All animals were sacrificed 24 h after the last defeat took place. (B) Animals underwent surgery at the age of 11 weeks and were subsequently allowed to recover from the viral infection for 3 weeks. After the recovery period, CSDS and behavioral testing was performed as described in experiment 1. (C) 7 days prior to the start of the CSDS period, all animals from experiment 3 were treated with either vehicle or CTEP per os. Treatment took place every 48 h immediately before the defeat or handling procedure. CSDS and behavioral testing was performed as described in experiment 1.

Experiment 3

In the third experiment, we investigated the effects of an inverse agonist to mGluR5 on chronic social stress (Figure 1C). Here, a total of 48 C57BL/6N mice were randomly divided into 2 × 2 groups (Control vehicle, control CTEP, CSDS vehicle, CSDS CTEP; n = 12/group) and subjected to the chronic stress procedure described below. Oral administration of the inverse mGluR5 agonist CTEP (F. Hoffmann-La Roche, Basel, Switzerland) commenced 7 days prior to the start of the CSDS paradigm to establish stable baseline receptor occupancy and blockade. Treatment by CTEP was performed as described previously (Lindemann et al., 2011; Michalon et al., 2012). Briefly, CTEP was formulated as a microsuspension in vehicle (0.9% NaCl, 0.3% Tween-80). Chronic treatment consisted in once per 48 h dosing at 2 mg/kg per os in a volume of 10 ml/kg. Gavaging took place immediately before the daily defeat or handling procedure to minimize confounding effects of oral drug administration. Behavioral tests were performed as in experiment 1.

Social defeat stress procedure

The CSDS paradigm lasted for 21 days and was conducted as described previously (Wagner et al., 2011). Briefly, the experimental mice were introduced into the home cage (45 cm × 25 cm) of a dominant resident mouse and defeated shortly after. When the defeat was achieved, the animals were separated by a wire mesh, preventing physical but allowing sensory contact for 24 h. Each day, stressed animals were defeated by another unfamiliar, dominant resident mouse, in order to exclude a repeated encounter throughout the experiment. The daily defeat was performed between 11:00 AM and 04:00 PM; varying starting times reduced the predictability of the stressor and therefore minimized a potential habituation effect. Experimental mice were always defeated by resident males during the entire stress period. Control mice were housed in their home cages during the course of experiment. Both stress and control animals were handled daily during the stress procedure; body weight was assessed at the beginning of the experiment as well as before the sacrifice.

Sampling procedure

Animals from experiment 1 and 3 were sacrificed by decapitation following quick anesthesia by isoflurane 24 h after the last defeat. Basal trunk blood samples were

collected in 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany). Blood samples were kept on ice and later centrifuged at 8000 rpm at 4 °C for 15 min. Plasma was transferred to new, labeled tubes and stored at -20 °C until determination of corticosterone by radioimmunoassay (MP Biomedicals Inc; sensitivity 12.5 ng/ml). Brains were removed and flash frozen in methyl butane and stored at -80 °C until processing for *in situ* hybridization. Adrenal glands were removed, dissected from fat and weighed.

For experiment 2, basal blood samples from all animals were collected by tail cut 24 h after the last defeat (Fluttert et al., 2000). Samples were gathered in 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany) and processed as described above. The animals then were deeply anesthetized with ketamine/Rompun and perfused intracardially with 4% paraformaldehyde. Brains were removed, postfixed overnight in 4% paraformaldehyde following overnight incubation in 30% sucrose solution at 4 °C, and then stored at -80 °C until processing for *in situ* hybridization. Adrenal glands were removed, dissected from fat and weighed.

Behavioral testing

Behavioral tests were performed between 08:00 AM and 12:00 PM in the same room where the animals were housed. All tests were described and validated previously (Wagner et al., 2011; Wagner et al., 2012; Wagner et al., 2013). Tests were recorded and analyzed using the video tracking software AnyMaze (Anymaze 4.20, Stoelting, IL, USA).

Open field test

The Open field (OF) test was performed on day 15 of the stress procedure. Testing was performed in an open field arena made of gray polyvinyl chloride (50 cm × 50 cm × 50 cm) that was evenly illuminated during testing (15 lux). The total test time was 15 min, and parameter of interest was the total distance traveled.

Social avoidance test

The social avoidance test is described in detail elsewhere (Golden et al., 2011). Briefly, animals were allowed to explore the open field arena for 2.5 min with an empty wire mesh cage placed at one side of the apparatus. In a second stage, the animals were confronted with an unfamiliar CD1 resident mouse in the wire mesh cage for another

2.5 min. The ratio between the time in the interaction zone of the no-target trial and the time in the interaction zone of the target trial serves as a marker for disturbed social behavior associated with depressive disorders. Animals that did not explore the interaction zone at all were excluded from the analysis.

Elevated plus maze

The elevated plus-maze (EPM) was conducted to display changes in anxiety-related behavior. The device consisted of a plus-shaped platform with two opposing open arms (30 cm × 5 cm × 0.5 cm) and two opposing enclosed arms (30 cm × 5 cm × 15 cm), made of gray PVC, which were connected by a central area (5 cm × 5 cm). The whole device was elevated 50 cm above the floor. The illumination was 25 lux in the open arms and less than 10 lux in the closed arms. Testing duration was 10 min and mice were placed into the center zone facing one of the enclosed arms at the start of the test. The time spent in the open arms compared to the total arm time were analyzed. Animals that fell off the open arm of the apparatus were excluded from the analysis.

Female urine sniffing test

In this test, the mice were habituated to a sterile cotton swab inserted into their home cage 1 h before the start of the first stage. In the first stage, mice were exposed to a cotton swab dipped in water for 3 min and sniffing time was scored. After an inter trial interval of 45 min, mice were exposed to a cotton swab dipped in urine from estrous females of the same mouse strain. Again, total sniffing time was scored. The test was performed in a dark environment (<3 lux). Animals that escaped from the apparatus in any trial were excluded from the analysis.

Forced swim test

In the forced swim test (FST), each mouse was put into a 2 liter glass beaker (diameter: 13 cm, height: 24 cm) filled with tap water (21 ± 1 °C) to a height of 15 cm, so that the mouse could not touch the bottom with its hind paws or tail. Testing duration was 5 min. Time spent immobile (Floating) was scored by an experienced observer, blind to treatment or condition of the animals.

Acute stress response

The FST also served as an acute stressor in order to determine the stress response by measuring corticosterone plasma concentrations. After the FST, all mice were towel-dried and placed into their home cage to recover from the acute stressor. Blood samples were taken by tail cut 30 min (Stress response) and 90 min (Stress recovery) after the onset of the FST. Samples were processed as described above and stored at -20 °C until determination of corticosterone by radioimmunoassay (MP Biomedicals Inc; sensitivity 12.5 ng/ml).

In situ *hybridization*

Frozen brains were sectioned at -20 °C in a cryostat microtome at 18 µm (Experiment 1 & 3) or 20 µm (Experiment 2), thaw mounted on Super Frost Plus slides, dried and stored at -80 °C. *In situ* hybridization using a ^{35}S UTP labeled ribonucleotide probes (Homer1a, Homer1b/c, corticotropin-releasing hormone (CRH), Glucocorticoid receptor (GR), mineralocorticoid receptor (MR)) was performed as described previously (Schmidt et al., 2007, Wagner et al. 2011, Wagner et al., 2013). Briefly, prepared sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. The antisense cRNA riboprobes were transcribed from a linearized plasmid. Tissue sections were saturated with 100 µl of hybridization buffer containing approximately $3 - 5 \times 10^6$ cpm ^{35}S labeled riboprobe. Brain sections were coverslipped and incubated overnight at 55 °C. The following day, the sections were rinsed in 4 × SSC (Standard saline citrate), treated with RNAse A (20 mg/l) and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1 × SSC for 1 h at 65 °C and dehydrated through increasing concentrations of ethanol. The slides were exposed to Kodak Biomax MR films (Eastman Kodak Co., Rochester, NY) and developed. Autoradiographs were digitized, and expression was determined by optical densitometry utilizing the freely available NIH ImageJ software. The mean of two measurements of two different brain slices was calculated for each animal. The data were analyzed blindly, always subtracting the background signal of a nearby structure not expressing the gene of interest from the measurements.

Viral overexpression of Homer1a

Viral overexpression was performed as described previously (Wagner et al., 2013). We used an adeno-associated bicistronic AAV1/2 vector (GeneDetect) containing the CAG-Homer1a-IRES-EGFP-WPRE-BGH-polyA expression cassette (Containing coding sequence of Homer1a NCBI CCDS ID CCDS26687). For the control group, we used the same vector construct expressing only EGFP. Virus production, amplification, and purification were performed by GeneDetect. Mice were anesthetized with isoflurane, and 0.5 µl of either AAVHomer1a or AAV-EGFP (Titers: 1.2×10^{12} genomic particles/ml) were bilaterally injected in the dorsal hippocampus at 0.06 µl/min by glass capillaries with tip resistance of 2 - 4 MΩ in a stereotactic apparatus. The following coordinates were used: 1.9 mm posterior to bregma, 1.3 mm lateral from midline, and 1.3/1.8 mm below the surface of the skull, targeting the CA1 and DG region of the dorsal hippocampus. After surgery, mice were treated for 5 days with Metacam via drinking water. Behavioral testing started 4 weeks after virus injection. Successful targeting and quantification of Homer1a overexpression was achieved by *in situ* hybridization using the riboprobe described above. Animals that were not infected bilaterally in both the CA1 and DG region were excluded from the analysis (n = 4).

Statistical analysis

The data presented are shown as means ± SEM, analyzed by the commercially available software SPSS 18.0. For all experiments, two-way ANOVAs were applied to the data as appropriate. Significant main effects and/or interactions were followed by Fisher's LSD post hoc analysis when appropriate. In case the data was not normally distributed, an ln-transformation was applied to the dataset (Social avoidance test). A nominal level of significance $P < 0.05$ was accepted.

Results

Experiment 1

We investigated HPA axis activation in KO and WT animals that were exposed to chronic stress by measuring circulating corticosterone both under basal conditions and after an acute challenge. ANOVA revealed a significant main condition effect in basal corticosterone levels ($F_{1,44} = 6.472$, $p < 0.05$), independent of the animals' genotype (Figure 2A). In response to an acute stressor, both a condition ($F_{1,44} = 43.232$, $p < 0.001$) and a genotype ($F_{1,44} = 15.796$, $p < 0.001$) main effect was detected (Figure 2B). Post hoc comparison revealed that CSDS increased the HPA axis response to a novel stressor both in WT ($p < 0.01$) and KO ($p < 0.05$) animals. Furthermore, KO mice showed a significantly increased corticosterone response irrespective of the condition (Control: $p < 0.001$; CSDS: $p < 0.001$). At 90 min after the onset of the stressor, ANOVA revealed a main condition effect ($F_{1,44} = 6.883$, $p < 0.05$) as well as a significant genotype × condition interaction ($F_{1,44} = 14.829$, $p < 0.001$) (Figure 2C). Further post hoc comparison showed that under control conditions, KO animals display lower corticosterone values compared to their WT littermates ($p < 0.05$). KOs that underwent the CSDS procedure recover significantly less from the acute stressor than both the stressed WT group ($p < 0.05$) and the respective control KO group ($p < 0.001$). These alterations in circulating corticosterone are accompanied by a marked increase in adrenal gland size in KO animals ($F_{1,45} = 81.040$, $p < 0.001$) both under control ($p < 0.001$) and stress ($p < 0.001$) conditions (Figure 2D). Additionally, CSDS also significantly increased adrenal size in both genotypes (Main condition effect: $F_{1,45} = 82.163$, $p < 0.001$; WT: $p < 0.001$, KO: $p < 0.001$).

Analysis of the gene expression of main HPA axis modulators in the hippocampus and the paraventricular nucleus of the hypothalamus (PVN) such as GR, MR and CRH revealed that CSDS induced an increase in CRH mRNA in the PVN irrespective of the genotype (Condition effect: $F_{1,43} = 11.264$, $p < 0.01$; WT: $p < 0.05$, KO: $p < 0.05$) (Figure 2E). Concurrently, KO animals show significantly reduced CRH mRNA levels compared to their WT littermates under basal conditions (Main genotype effect: $F_{1,43} = 10.744$, $p < 0.01$; control: $p < 0.01$) but not after CSDS exposure ($p = 0.075$). ANOVA analysis of mRNA expression levels of GR in the CA1 region of the ventral hippocampus revealed a condition × genotype interaction ($F_{1,44} = 7.379$, $p < 0.01$) as well as a condition ($F_{1,44} = 12.908$, $p < 0.001$) and a genotype effect ($F_{1,44} = 12.290$, $p < 0.001$) (Figure 2F). Post

hoc analysis showed that stressed KO mice show significantly reduced GR levels compared to both unstressed KO animals ($p < 0.01$) and to stressed WT mice ($p < 0.001$). The complete results of the gene expression data are listed in supplemental table 1.

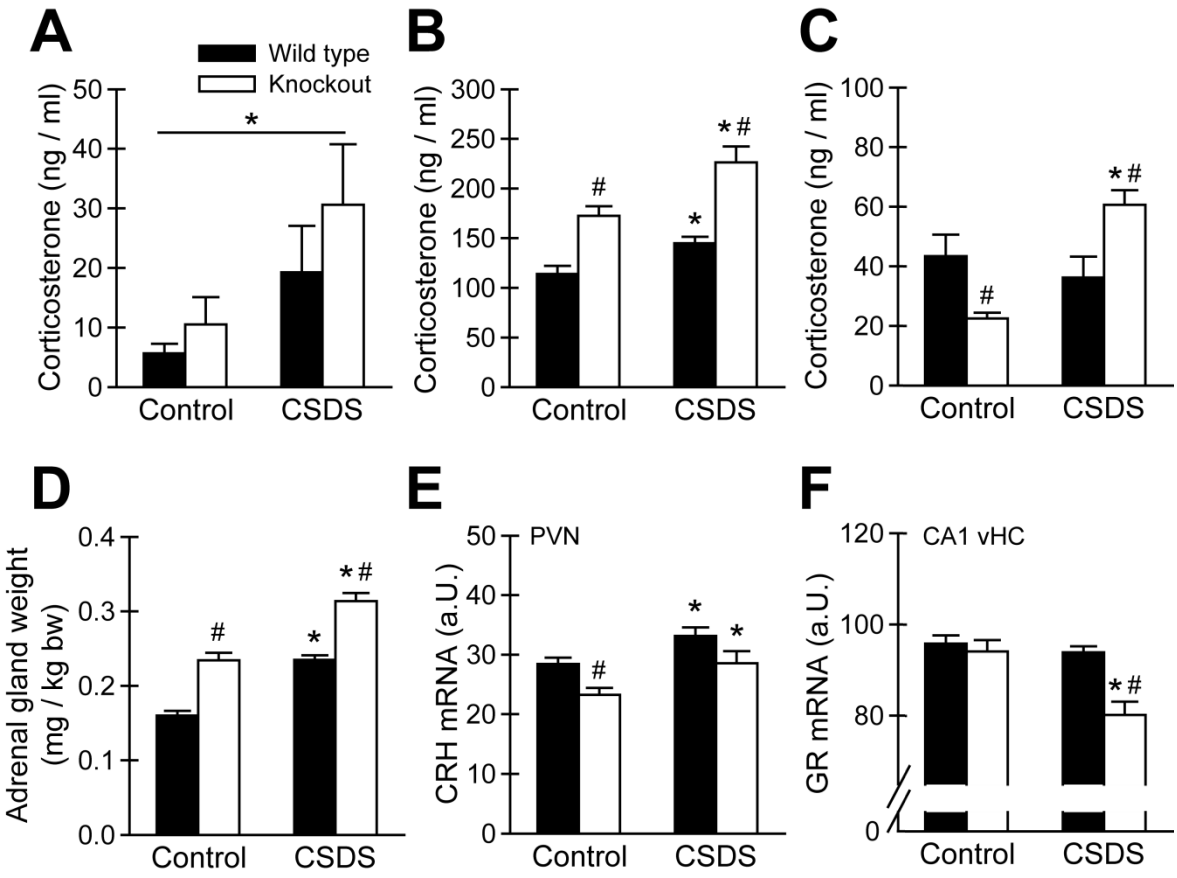


Figure 2: Activity of the hypothalamic-pituitary-adrenal (HPA) axis is severely deteriorated in KO mice. (A) Both WT and KO animals showed elevated corticosterone levels in response to CSDS. (B) The corticosterone response to a novel acute stressor was increased in mice that were exposed to CSDS. At the same time, KO mice showed a strongly enhanced response to the stressor irrespective of the condition. (C) Under basal conditions, KO animals displayed an improved recovery from the stressor. However, when challenged by CSDS, KO mice showed significantly increased corticosterone levels, indicating a disrupted HPA axis regulation in stressed KO animals. (D) Analogous to the corticosterone response, CSDS induced an increase in adrenal glands weight in both genotypes. However, KO mice already possessed enlarged adrenal glands under basal condition and this effect was also present in the stressed group. (E) In the paraventricular nucleus of the hypothalamus (PVN), corticotropin-releasing hormone (CRH) expression was increased in response to CSDS. At the same time, KO animals showed significantly lower CRH levels under basal conditions. This effect is not significant in the stressed groups. (F) mRNA levels of glucocorticoid receptor (GR) in the CA1 region of the ventral hippocampus were reduced in KO mice in response to CSDS. * Significant from control, $p < 0.05$, # Significant from WT, $p < 0.05$. Other abbreviations as in figure 1.

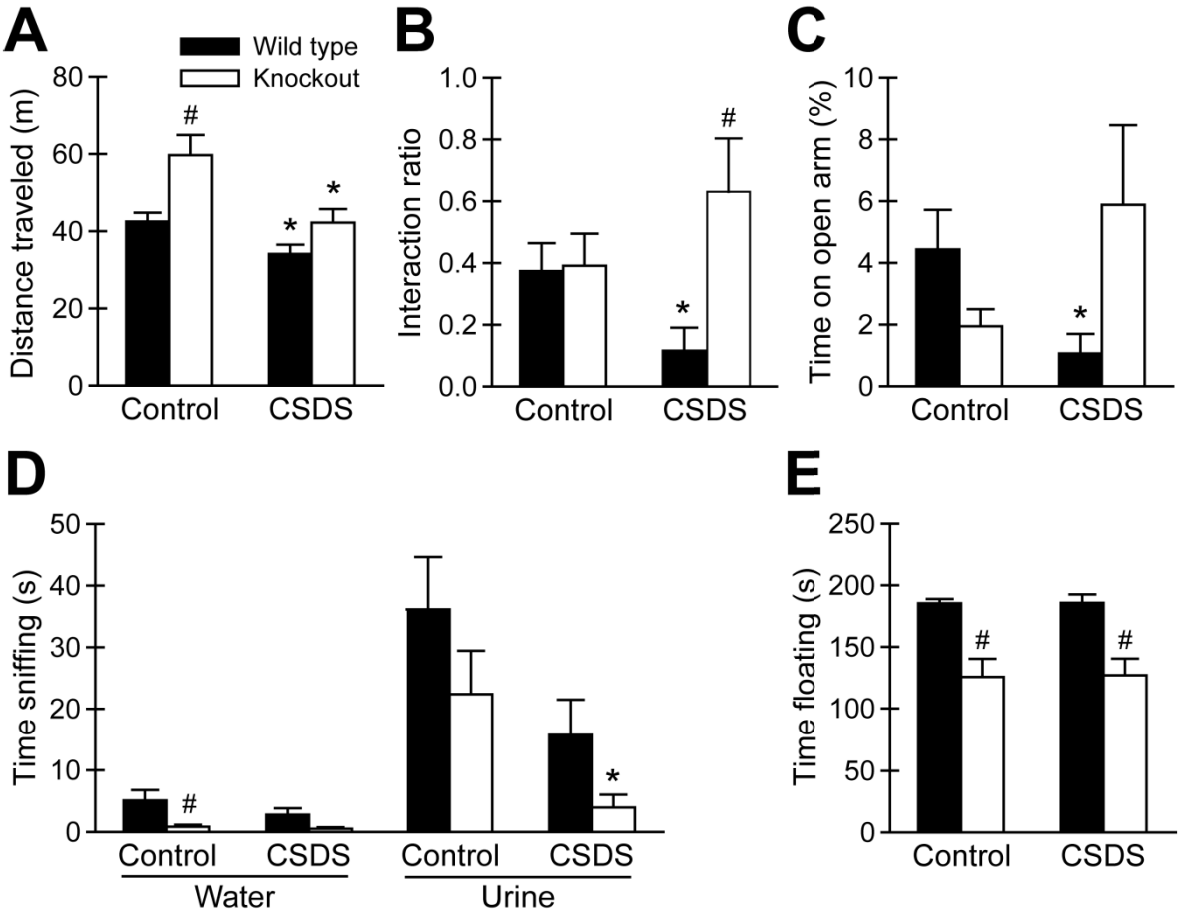


Figure 3: Behavioral profile of KO mice during CSDS. (A) While CSDS resulted in a reduction of locomotion in both groups, a marked hyperactivity was detected in KO mice under basal conditions which was severely affected by CSDS. (B) In the social avoidance (SA) test, CSDS resulted in a reduction of interaction in WT but not in KO mice, which showed a significantly increased interaction ratio compared to their stressed WT littermates. (C) In the elevated plus maze (EPM) WT mice displayed an increased anxiety-related behavior, which was not present in KO mice. (D) In the water trial of the female urine sniffing test (FUST), animals with a Homer1 deletion showed a reduced interest in the presented cotton tip. This effect was significant under basal but not under CSDS conditions. In the urine trial, CSDS had a strong effect on sniffing time, which was highly significant in KO mice. (E) Independent of CSDS, KO mice exhibited their hyperactive phenotype in the forced swim test (FST) as indicated by reduced floating times. * Significant from control, $p < 0.05$, # Significant from WT, $p < 0.05$. Other abbreviations as in figure 1.

Deletion of Homer1 resulted in considerable changes in the animals' behavior. In the OF test, CSDS reduced total locomotion in both genotypes (ANOVA main condition effect: $F_{1,43} = 13.129$, $p < 0.01$; WT: $p < 0.05$, KO: $p < 0.05$), while KO animals displayed a strong increase in locomotion (Genotype effect: $F_{1,43} = 12.630$, $p < 0.01$) under basal ($p < 0.01$) but not under CSDS conditions ($p = 0.071$) (Figure 3A). In the SA test, ANOVA revealed both a genotype ($F_{1,37} = 5.337$, $p < 0.05$) and a significant interaction effect ($F_{1,37} = 4.644$, $p < 0.05$) (Figure 3B).

Further post hoc analysis indicated that WT animals displayed a reduced social interaction ratio when under the effects of CSDS ($p < 0.05$) while interaction ratios of stressed KO mice were not significantly reduced compared to control conditions ($p = 0.256$). This is supported by the comparison of stressed WT and KO mice, where deletion of Homer1 led to a significantly increased interaction ratio ($p < 0.05$). With respect to anxiety-related behavior, ANOVA revealed a significant interaction between genotype and condition ($F_{1,37} = 5.205$, $p < 0.05$) (Figure 3C). In WT animals, CSDS reduced the time on the open arm ($p < 0.05$) while KO animals were not affected. A strong genotype difference was also apparent in the FUST, where KO mice spent significantly less time with the presented cotton tip in the water trial ($F_{1,45} = 9.269$, $p < 0.01$) (Figure 3D). This effect was significant under basal conditions ($p < 0.05$) but not after CSDS exposure ($p = 0.053$). In the urine trial, ANOVA revealed a condition effect ($F_{1,45} = 9.185$, $p < 0.01$). Here, we measured a reduction in sniffing time of stressed mice compared to their non-stressed littermates, which was significant in KO ($p < 0.05$) but not in WT animals ($p = 0.059$). The aforementioned hyperactive phenotype was also apparent in the FST, where KO mice spent significantly less time floating, thereby exhibiting a more active stress coping strategy (ANOVA genotype effect: $F_{1,45} = 32.662$, $p < 0.001$; control: $p < 0.01$; CSDS: $p < 0.01$) (Figure 3E). There was no effect of the stress exposure on the stress coping behavior of either genotype.

Experiment 2

The injection site as well as qualitative and quantitative analysis of the viral overexpression can be found in supplemental figure 1. Overexpression of Homer1a in the hippocampus did not change basal levels of circulating corticosterone, while ANOVA revealed a significant effect of CSDS on basal corticosterone ($F_{1,43} = 9.273$, $p < 0.01$) (Figure 4A). This effect reached post hoc significance in Homer1a OE mice ($p < 0.05$) but not in Empty mice ($p = 0.066$). For the stress response (Figure 4B), ANOVA revealed both a condition ($F_{1,44} = 61.134$, $p < 0.001$) and a condition × AAV interaction ($F_{1,44} = 4.845$, $p < 0.05$): Empty animals show a significantly increased corticosterone response when under the effects of CSDS ($p < 0.001$), while Homer1a OE animals already show an increased response under basal conditions compared to Empty animals ($p < 0.05$). CSDS was not able to further increase the corticosterone response, possibly due to a ceiling

effect. After 90 min of recovery, both Homer1a OE and Empty animals showed a disturbed HPA axis recovery indicated by significantly increased corticosterone levels in the CSDS groups (ANOVA condition effect: $F_{1,44} = 14.285$, $p < 0.01$; Empty: $p < 0.01$, Homer1a OE: $p < 0.05$) (Figure 4C).

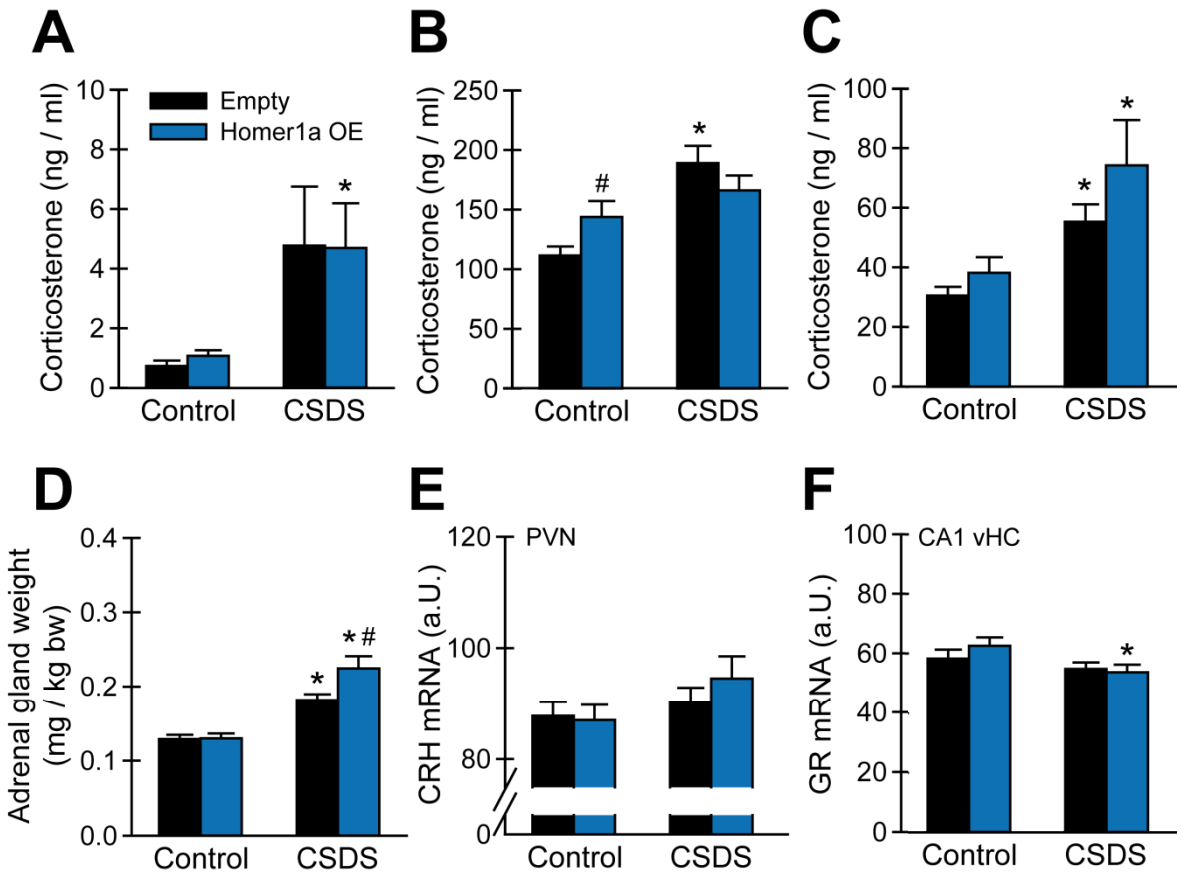


Figure 4: Overexpression of Homer1a in the hippocampus leads to HPA axis dysregulation. (A) Basal levels of circulating corticosterone were increased in response to CSDS, with a significant post hoc effect in Homer1a OE mice. (B) Under control conditions, Homer1a OE led to a hyperactivation of the HPA axis compared to Empty animals. This effect was not apparent in mice that underwent CSDS, possibly due to a ceiling effect, since CSDS strongly enhanced the corticosterone response to a novel stressor. (C) Recovery from a novel stressor was deteriorated due to the exposure to chronic stress, independent of Homer1a. (D) Under control conditions, overexpression of Homer1a did not affect adrenal gland size. However, the increase in size in response to CSDS was strongly enhanced in Homer1a OE animals compared to Empty littermates. (E) mRNA levels of CRH were not altered in this experiment. (F) CSDS resulted in a reduction of GR mRNA in the CA1 region of the ventral hippocampus. This effect reached post hoc significance in Homer1 OE mice but not in Empty animals. * Significant from control, $p < 0.05$, # Significant from Empty, $p < 0.05$. Other abbreviations as in figures 1 & 2.

Interestingly, Homer1a OE mice had significantly bigger adrenal glands when exposed to CSDS compared to Empty mice that were stressed (Figure 4D). Here, ANOVA revealed a condition ($F_{1,44} = 61.134$, $p < 0.001$) as well as an AAV ($F_{1,44} = 5.365$, $p < 0.05$) and a

condition × AAV interaction ($F_{1,44} = 4.845$, $p < 0.05$). Further post hoc testing confirmed that CSDS increased adrenal gland sizes in both AAV groups (Empty: $p < 0.001$; Homer1a OE: $p < 0.001$), but in stressed Homer1a OE animals, this increase was significantly bigger compared to stress Empty animals ($p < 0.05$). These HPA axis alterations were not accompanied by gene expression differences of CRH in the PVN, where ANOVA did not find a significant effect of either condition or AAV treatment (Figure 4E). Concerning GR expression in the ventral hippocampus, we found a significant reduction of GR mRNA in the CA1 region (ANOVA condition effect: $F_{1,44} = 5.100$, $p < 0.05$), which was significant in Homer1a OE ($p < 0.05$) but not in Empty mice (Figure 4F). A comprehensive overview of expression levels in HPA axis related genes can be found in supplemental table 2.

While being exposed to CSDS, mice overexpressing Homer1a in the hippocampus show a significant reduction in locomotion in the OF test (Figure 5A). Here, ANOVA revealed a condition effect ($F_{1,44} = 6.722$, $p < 0.05$) with post hocs showing that this reduction was significant in Homer1a OE mice ($p < 0.05$) but not in Empty animals ($p = 0.527$). Similar results were obtained in the SA test, where CSDS led to a reduced interaction ratio (ANOVA condition: $F_{1,44} = 5.171$, $p < 0.05$) which reached significance in Homer1a OE mice ($p < 0.05$) (Figure 5B). We could not detect an anxiety-related phenotype in the EPM that was connected to either CSDS or AAV treatment in this experiment (Figure 5C). The FUST revealed a significant stress effect in both the water ($F_{1,45} = 5.863$, $p < 0.05$) and the urine trial ($F_{1,44} = 27.368$, $p < 0.001$) (Figure 5D). In the water trial, post hoc test failed to reach significance, but both AAV groups showed a significant reduction in urine sniffing time when exposed to CSDS compared to the respective control groups (Empty: $p < 0.001$; Homer1a OE: $p < 0.01$). There was no difference between the AAV treatments. While under the effects of CSDS, overexpression of Homer1a in the hippocampus led to an increased behavioral despair and less active stress coping behavior as depicted by increased floating time in the FST (Figure 5E). Here, ANOVA revealed a condition ($F_{1,43} = 7.045$, $p < 0.05$) , an AAV ($F_{1,43} = 6.185$, $p < 0.05$) and a condition × AAV interaction effect ($F_{1,43} = 5.496$, $p < 0.05$). Following post hoc analysis, Homer1a OE mice showed significantly increased floating time compared to both their respective control ($p < 0.001$) as well as to stressed Empty mice ($p < 0.05$).

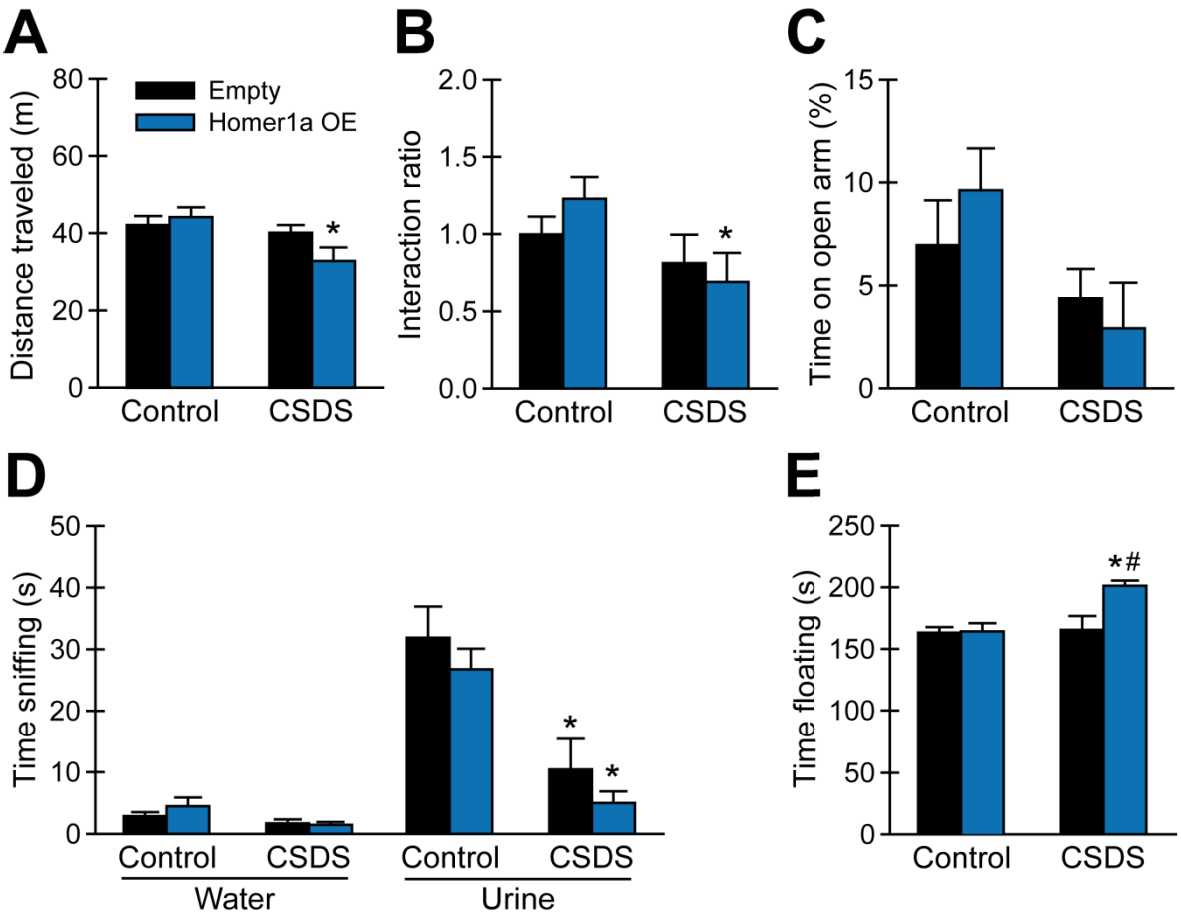


Figure 5: Homer1a OE mice exhibit increased behavioral vulnerability to CSDS. (A) While CSDS did not lead to a reduction in locomotion in Empty animals, overexpression of Homer1a affected the animals' behavior, indication a more pronounced susceptibility to CSDS in this parameter. (B) This effect was also apparent in the SA test, where only Homer1a OE mice showed a reduction in social interaction when stressed, but not Empty mice. (C) There was no significant anxiety-related effect visible in the EPM in either AAV group. (D) During the water trial of the FUST, all subgroups showed a comparable interest in the presented cotton tip. In the urine trial, however, CSDS led to a significant reduction in sniffing time in both AAV groups. (E) While under the effects of CSDS, Homer1a OE mice elicited less active stress coping behavior in the FST, further indicating a susceptibility to CSDS which leads to behavioral despair. * Significant from control, $p < 0.05$, # Significant from Empty, $p < 0.05$. Other abbreviations as in figures 1 & 3.

Experiment 3

Chronic treatment with CTEP did not alter the HPA axis response to CSDS in any of the examined parameters. We could detect robust CSDS effects both at basal circulating corticosterone ($F_{1,42} = 13.520$, $p < 0.01$; Vehicle: $p < 0.05$, CTEP: $p < 0.05$) and the corticosterone response to a novel stressor ($F_{1,44} = 30.317$, $p < 0.001$; Vehicle: $p < 0.001$, CTEP: $p < 0.01$) (Figure 6A, B). Treatment with CTEP did not affect these parameters both under basal and stress conditions. Recovery from the novel stressor was also disturbed in

animals that underwent CSDS, but was not influenced by CTEP treatment as indicated by an ANOVA condition effect ($F_{1,44} = 5.997$, $p < 0.05$) (Figure 6C). This effect could not be attributed to a specific treatment group as the condition effect did not result in significant post hoc tests. On the physiological level, CSDS resulted in increased adrenal glands (ANOVA condition effect: $F_{1,44} = 140.316$, $p < 0.001$; Vehicle: $p < 0.001$, CTEP: $p < 0.001$) with no effect of CTEP treatment (Figure 6D). Gene expression analysis in the PVN revealed that CRH mRNA levels were elevated in response to CSDS (ANOVA condition effect: $F_{1,44} = 8.894$, $p < 0.01$) (Figure 6E), with post hoc tests showing that this effect was significant in vehicle treated animals ($p < 0.05$) but failed to reach significance in the CTEP treatment group ($p = 0.053$). Interestingly, CTEP treatment induced an increase in GR mRNA levels in the CA1 region of ventral hippocampus, which was significant in stressed animals (ANOVA treatment effect: $F_{1,44} = 8.372$, $p < 0.01$; Control: $p = 0.063$, CSDS: $p < 0.05$) (Figure 6F). Also, CSDS resulted in a reduction of GR mRNA (ANOVA condition effect: ($F_{1,44} = 5.498$, $p < 0.05$), yet did not reach significance after post hoc analysis. ANOVA analysis of Homer1b/c expression levels in the CA1 region of the dorsal hippocampus revealed both a condition effect ($F_{1,44} = 6.610$, $p < 0.05$) and a condition × treatment interaction ($F_{1,44} = 4.404$, $p < 0.05$) (Figure 6G). Further post hoc analysis showed that in vehicle treated animals, Homer1b/c levels were increased ($p < 0.05$), whereas CTEP was able to abolish this effect, with significantly reduced mRNA levels compared to vehicle treated mice that underwent CSDS ($p < 0.01$). Expression data of all investigated HPA axis related genes and Homer1 isoforms in the hippocampus and PVN can be found in supplemental table 3.

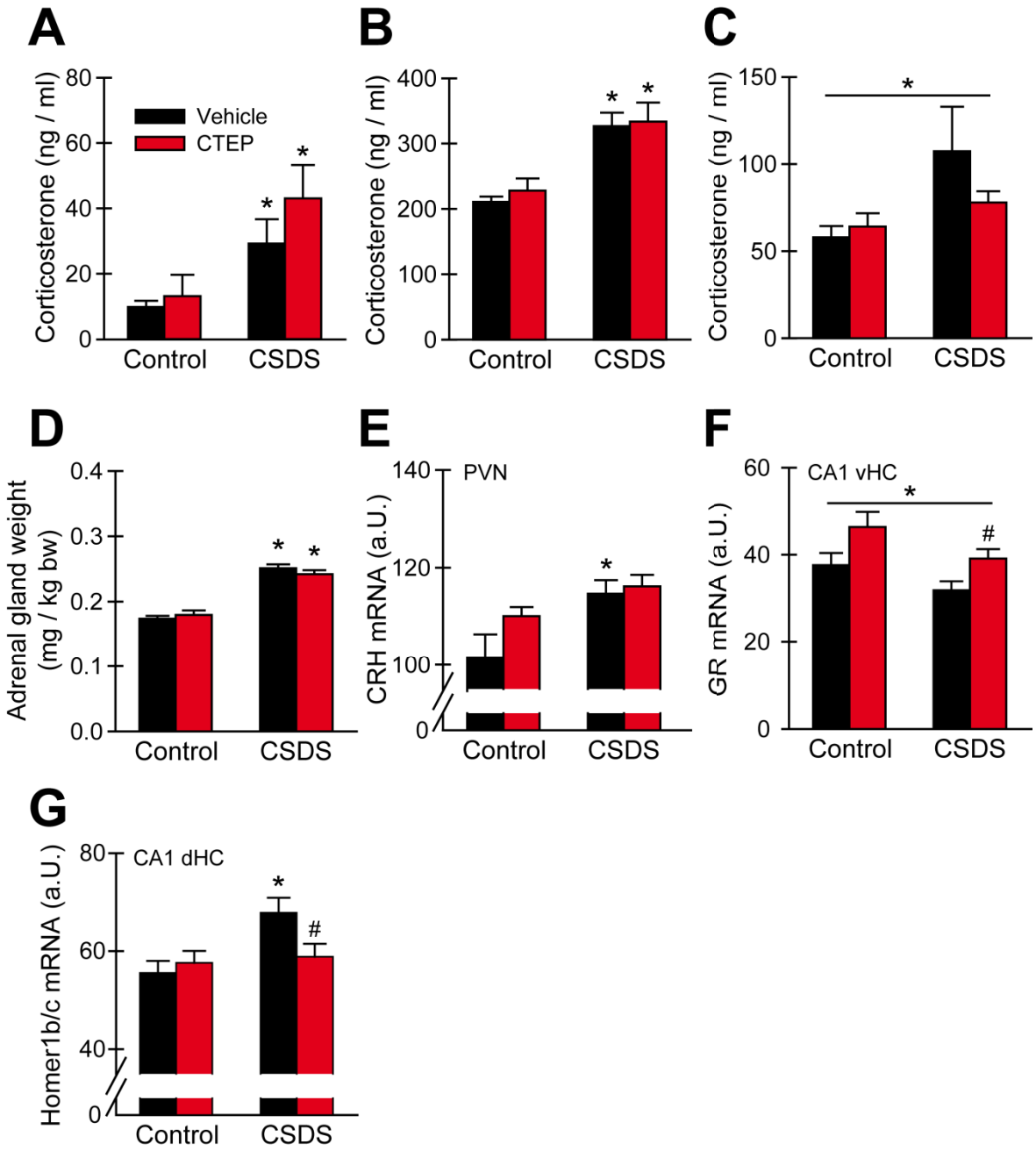


Figure 6: Treatment with CTEP does not alter HPA axis function. (A) Both treatment groups showed increased basal corticosterone levels when under the effects of CTEP. (B) Also, CSDS resulted in a stronger activation of the HPA axis in response to a novel stressor. Yet, CTEP did not affect this phenotype. (C) Recovery from the novel stressor was impaired in stressed animals. Again, there was no significant treatment effect. (D) Analogous the previous experiments, CSDS induced an increase in adrenal size that was also not affected by CTEP treatment. (E) In stressed vehicle treated animals, CRH mRNA levels in the PVN were elevated compared to their respective control group. CTEP treated animals did not show a significant alteration in CRH levels, possibly due to slightly increased basal levels of CRH. (F) Expression of GR in the CA1 region of the ventral hippocampus was reduced in stressed animals, but CTEP increased overall expression independent of the condition. (G) Increased mRNA levels of Homer1b/c in the CA1 region of the dorsal hippocampus in response to CSDS were rescued by CTEP treatment. * Significant from control, $p < 0.05$, # Significant from vehicle, $p < 0.05$. Other abbreviations as in figures 1, 2 & 3.

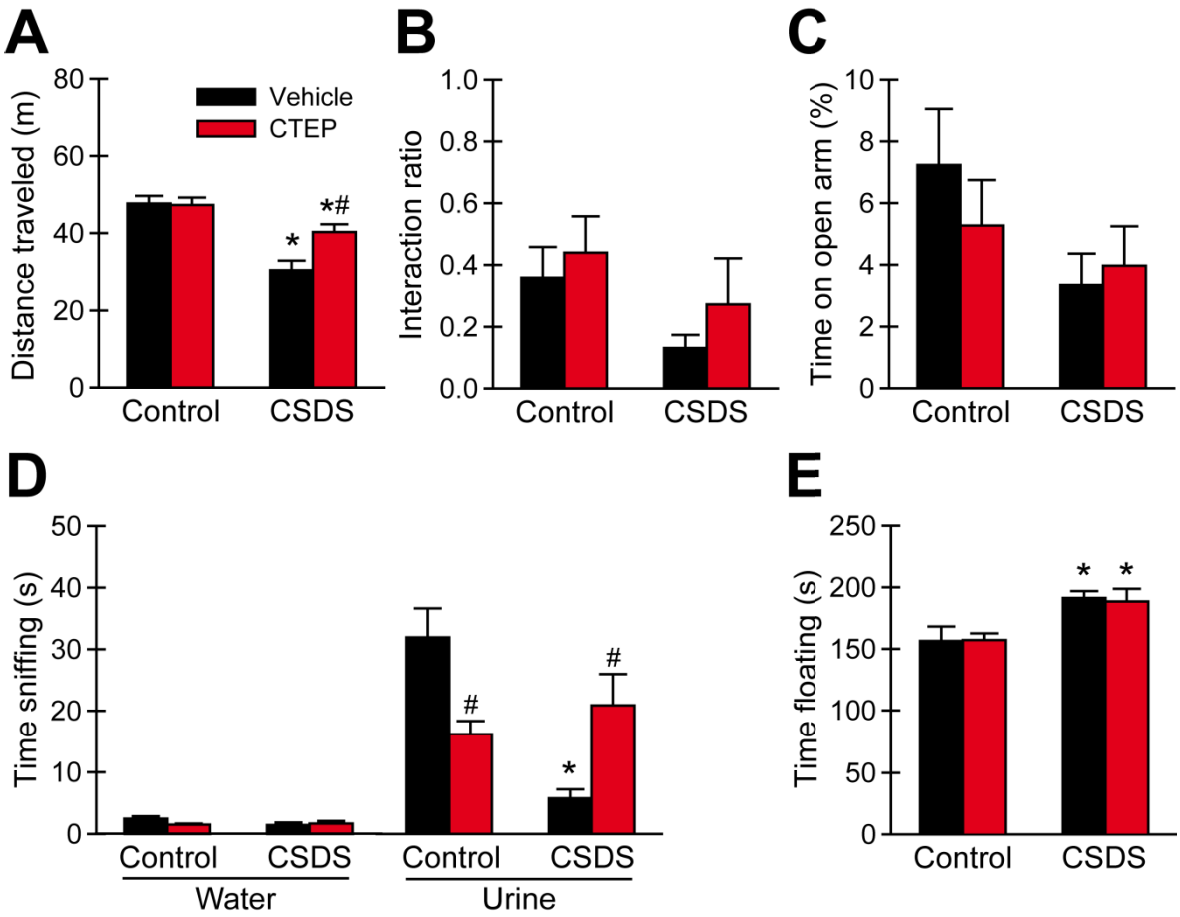


Figure 7: Treatment with CTEP can reverse stress-induced behavioral impairments. (A) While vehicle treated animals displayed a strong decrease in locomotion when being stressed, CTEP treatment was able to counteract this phenotype and significantly enhanced locomotion in the CSDS group. (B) There was no effect of CSDS or CTEP in the SA test. (C) Also, we could not detect an anxiety-like phenotype in this experiment as depicted by the results from the EPM. (D) In the FUST, the water trial did not reveal any differences between treatment and condition groups. However, CTEP treated animals expressed less interest in the urine dipped cotton tip under control conditions. In contrast, CSDS did not affect the anhedonic phenotype of CTEP animals, rendering them resilient to the stress-induced reduction in sniffing time that was present in the vehicle treated group. (E) While CSDS led to a decrease in active stress coping behavior in the FST, CTEP did not influence this behavioral parameter. * Significant from control, $p < 0.05$, # Significant from vehicle, $p < 0.05$. Other abbreviations as in figures 1 & 3.

A stress-induced reduction of locomotion in the OF test was reversed by chronic CTEP treatment (Figure 7A). Here, ANOVA revealed a condition effect ($F_{1,43} = 32.861$, $p < 0.001$) as well as a treatment effect ($F_{1,43} = 4.981$, $p < 0.05$) and a condition × treatment interaction ($F_{1,43} = 5.971$, $p < 0.05$). Post hoc testing confirmed that on the one hand, CSDS reduced locomotion in the vehicle treated group compared to control conditions ($p < 0.001$), while treatment with CTEP did not have an effect under basal conditions, but reversed the stress-induced effect to a large extend ($p < 0.01$). Yet, locomotion was still reduced due to CSDS in CTEP treated animals compared to their control littermates

($p < 0.05$). Both in the SA test (Figure 7B) and the EPM (Figure 7C), we could not detect a significant stress or treatment effect. However, regarding anhedonic behavior, CTEP treatment had differential effects on the animals. While there was no difference between either subgroups in the water trial of the FUST, ANOVA revealed both a condition ($F_{1,43} = 8.349$, $p < 0.01$) and a condition × treatment interaction effect ($F_{1,43} = 17.281$, $p < 0.001$) in the urine trial (Figure 7D). Further post hoc tests indicated that CTEP reduces the interest in female urine under basal conditions ($p < 0.01$). Yet, while vehicle treated mice that underwent CSDS showed a strong reduction in sniffing time ($p < 0.001$), this effect was reversed by the CTEP treatment in the same condition group ($p < 0.05$). Regarding active stress coping behavior, we could detect a robust effect of CSDS ($F_{1,44} = 14.109$, $p < 0.01$; Vehicle: $p < 0.05$, CTEP: $p < 0.05$) with no influence of CTEP on this parameter.

Discussion

In the current study, we provide strong evidence for the involvement of Homer1/mGluR5 signaling in the regulation of HPA axis activity and show that manipulation of this signaling pathway can profoundly alter the neuroendocrine and behavioral consequences of chronic stress. First, we could show that a total knockout of Homer1 leads to considerable consequences on various levels, which are related to, but rather independent from chronic stress exposure. Furthermore, we demonstrate that an imbalance of the aforementioned signaling pathway in the hippocampus by means of overexpressing Homer1a increases the vulnerability to chronic stress on the physiological, neuroendocrine and behavioral level. On the other hand, we were also able to rescue some stress-induced behavioral alterations by chronic administration of the novel, orally bioavailable mGluR5 antagonist CTEP without interfering with HPA axis function. These findings propose the mGluR5/Homer1 signaling pathway as a prominent target for development of novel treatment strategies, especially with respect to stress-induced pathologies such as depression.

In this study, we are the first to show a major disturbance of HPA axis activity in mice that are deficient in Homer1. This is evidenced on the one hand on the physiological level, where Homer1KO mice show enlarged adrenal glands, which is in line with previous reports (Grinevich et al., 2011). On the other hand, we could also show that corticosterone release in response to stress is severely altered in these animals. A hyperactive corticosterone response induced by CSDS is frequently observed in this paradigm (Wagner et al., 2011; Wang et al., 2011a; Hartmann et al., 2012) and deletion of Homer1 further increased this effect, indicating a prominent regulatory role of this glutamatergic pathway in the feedback regulation of the HPA axis. In contrast to previous reports, we could also detect various alterations in central gene expression patterns related to HPA axis feedback regulation (Grinevich et al., 2012). Grinevich and colleagues suggest that deletion of Homer1 results in altered steroidogenesis on the level of the adrenal cortex, where stimulation by ACTH lead to an increased corticosterone response, while central HPA axis regulation was not affected (Grinevich et al., 2011). In our study, CRH mRNA levels in the PVN were reduced in Homer1KO mice, while GR levels in the hippocampus were exclusively reduced in response to CSDS, a phenotype which may

readily contribute to the reduced feedback ability of these mice when chronically stressed.

We also observed a strong hyperactive phenotype due to Homer1 deletion, which has also been indicated in previous studies that employed this mouse model (Szumlinski et al., 2005; Jaubert et al., 2007). More precisely, these hyperactive behaviors in general led to a reversal of the CSDS induced phenotype, which was mostly visible in locomotive and social behavior. However, we also detected a reduced interest in interacting with novel stimuli in KO animals, such as in the FUST. These behavioral patterns may be ascribed to an attention deficit hyperactivity disorder (ADHD)-like phenotype (Sagvolden et al., 2005), which has previously been linked to altered Homer1 expression profiles in the prefrontal cortex and the hippocampus (Hong et al., 2009; Hong et al., 2011). It is important to note that based on the present data, we are not able to discern immediate effects of Homer1 deletion from developmental effects that originate in earlier stages of the animal's life. Indeed, Homer1 has been shown to be strongly expressed in developing tissues (Shiraishi-Yamaguchi and Furuichi, 2007) and a total knockout is therefore likely to exert major effects on these animals before the CSDS procedure started. Nonetheless, these findings indicate the importance of Homer1-mediated signaling in the response to CSDS, and we therefore continued to investigate its role by selectively overexpressing the immediate early form Homer1a specifically in the hippocampus.

A specific overexpression of Homer1a in the hippocampus and the subsequent alterations of mGluR5/Homer1b/c mediated signaling led to changes in HPA axis activity both under basal and under CSDS conditions, thereby indicating an increase in vulnerability to both acute and chronic stress. This phenotype is not only present on the neuroendocrine and physiological level but also reflected in different behavioral parameters. Here, Homer1a overexpression predominantly increased the effect of CSDS on the animals, further supporting the hypothesis of detrimental gene by environment interactions involving Homer1 that may ultimately contribute to the emergence of depression (Rietschel et al., 2010). Activation of Homer1a gene transcription is a rapid and plastic process in response to synaptic activity (Brakeman et al., 1997; Kato et al., 1997; de Bartolomeis and Iasevoli, 2003). We therefore hypothesize that repeated transcriptional activation of this immediate early gene in response to the daily defeat sessions induces counterregulatory changes in the central stress systems, including the upregulation of Homer1b/c (See

Chapter 2, but also Berton et al., 2006) as well as alterations in GR and MR expression levels. These disturbances, in turn, may contribute to the vulnerable behavioral and neuroendocrine phenotypes that we observed under the influence of CSDS. Prolonged ligand-independent activation of mGluR5 via abundant Homer1a protein levels also severely affects IP_3 receptor activation and subsequent downstream signaling (Ango et al., 2001, Kammermeier, 2008). The continuous presence of Homer1a may therefore profoundly change neuronal signaling pathways that may in turn render the organism more vulnerable to chronic stress. In addition, it has previously been shown that interactions between NMDA and mGluR5 are mediated by the PSD95/Shank/Homer1 complex (Hayashi et al., 2009; Bertaso et al., 2010), and Homer1a was demonstrated to be a key modulator of mGluR5 coupling to effector targets that produce excitatory postsynaptic currents (Kammermeier and Worley, 2007). Given the increasing body of evidence that imply NMDA receptor targeting agents as potential novel, rapid-acting antidepressant treatment option (Kavalali and Monteggia, 2012; Krystal et al., 2013), a Homer1a mediated overactivation of this signaling pathway may profoundly affect antidepressant treatment efficacy. The development of new drugs that target this system, mainly the mGluR5, may therefore be of great value and importance (Krystal et al., 2010; Sanacora et al., 2012).

Consequently, we administered the novel, bioavailable mGluR5 antagonist CTEP (Lindemann et al., 2011) to mice that were subjected to chronic stress. Under basal conditions, CTEP did not have any detrimental effects on the physiological or neuroendocrine level. Also, blockade of mGluR5 signaling over the course of the stress exposure did not affect HPA axis function or modulation, since both treatment groups showed similar corticosterone profiles under all measured conditions. These findings indicate that while modulation of Homer1 function can prime HPA axis activity towards a more sensitive phenotype, this cannot be counteracted by blockade of mGluR5 signaling, thereby suggesting alternative signaling pathways in the PSD that lead to the observed functional changes in HPA axis function. However, CTEP did have beneficial effects on the behavioral phenotype of stressed animals. Here, we could demonstrate that stress-induced anhedonia and reduced locomotion was rescued in animals that received CTEP. Thus, while CTEP did not reverse all stress-induced phenotypes, it showed therapeutic value in some behavioral parameters. These results strengthen the idea of combining

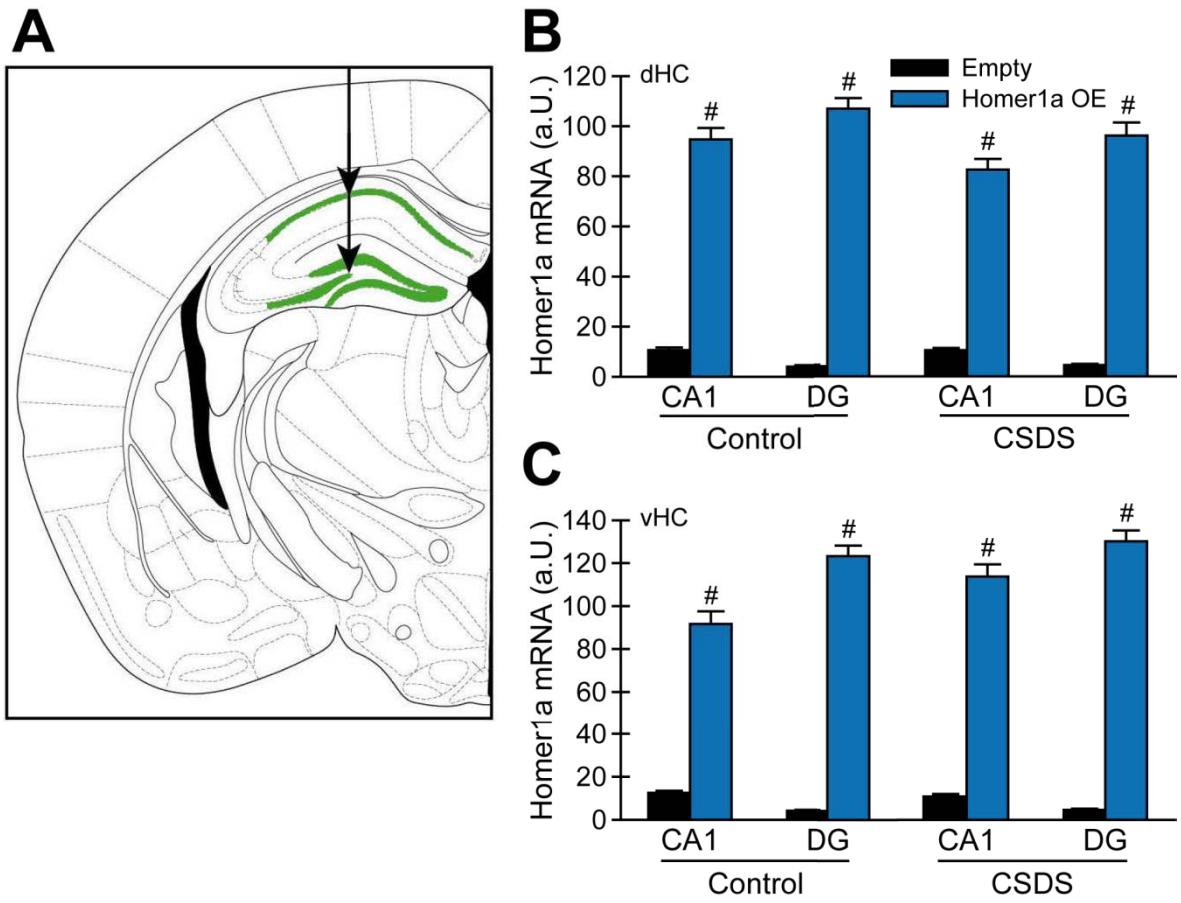
different antidepressant treatments to maximize therapeutic efficacy (Palaniyappan et al., 2009; Connolly and Thase, 2011). Indeed, CTEP may serve as a basis for future antidepressants that specifically target the glutamate system, since its pharmacokinetic properties are significantly improved from previous mGluR5 antagonists such as MPEP and MTEP (Anderson et al., 2003; Busse et al., 2004; Lindemann et al., 2011).

It should be kept in mind that comparing the behavioral and molecular results across experiments can only be done with reservations. Although the actual stress paradigms were performed analogous, there are some differences, which may impact certain parameters. In experiment 1, we used a knockout mouse line that was bred in house for >5 generations, which can already lead to a certain genetic drift that differentiates this animal batch from other C57BL/6 mice ordered from outside companies. In experiment 2, all animals underwent a stressful surgery and one week of treatment with an analgesic, and although the mice were allowed to recover for 3 weeks, we cannot rule out that this intervention also produces long-term consequences. In experiment 3, all animals were gavaged while undergoing the CSDS paradigm, which acts as a stressor in itself and may particularly influence the performance of the control groups but also enhance the effects of CSDS. For example, we detected different performances in some behavioral tests, such as the FST, where a strong effect of CSDS was apparent in the third but not in the other experiments. Also, this effect on floating behavior has not been reported before in context of this stress paradigm (Wagner et al., 2011; Wang et al., 2011a). Nonetheless, within one experiment, the results presented are consistent and of high technical quality and should therefore allow for valid interpretation.

In summary, our study provides compelling evidence for the involvement of the Homer1/mGluR5 signaling pathway in the emergence and regulation of stress-induced behavioral and neuroendocrine phenotypes. We could show that HPA axis function is strongly disturbed in animals that either carry a total knockout of Homer1 or overexpress Homer1a in the hippocampus. We could also demonstrate that increased levels of Homer1a lead to a stress-vulnerable behavioral phenotype, with blockade of mGluR5 by CTEP being able to recover the stress-induced behavioral alterations. These findings strongly support the glutamate hypothesis of depression (Sanacora et al., 2012) and further illustrate the importance of searching for novel antidepressant treatment strategies. With the present data indicating a major involvement of the Homer1/mGluR5

pathway in stress-related psychiatric disorder, further research to elucidate the contributing molecular mechanisms is highly warranted.

Supplemental Material



Supplemental figure 1: Injection site and quantification of Homer1a overexpression in the hippocampus. (A) The viral construct was injected in the CA1 and dentate gyrus (DG) region of the dorsal hippocampus. Virus spread after 6 weeks is depicted in green. (B) After 6 weeks, Homer1a mRNA was robustly increased in both injected subregions of the dorsal hippocampus (dHC) compared to control mice. (C) The ventral hippocampus (vHC) was also infected and showed a strong upregulation of Homer1a mRNA in both measured subregions. # Significant from Empty, $p < 0.05$

Gene symbol	Region	ANOVA effect			Expression levels (a.U.)			
		condition	genotype	interaction	WT control	KO control	WT CSDS	KO CSDS
MR	dHC CA1	n.s.	n.s.	n.s.	31.81 ± 2.28	30.07 ± 1.29	28.59 ± 1.63	34.62 ± 2.39
MR	dHC CA3	n.s.	n.s.	n.s.	21.25 ± 1.59	20.38 ± 0.83	20.05 ± 1.13	24.14 ± 1.54
MR	dHC DG	n.s.	n.s.	n.s.	48.40 ± 2.28	45.36 ± 1.51	46.44 ± 1.98	48.07 ± 1.92
MR	vHC CA1	n.s.	n.s.	n.s.	22.43 ± 1.00	24.14 ± 1.11	19.53 ± 2.09	23.51 ± 1.83
MR	vHC CA3	n.s.	n.s.	n.s.	19.83 ± 1.12	20.55 ± 0.85	16.87 ± 1.91	20.09 ± 1.50
MR	vHC DG	$p < 0.05$	n.s.	n.s.	38.24 ± 1.50	37.86 ± 1.20	32.20 ± 3.46	33.69 ± 2.17
GR	dHC CA1	n.s.	n.s.	n.s.	85.04 ± 1.57	82.60 ± 2.60	81.71 ± 2.19	78.17 ± 1.48
GR	dHC CA3	$p < 0.05$	n.s.	n.s.	54.20 ± 1.09	51.70 ± 2.63	50.28 ± 2.20	47.02 ± 1.51
GR	dHC DG	$p < 0.05$	n.s.	n.s.	94.06 ± 0.90	92.45 ± 2.64	90.92 ± 1.87	85.43 ± 1.35 *#
GR	vHC CA1	$p < 0.001$	$p < 0.001$	$p < 0.01$	95.85 ± 1.77	94.11 ± 2.52	93.92 ± 1.34	80.22 ± 2.90 *#
GR	vHC CA3	$p < 0.01$	n.s.	n.s.	65.20 ± 2.35	59.16 ± 2.64	63.70 ± 1.37	54.76 ± 2.61 #
GR	vHC DG	$p < 0.05$	$p < 0.01$	$p < 0.05$	94.76 ± 2.36	94.50 ± 2.84	93.15 ± 2.02	81.76 ± 2.31 *#
GR	PVN	n.s.	n.s.	n.s.	74.81 ± 2.40	73.22 ± 2.52	75.65 ± 2.47	71.70 ± 2.72
CRH	PVN	$p < 0.01$	$p < 0.01$	n.s.	28.45 ± 1.07	23.26 ± 1.18 #	33.15 ± 1.42 *	28.56 ± 2.03 *#

Supplemental table 1: Overview of gene expression analysis in experiment 1. Values are depicted in means ± SEM. MR: Mineralocorticoid receptor; GR: Glucocorticoid receptor; CRH: Corticotropin-releasing hormone; dHC: Dorsal hippocampus; vHC: Ventral hippocampus; n.s.: not significant; WT: Wild type; KO: Knockout; CSDS: Chronic social defeat stress. * Significant from control of same genotype, # Significant from Wild type of same condition.

Gene symbol	Region	ANOVA effect			Expression levels (a.U.)			
		condition	AAV	interaction	Empy control	H1a OE control	Empty CSDS	H1a OE CSDS
MR	dHC CA1	n.s.	$p < 0.05$	n.s.	35.71 ± 3.48	31.81 ± 2.58	41.14 ± 2.77	29.13 ± 4.14 #
MR	dHC CA3	n.s.	n.s.	n.s.	34.43 ± 3.97	32.37 ± 2.58	40.15 ± 3.09	30.95 ± 4.30
MR	dHC DG	n.s.	$p < 0.05$	n.s.	48.90 ± 4.69	47.04 ± 3.53	61.06 ± 2.50	43.82 ± 5.32 #
MR	vHC CA1	n.s.	n.s.	n.s.	64.13 ± 2.20	65.30 ± 2.50	60.07 ± 3.87	58.19 ± 3.41
MR	vHC CA3	n.s.	n.s.	n.s.	61.43 ± 2.63	63.49 ± 3.58	59.10 ± 4.70	58.58 ± 3.93
MR	vHC DG	n.s.	n.s.	n.s.	73.01 ± 3.75	76.68 ± 3.76	72.76 ± 5.74	67.15 ± 4.85
GR	dHC CA1	$p < 0.05$	n.s.	n.s.	46.27 ± 3.27	45.87 ± 2.40	40.76 ± 2.70	38.06 ± 2.48 *
GR	dHC CA3	n.s.	n.s.	n.s.	14.21 ± 1.39	13.06 ± 1.19	12.29 ± 1.29	11.35 ± 0.84
GR	dHC DG	n.s.	n.s.	n.s.	38.28 ± 3.13	40.78 ± 2.33	34.82 ± 2.30	35.07 ± 2.03
GR	vHC CA1	$p < 0.05$	n.s.	n.s.	58.16 ± 3.01	62.45 ± 2.87	54.65 ± 2.31	53.41 ± 2.85 *
GR	vHC CA3	$p < 0.05$	n.s.	n.s.	23.76 ± 1.98	24.80 ± 1.80	19.86 ± 1.18	20.77 ± 1.60
GR	vHC DG	$p < 0.05$	n.s.	n.s.	42.84 ± 3.28	45.73 ± 2.90	36.52 ± 2.52	40.13 ± 2.84
GR	PVN	$p < 0.01$	n.s.	n.s.	43.13 ± 2.98	42.68 ± 2.02	46.51 ± 1.37	51.45 ± 2.04 *
CRH	PVN	n.s.	n.s.	n.s.	87.70 ± 2.51	86.97 ± 2.74	90.18 ± 2.68	94.54 ± 3.98

Supplemental table 2: Overview of gene expression analysis in experiment 2. Values are depicted in means ± SEM. AAV: Adeno-associated virus; H1a OE: Homer1a overexpression; Other abbreviations as in supplemental table 1. * Significant from control of same AAV, # Significant from Empty of same condition.

Gene symbol	Region	ANOVA effect			Expression levels (a.U.)			
		condition	treatment	interaction	Vehicle control	CTEP control	Vehicle CSDS	CTEP CSDS
MR	dHC CA1	n.s.	n.s.	n.s.	81.76 ± 4.35	77.69 ± 4.37	74.59 ± 4.86	80.36 ± 5.43
MR	dHC CA3	n.s.	n.s.	n.s.	69.58 ± 4.83	65.99 ± 4.98	65.70 ± 4.94	70.81 ± 5.85
MR	dHC DG	n.s.	n.s.	n.s.	92.17 ± 4.68	92.48 ± 3.86	90.58 ± 4.29	93.82 ± 4.89
MR	vHC CA1	n.s.	n.s.	n.s.	20.57 ± 2.30	19.69 ± 2.61	21.38 ± 1.97	21.78 ± 3.22
MR	vHC CA3	n.s.	n.s.	n.s.	16.30 ± 1.63	15.26 ± 1.86	19.40 ± 1.26	17.42 ± 2.58
MR	vHC DG	n.s.	n.s.	n.s.	39.21 ± 3.05	38.38 ± 3.28	44.15 ± 1.86	38.39 ± 4.87
GR	dHC CA1	n.s.	n.s.	n.s.	61.07 ± 1.43	62.42 ± 1.64	63.97 ± 2.20	62.26 ± 3.73
GR	dHC CA3	n.s.	n.s.	n.s.	22.44 ± 1.38	23.08 ± 1.04	23.33 ± 1.06	24.24 ± 2.04
GR	dHC DG	n.s.	n.s.	n.s.	55.39 ± 2.12	59.13 ± 1.96	58.88 ± 1.83	58.87 ± 3,49
GR	vHC CA1	$p < 0.05$	$p < 0.01$	n.s.	37.62 ± 2.80	46.36 ± 3.48	31.90 ± 1.98	39.13 ± 2.15 #
GR	vHC CA3	$p < 0.05$	$p < 0.01$	n.s.	14.38 ± 1.03	18.32 ± 1.32 #	12.45 ± 1.15	15.47 ± 0.87 #
GR	vHC DG	n.s.	$p < 0.02$	n.s.	32.15 ± 2.04	41.03 ± 2.65 #	30.28 ± 1.77	34.10 ± 2.49
GR	PVN	n.s.	n.s.	n.s.	66.93 ± 2.57	65.69 ± 2.73	60.97 ± 3.30	66.67 ± 2.72
CRH	PVN	$p < 0.01$	n.s.	n.s.	101.56 ± 4.74	110.05 ± 1.88	114.63 ± 2.81 *	116.16 ± 2.34
Homer1a	dHC CA1	n.s.	n.s.	n.s.	33.96 ± 2.84	32.85 ± 2.57	33.43 ± 4.50	34.97 ± 3.65
Homer1a	dHC CA3	n.s.	n.s.	n.s.	19.59 ± 1.81	19.08 ± 1.44	20.09 ± 3.18	20.81 ± 2.61
Homer1a	dHC DG	n.s.	n.s.	n.s.	15.83 ± 1.45	16.28 ± 1.17	18.05 ± 1.88	18.22 ± 1.44
Homer1a	vHC CA1	n.s.	n.s.	$p < 0.05$	37.77 ± 4.52	33.19 ± 3.55	31.29 ± 3.89	42.63 ± 3.24 #
Homer1a	vHC CA3	n.s.	n.s.	n.s.	27.16 ± 3.22	25.34 ± 2.85	24.27 ± 2.67	29.63 ± 2.56
Homer1a	vHC DG	n.s.	n.s.	n.s.	18.73 ± 2.19	16.24 ± 1.68	17.52 ± 1.93	22.14 ± 1.71
Homer1b/c	dHC CA1	$p < 0.05$	n.s.	$p < 0.05$	55.55 ± 2.45	57.62 ± 2.44	67.85 ± 3.04 *	58.86 ± 2.65 #
Homer1b/c	dHC CA3	n.s.	n.s.	n.s.	42.61 ± 2.35	45.54 ± 2.12	50.29 ± 2.99	45.88 ± 2.47
Homer1b/c	dHC DG	n.s.	n.s.	n.s.	23.78 ± 1.69	26.29 ± 1.51	29,65 ± 2,62	25.66 ± 1.56
Homer1b/c	vHC CA1	n.s.	n.s.	n.s.	63.93 ± 2.05	59.34 ± 2.65	60.65 ± 3.31	63.81 ± 2.77
Homer1b/c	vHC CA3	n.s.	n.s.	n.s.	54.94 ± 1.91	53.01 ± 2.77	57.78 ± 2.07	55.76 ± 2.73
Homer1b/c	vHC DG	n.s.	n.s.	n.s.	30.00 ± 1.61	29.39 ± 1.36	31.05 ± 1.30	28.64 ± 1.66

Supplemental table 3: Overview of gene expression analysis in experiment 3. Values are depicted in means ± SEM. Abbreviations as in supplemental table 1. * Significant from control of same treatment.

3. General discussion

Besides the monoaminergic systems, other signaling circuits have received growing attention with respect to mood disorders in recent years. In particular, the main excitatory neurotransmitter glutamate plays a decisive role in superordinate systems that, if malfunctioning, may contribute to the development of depression (Mathews et al., 2012; Sanacora et al., 2012). Since chronic stress is a commonly accepted risk factor for mood disorders such as depression, it is of particular importance to understand the molecular mechanisms that are induced by stress and promote psychopathology. However, to date, the impact of stress, especially chronic stress, on glutamatergic neurotransmission is still not well understood (Popoli et al., 2012).

The current thesis now provides a large body of evidence that Homer1, a postsynaptic scaffold protein, which mainly links group I mGluRs to intracellular targets that modulate Ca^{2+} release, is crucially involved in mediating the behavioral and neuroendocrine consequences of stress in the central nervous system. At first, we further validated the well-established chronic social defeat stress paradigm with respect to both immediate and long-term consequences. Concurrently, we tested whether currently prescribed antidepressant treatment can alleviate the stress-induced symptoms (Chapter 1). We then extensively investigated the role of different Homer1 isoforms with respect to behavior, endocrinology and central gene expression profiles under both acute (Chapter 3) and chronic social stress conditions (Chapters 2, 5) and also elucidated its function in reward behavior (Chapter 4). By including both pharmacological and virally mediated gene expression modulation, we could demonstrate the functional relevance of Homer1 for stress-induced memory impairments, HPA axis dysregulation and behavioral alterations such as anhedonia. In a clinical context, these symptoms are important markers for major depression (Ising and Holsboer, 2006; Fairhall et al., 2010; Der-Avakian and Markou, 2012), which leads to the conclusion that Homer1 and its interaction partners, including group I mGluRs, may be critically involved in the development of such mood disorders.

3.1. Social defeat stress

In this thesis, we applied social defeat stress as the main stressor for all experiments. In support of the existing literature, we could convincingly demonstrate that defeat stress exerts a major impact on the individual. Single defeat events lead to a strong activation of the HPA axis and a subsequent increase in circulating corticosterone. However, the behavioral consequences of this acute stressor are highly specific with respect to memory processes and can only be observed in a limited time window (Chapter 3). In contrast, chronic application of social defeat has a multitude of consequences on different levels, including physiology, behavior and neuroendocrinology (Chapters 1, 2 & 5). Further strengthening the viability of this stressor model, the effects observed in this thesis are highly reproducible across experiments, both on the acute and chronic level and also confirm effects that have previously been published (Wagner et al., 2011; Wang et al., 2011a; Hartmann et al., 2012).

In contrast to more artificial stressors typically used in e.g. CUMS models, this type of social stressor is also of high etiological value, since social conflict is commonly encountered in natural settings. Additionally, the severity of the stressor and its perception is unpredictable and highly variable for the affected experimental animal, because it is largely based on the interaction of the conspecifics, which also reduces a potential bias caused by the investigator. Nonetheless, when comparing this paradigm with other social defeat models, it becomes clear that there is certain variability especially in the behavioral consequences that are most likely due to the different length and intensity of the applied defeat (Venzala et al., 2012). More precisely, we applied 21 relatively short bouts of physical defeat that usually lasted less than 1 min, followed by 24 h of sensory exposure to the dominant resident. In our experience, this protocol significantly reduces the confounding effects of physical injuries, which are more likely to occur during prolonged defeat sessions. In contrast, Nestler and colleagues usually apply ten defeat sessions of 5 to 10 min length, followed by sensory contact similar to the paradigm used in this thesis (Krishnan and Nestler, 2008; Golden et al., 2011). As a main readout, social avoidance towards an unfamiliar conspecific is measured. This behavioral phenotype is readily inducible after ten days of social defeat, long-lasting and reversible by antidepressant treatment, thereby providing good face and predictive validity (Berton et al., 2006; Covington, III et al., 2011). Based on the social avoidance behavior, the

chronic social defeat model has been extensively used to investigate basic molecular principles of stress vulnerability, to identify important brain structures involved in these processes, as well as to evaluate the efficacy of novel potential antidepressant compounds (Nestler and Hyman, 2010). However, social avoidance alone may not sufficiently reflect the complex disease phenotype of depression. Consequently, more elaborate tests are needed to appropriately model the human situation (Dzirasa and Covington, III, 2012). In this thesis, we reinforce the validity of the 21-day social defeat paradigm by providing additional endophenotypes that are related to psychiatric disorders, such as disturbed HPA axis function and central gene expression alterations, which resemble the situation in patients suffering from depression (Holsboer, 2000; Ising et al., 2007). We also induce lasting changes on the behavioral level that not only include social avoidance but also encompass locomotion, anhedonia, and anxiety-related behavior, thereby providing good face and construct validity.

Nevertheless, given the immense complexity of depression pathology and the lack of understanding of the neurophysiological underpinnings of this disease, social defeat is only able to model certain aspects of depression in rodents. It is therefore of utmost importance that these models are constantly reviewed and refined in light of new findings in the field of psychiatric research. For example, functional magnetic resonance imaging has been used to identify abnormalities of neural structures in patients and thereby provides new connecting factors that need to be taken into account for the improvement of animal models (Sheline et al., 2010; Hasler and Northoff, 2011). As shown in this thesis, our social defeat stress model provides an excellent framework for the incorporation of such novel therapeutic, pharmacologic, and genetic approaches to investigate the neurophysiological basics of mood disorders.

3.2. Homer1 is involved in the stress response

The postsynaptic scaffold Homer1 has been thoroughly investigated with respect to its molecular function (Brakeman et al., 1997; Xiao et al., 2000). Also, a prominent role in neuroplasticity, in particular synaptic remodeling has been proposed (Ango et al., 2000; Foa et al., 2001). The consequences of Homer1 manipulation with respect to behavioral alterations have already been implicated in drug addiction and, to a lesser extent, animal models of schizophrenia (Szumlinski et al., 2004; Szumlinski et al., 2005; Szumlinski et al., 2006). Yet, the role of Homer1 in the response to acute and chronic stress and its relevance for stress-related disorders such as depression has not been extensively addressed so far. The current thesis now provides multiple lines of evidence that Homer1 plays a crucial role in the mediation of the stress response, thereby substantially expanding the current knowledge about the involvement of the glutamatergic system in stress-related psychopathology (Popoli et al., 2012).

Previous reports made mention of an upregulation of Homer1 after chronic stress in two microarray studies but did not further investigate this signaling pathway (Berton et al., 2006; Schmidt et al., 2010). We now thoroughly replicated the reported regulation of hippocampal Homer1 in response to chronic social defeat stress in several independent samples using different technical approaches such as qPCR, microarray and *in situ* hybridization (Chapter 2). In addition, a more in-depth analysis of the different Homer1 isoforms was performed and revealed that a single defeat session induces transcription of the IEG Homer1a and a subsequent downregulation of the constitutively expressed long isoforms Homer1b/c (Chapter 3). Together with the results from the chronic stress experiments, where Homer1a was not significantly regulated at the end of the CSDS period, while Homer1b/c expression was increased, it becomes apparent that different Homer1 isoforms are dynamically regulated by social stress.

Based on these results, it is likely that a repeated activation of this transcriptional cascade induced by an acute stressor, as apparent during the CSDS paradigm, leads to a counterregulatory upregulation of Homer1b/c that on the one hand neutralizes the acute stress-induced downregulation, but on the other hand causes a severe imbalance of this signaling pathway under basal conditions (Figure 3.1). This hypothesis is further supported by data from our lab (Chapter 3) and others (Tronson et al., 2010), showing that Homer1b/c coupling to the mGluR5 is reduced in response to stress. It is therefore

likely that, in the long term, this deficit is compensated by increased overall levels of long Homer1 isoforms.

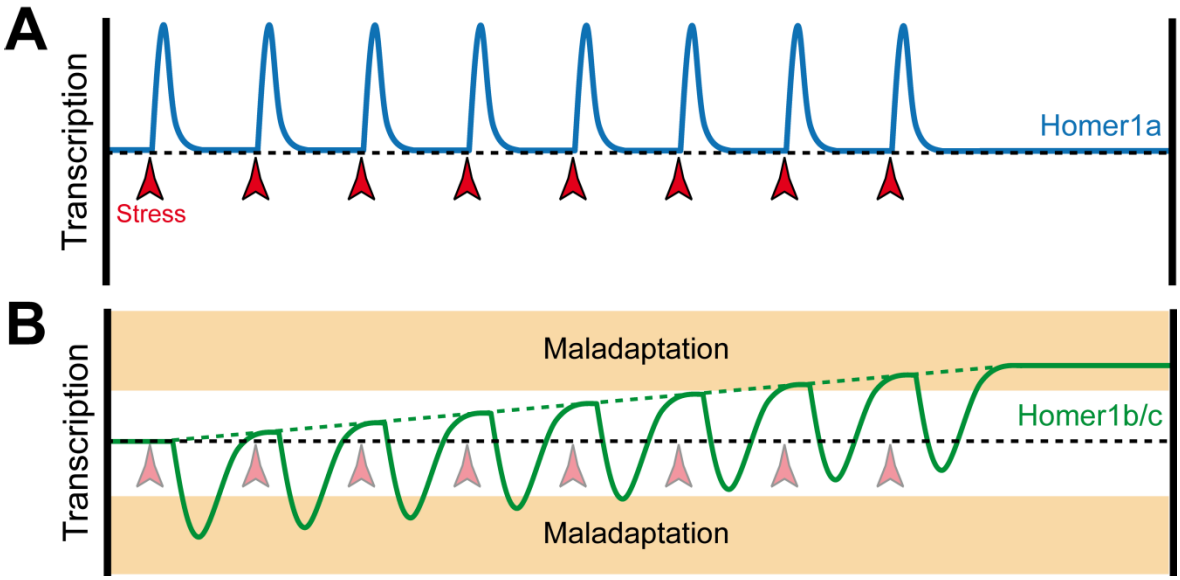


Figure 3.1: Schematic hypothesis of transcriptional Homer1 regulation in response to stress. (A) Homer1a transcription is rapidly induced by stress, but quickly returns to basal levels (Dotted black line). (B) Homer1b/c levels decrease in response to stress after a slight delay, possibly in a direct reaction to increased Homer1a levels. As adaptational process, Homer1b/c transcription levels are steadily increased after several stress exposures, which consequently leads to increased basal Homer1b/c levels (Dotted green line). These, in turn, may have maladaptive consequences under specific conditions.

Interestingly, the regulatory effects described above do not appear to be driven by GR signaling or corticosterone, but may rather be an intrinsic effect of augmented mGluR5 activation, most likely due to ligand-independent signaling induced by Homer1a (Ango et al., 2001). This is supported by the fact that GR activation alone did not induce a modulation of Homer1b/c transcription, nor did blockade of the GR during the defeat reverse the memory deficits induced by stress (Chapter 3). In contrast, blockade of mGluR5 activity during the stress period was able to rescue the acute stress-induced behavioral effects (Chapter 3) as well as the upregulation of Homer1b/c after CSDS (Chapter 5). Furthermore, antagonizing mGluR5 positively modified the behavioral phenotype of chronically stressed mice, supporting a possible potential of this drug class as a treatment option for psychiatric disorders (Palucha and Pilc, 2007; Pilc et al., 2008; Piers et al., 2012).

A popular concept regarding the IEG Homer1a is the idea that it acts as a dominant negative to long Homer1 isoforms (Brakeman et al., 1997). This hypothesis would suggest

that overexpression of Homer1a on the one hand should exert similar effects as a blockade of mGluR5, and on the other hand have opposite consequences compared to an overexpression of Homer1b/c. Our current data now indicates that the simple concept of Homer1a acting in a completely contrary direction to Homer1b/c cannot be fully sustained. Indeed, the complex behavioral and neuroendocrine alterations, which arise from the manipulation of the mGluR5/Homer1 pathway, are likely to be embedded in a bigger framework of interactions that include both main Homer1 isoforms as well as other postsynaptic scaffolds and effectors such as Shank or Preso1 (Hayashi et al., 2009; Hu et al., 2012).

The HPA axis is a major regulatory system of the stress response and its dysfunction is one of the most prominent neurobiological findings in depression (Holsboer, 2000). In addition, recent studies have suggested an important role of the HPA axis in the etiology of treatment resistance (Zobel et al., 1999; Bauer et al., 2003; Juruena et al., 2009). In this thesis, we now provide evidence that Homer1 plays an important role in the feedback regulation of the HPA axis. Previous reports have demonstrated that a total knockout of Homer1 leads to an increased adrenal cortex size, and to an elevated corticosterone response to ACTH stimulation (Grinevich et al., 2011). Indeed, we replicated and further expanded these findings by showing that Homer1KO mice exhibit increased HPA axis responsiveness to a stressful challenge (Chapter 5). Furthermore, we could sensitize HPA axis activation by overexpressing Homer1a in the hippocampus, indicating an important role of glutamate signaling in HPA axis regulation. However, blockade of mGluR5 did not affect this endocrine phenotype, which suggests an alternative molecular pathway leading to HPA axis feedback disturbances that involves Homer1, but not mGluR5. In line with this hypothesize, deletion of Homer1 also led to alterations in the GR expression profile in the hippocampus, further suggesting that a modulation of this pathway may have profound effects on HPA axis feedback regulation (Chapter 5).

3.3. Implications for novel treatment options

Glutamate signaling has been strongly implicated in the emergence of mood disorders in several studies (Sanacora et al., 2012; Krystal et al., 2013). In this context, the Homer1 knockout mouse line was suggested as model for schizophrenia (Szumlinski et al., 2005). However, the complex phenotype of this mouse line has not been completely disentangled so far, thereby only providing limited insight into the origin of the observed alterations. Since Homer1 is critically involved in early development, these effects may likely arise during early life and, due to their severity, may not be counterregulated during later stages of life (Shiraishi et al., 2004). To-date, animal studies have mainly focused on the role of Homer1 in memory processes such as fear memory or reward associated learning (Szumlinski et al., 2004; Inoue et al., 2009; Tronson et al., 2010), utilizing conditional Homer1a knockout animals or pharmacological methods to interfere with Homer1 function. In particular, studies that examine reward associated behaviors have mainly focused on the prefrontal cortex as the target region for their manipulations (Kalivas, 2004; Murray and Wise, 2010). We have now added new evidence that hippocampal glutamate signaling via Homer1 is also involved in incentive motivational behavior (Chapter 4). Together with the extensive dissection of hippocampal Homer1 in spatial memory processes (Chapter 3), and its involvement in chronic stress-mediated anhedonia and locomotion impairments (Chapter 5), we conclude that Homer1 and its signaling pathway are profoundly involved in stress-related mood disorders.

Based on findings that ketamine, a potent NMDA receptor antagonist, provide rapid antidepressant effects both in clinical trials (Zarate, Jr. et al., 2006; Price et al., 2009) and animal studies (Garcia et al., 2008a; Garcia et al., 2008b), research has aimed to discover mechanisms and compounds that provide comparable efficacy without the negative side effects associated with ketamine treatment (Tsai, 2007). Most advancements in this field are based upon direct manipulation of the NMDA receptor, for example by antagonizing specific subunits that consequently prevent natural signaling (Burgdorf et al., 2013). Interestingly, potentiation of NMDA receptor function has also been ascribed to antidepressant effects (Huang et al., 2013), indicating that there is a complex molecular context that goes beyond mere (ant)agonistic actions. It has, for example, been demonstrated that intracellular signaling cascades can change the synapses' ability to express plasticity and thereby profoundly alter the neuronal characteristics of affected

regions (Abraham, 2008). This concept of metaplasticity has already been implicated in mGluR mediated long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus (Rush et al., 2002; Gisabella et al., 2003), and it can be hypothesized that it also applies to NMDA receptor mediated signaling pathways. In this regard, a molecular link between group I mGluRs, Homer1, and NMDA receptor signaling has been established in various studies (Naisbitt et al., 1999; Pilc et al., 2008; Hu et al., 2012) and within this pathway, Homer1 may indeed be a crucial mediator of behavioral metaplasticity in response to environmental stimuli such as stress or pharmacological intervention (Schmidt et al., 2013). In fact, it has been shown that Homer1 plays a critical role in formation and composition of the PSD complex as well as in group I mGluR membrane expression (Ango et al., 2000; Ango et al., 2002; Feng et al., 2002; de Bartolomeis and Iasevoli, 2003). In addition, the IEG Homer1a is induced upon external stimuli and may significantly alter the connectivity between mGluRs and NMDA receptors (Kammermeier and Worley, 2007; Bertaso et al., 2010). This highly dynamic system in the glutamatergic PSD, with Homer1 as a scaffolding protein that is centrally involved in these mechanisms, therefore represents a valuable target for further research focusing on glutamate signaling as well as a novel treatment method for mood disorders.

3.4. Summary and future perspectives

The current thesis constitutes a compelling basis for the implication of Homer1 in stress-related disorders. We thoroughly explored the function of Homer1 and mGluR5 in the context of environmental challenges, and could provide strong evidence that Homer1 is involved in both the behavioral and neuroendocrine response to acute and chronic stress and also further elucidated the efficacy of mGluR5 antagonists as potential antidepressant treatment option. The complex molecular mechanisms in the PSD of glutamatergic synapses, where Homer1 exerts its actions, are however not completely understood (Popoli et al., 2012), since novel interaction partners of postsynaptic receptors and scaffolds are constantly being discovered and add further complexity to the intricate framework that provides functional excitatory signaling (Hu et al., 2012). An important path that would be worthwhile pursuing in the context of mood disorders, such as depression, includes the interaction between Homer1 isoforms and ionotropic glutamate receptors. Especially the molecular mechanisms of NMDA receptor mediated signaling that lead to the antidepressant action of compounds targeting this receptor need to be explored in more detail (Kavalali and Monteggia, 2012). The molecular action of potential novel compounds that are considered suitable to enter clinical trials need to be clarified to minimize the negative side effects observed when manipulating NMDA receptor function (Hashimoto et al., 2013; Lakhan et al., 2013). Furthermore, given the growing body of evidence presented in this thesis and elsewhere (Kendell et al., 2005; Sanacora et al., 2008; Krystal et al., 2010; Krystal et al., 2013), the combination of compounds that simultaneously modulate group I mGluRs and NMDA receptors may represent a novel, fast acting option to treat depression. In this regard, Homer1, as the main scaffold that postsynaptically links these receptors, may play a crucial role in the mediation of these effects (de Bartolomeis et al., 2013).

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5. Curriculum Vitae

Personal information

Name	Klaus Wagner
Date of birth	July 19th, 1983
Place of birth	München, Germany
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Education

since 10/2009	PhD studies in the research group ´Neurobiology of Stress´ at the Max Planck Institute of Psychiatry, Munich
09/2004 - 09/2009	Studies of Biology (Diploma, 1,0) at the Ludwig-Maximilians-University Munich Major: Neuroscience Minors: Biology of the cell; Toxicology and pharmacology

Work experience

07/2006 - 12/2008	Internship at Micromet AG, Munich Cell culture, fermentation

Awards and Scholarships

2007	*Miami Exchange Program* of the Max-Planck-Institute of Neurobiology
2009	Poster price award, 26. Symposium der AGNP, Munich
2011	Terje Sagvolden Travel Award, 43. EBBS Meeting, Sevilla, Spain
2012	DAAD travel stipend, SfN meeting 2012, New Orleans, USA Travel stipend GlaxoSmithKline Stiftung, 32. EWCBR Meeting, Villars-sur-Ollon, Switzerland
2013	FENS Travel Award, SfN meeting 2013, San Diego, USA Peter-Hans-Hofschneider Preis für molekulare Medizin der Max Planck Gesellschaft

6. Publications

2013 **Wagner KV**, Hartmann J, Mangold K, Wang XD, Labermaier C, Liebl C, Wolf M, Gassen NC, Holsboer F, Rein T, Müller MB and Schmidt MV: Homer1 mediates acute stress-induced cognitive deficits in the dorsal hippocampus.
Journal of Neuroscience 2013 Feb 27;33(9):3857-64

Wang XD, Su YA, **Wagner KV**, Avrabos C, Scharf SH, Hartmann J, Wolf M, Liebl C, Kühne C, Wurst W, Holsboer F, Eder M, Deussing JM, Müller MB and Schmidt MV: Nectin-3 Links CRHR1 Signaling to Stress-Induced Memory Deficits and Spine Loss.
Nature Neuroscience 16 (6), 706-713

2012 **Wagner KV**, Marinescu D, Hartmann J, Wang XD, Labermaier C, Scharf SH, Liebl C, Uhr M, Holsboer F, Müller MB and Schmidt MV: Differences in FKBP51 Regulation following chronic social defeat stress correlate with individual stress sensitivity: influence of paroxetine treatment.
Neuropsychopharmacology 37:2797–2808

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8. Assertion / Eidesstattliche Versicherung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation

'Homer has the blues - Involvement of Homer1 in stress-induced psychopathology´

selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum oder annähernd übernommen worden sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Zu den Manuskripten habe ich wie folgt beigetragen:

Wagner KV, Marinescu D, Hartmann J, Wang XD, Labermaier C, Scharf SH, Liebl C, Uhr M, Holsboer F, Müller MB, Schmidt MV (2012). Differences in FKBP51 Regulation Following Chronic Social Defeat Stress Correlate with Individual Stress Sensitivity: Influence of Paroxetine Treatment. *Neuropsychopharmacology 2012 Dec;37(13):2797-808*

- Studiendesign und –planung: In Zusammenarbeit mit MBM, MVS
- Durchführung der Experimente: In Zusammenarbeit mit DM, JH, CL
- Datenanalyse: in Zusammenarbeit mit SHS
- Verfassen des Manuskripts: In Zusammenarbeit mit MVS

Wagner KV, Labermaier C, Webhofer C, Hartmann J, Weber P, Turck C, Binder EB, Müller MB, Schmidt MV: Hippocampal Homer1 is regulated by chronic social defeat stress. *In preparation*

- Studiendesign und –planung: In Zusammenarbeit mit MVS
- Durchführung der Experimente: In Zusammenarbeit mit JH, CL, CW
- Datenanalyse: in Zusammenarbeit mit PW, EBB
- Verfassen des Manuskripts: In Zusammenarbeit mit MVS

Wagner KV, Hartmann J, Mangold K, Wang XD, Labermaier C, Liebl C, Wolf M, Gassen NC, Holsboer F, Rein T, Müller MB, Schmidt MV (2013): Homer1 mediates acute stress-induced cognitive deficits in the dorsal hippocampus. *The Journal of Neuroscience, 2013 Feb 27, 33(9):3857–3864*

- Studiendesign und –planung: In Zusammenarbeit mit MVS
- Durchführung der Experimente: In Zusammenarbeit mit JH, KM, XDW, CL, MW
- Datenanalyse: Selbst durchgeführt
- Verfassen des Manuskripts: In Zusammenarbeit mit MVS

Wagner KV, Häusl AS, Pöhlmann ML, Hartmann J, Labermaier C, Müller MB, Schmidt MV: Hippocampal Homer1 levels influence motivational behavior in an operant conditioning task. *Manuscript submitted*

- Studiendesign und –planung: In Zusammenarbeit mit MVS
- Durchführung der Experimente: In Zusammenarbeit mit ASH, MLP
- Datenanalyse: in Zusammenarbeit mit ASH, MLP
- Verfassen des Manuskripts: In Zusammenarbeit mit MVS

Wagner KV, Hartmann J, Häusl AS, Pühlmann ML, Zhao G, Labermaier C, Wang XD, Santarelli S, Kohl C, Lindemann L, Jaschke G, Wettstein JG, Holsboer F, Müller MB, Schmidt MV: Homer1/mGluR5 signaling moderates vulnerability to chronic social stress. *Manuscript in preparation*

- Studiendesign und –planung: Selbst durchgeführt
- Durchführung der Experimente: In Zusammenarbeit mit ASH, MLP, JH, GZ, CL, XDW, SS, CK
- Bereitstellung des Antagonisten durch: LL, GJ, JGW
- Datenanalyse: Selbst durchgeführt
- Verfassen des Manuskripts: In Zusammenarbeit mit MVS

I hereby confirm that the dissertation

'Homer has the blues - Involvement of Homer1 in stress-induced psychopathology'

is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, Juli 2013

Klaus Wagner

Hiermit bestätige ich die von Herrn Wagner angegebenen Beiträge zu den einzelnen Publikationen

München, Juli 2013

Mathias V. Schmidt