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MEDIATORS OF SYNAPTIC ACTIVITY IN ANXIETY- AND DEPRESSION-RELATED BEHAVIORS

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About the cover: The front cover image shows spines along the dendritic process of a pyramidal cell in the mouse prefrontal cortex, visualized through Golgi-Cox staining. As discussed and diagramed in this thesis, these spines are the site of synaptic transmission and communication between neurons in excitatory cells. Stress can lead to the loss of spines and atrophy of the dendrites in this region, and antidepressants can reverse these effects. The back cover uses lower magnificantion to show both the cell bodies and dendrites of similar pyramidal excitatory cells.

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Dedicated to my Mom, Dad, and brother,
For crossing the Atlantic to hear me talk science.

ABSTRACT

Depression and anxiety are leading causes of years lost to disability, despite antidepressants being among the most commonly prescribed medications. Most currently prescribed antidepressants were found serendipitously rather than from an understanding of the biological mechanisms underlying depression. Recent evidence supports instead the antidepressant efficacy of glutamate-targeting drugs, such as ketamine, which promote plastic changes in synaptic structure and function.

Here we employed pharmacological and genetic approaches to study the role of various molecules known to mediate synaptic activity and plasticity in baseline depression- and anxiety-related behaviors and antidepressant-like effects in mice. Specifically, we examined voltage-gated potassium channels (Kv4.2) known to regulate dendritic excitability, a molecule of the postsynaptic density (PSD-95), and glutamatergic receptors, including the GluA1 subunit of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and its phosphorylation sites, and the GluN1, GluN2B, and GluN2A subunits of *N*-methyl-D-aspartate (NMDA) receptor. We employed traditional assays for murine depression-related behavior, including the forced swim test (FST), and a novel test for responses to repeated swim stress.

We found that mice with constitutive deletions to GluA1, GluN2A and PSD-95 showed reduced depression-related behaviors in the FST, but similar behavioural effects were not observed in mice with a conditional cell-type specific knockout of NMDAR subunits. However, pharmacological blockade of GluN2B, via administration of Ro 25-6981, reduced depression-like behavior in the FST. This antidepressant-like effect was replicated by microinfusion of Ro 25-6981 into the medial prefrontal cortex (mPFC). By contrast, neither lentiviral-mediated knockdown of GluN2B nor conditional GluN2B deletion in forebrain pyramidal neurons or interneurons was sufficient to reduce depression-like behavior in the FST. Pharmacological GluN2B antagonism via Ro 25-6981 similarly reduced despair-like behavior during a repeated swim stress, and, unlike spontaneous swim measures, this effect was replicated by genetic deletion of GluN2B on pyramidal cells, but not interneurons. Collectively, these results suggest that multiple synaptically expressed molecules mediate depression-related behavior. GluN2B-containing NMDARs play a role in mediating depression-related behaviors during acute and repeated stressors, depending upon the pharmacological or genetic manipulations used, cell-type and brain region localization.

Together this suggests that multiple synaptic proteins are important in depression-related behavior. However, reduction of GluN2B receptors does not *per se* lead to changes in depression-related behaviors. Here we suggest that it is the NMDAR-antagonist induced rise in extracellular glutamate and subsequent increase in synaptic transmission, such as through AMPARs, that is necessary for an antidepressant-like response and is lacking in the genetic deletions of GluN2B. In the repeated stress procedure our data supports a role of GluN2B transmission selective to pyramidal cells in mediating the behavioural alterations that lead to despair-like behavior. This would fit with previous data showing systemic NMDAR antagonism reduced atrophy in these same cell types, and suggests that it is the pyramidal cell NMDARs that mediate the morphological and behavioural effects of repeated stress.

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

AMPAR	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
BLA	Basolateral Amygdala
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
DH	Dorsal Hippocampus
EAAT	Excitatory Amino Acid Transporter
EPM	Elevated Plus Maze
FST	Forced Swim Test
GABA	Gamma-aminobutyric acid
GLAST	GLutamate ASpartate Transporter
GLT1	Glutamate Transporter 1
L/D	L/D emergence task
MDD	Major Depressive Disorder
mPFC	Medial Prefrontal Cortex
NMDAR	*N*-methyl-D-aspartate receptor
NIH	Novelty-induced Hypophagia
NSF	Novelty-suppressed feeding
OF	Novel Open Field
OFC	Orbital Frontal Cortex
PKA	Protein kinase A
PKC	Protein kinase C
PSD-95	Postsynaptic Density Protein 95
riFS	Repeated Inescapable Forced Swim
reFS	Repeated Escapable Forced Swim
sgACC	Subgenual Anterior Cingulate Cortex
SIH	Stress-induced Hyperthermia
TST	Tail Suspension Test
VGLUT	Vesicular Glutamate Transporter

1 INTRODUCTION

Despite current treatments, neuropsychiatric disorders remain challenging conditions to manage. In the case of depression, antidepressants are one of the most prescribed medications, and yet depression remains one of the leading causes of years lost to disability (Murray and Lopez, 1997, Wong and Licinio, 2001, 2004). In part, this is due to our lack of understanding of the biological mechanisms underlying the etiology and treatment of these disorders. The vast majority of treatments used today were found serendipitously with little understanding of their mechanism of action.

The discovery of mood-elevating effects of the tricyclic antidepressants, monoamine oxidase inhibitors, and later selective serotonin reuptake inhibitors was a significant advance in the treatment of depression and these drugs are still the main therapy used today. The shared ability of these medications to increase levels of monoaminergic neurotransmitters such as serotonin, norepinephrine, and dopamine, led to the monoaminergic theory of depression, suggesting that depression was caused by a depletion of these neurotransmitters (Bunney and Davis, 1965). This hypothesis led to significant advances in our understanding of the biological mechanisms of depression, including predisposing genetic and environmental factors. These treatments were also important to the development of rodent-based assays of antidepressant-like response and depression-related behaviors that enabled the discovery of new molecular targets.

However, while these treatments immediately increase monoamines, relief from symptoms does not occur for weeks or months, and are effective in only a subset of patients (Rosenzweig-Lipson et al., 2007). Additionally, experimentally reducing monoaminergic levels does not induce depression in all subjects (Moore et al., 2000, Neumeister et al., 2005), while other effective antidepressant treatments, such as tianeptine, decrease serotonin levels (Datla and Curzon, 1993). There is also a wealth of effective treatments with diverse mechanisms that do not directly work on monoamines such as cognitive behavioral therapy, sleep deprivation, electroconvulsive shock therapy, and deep brain stimulation. This complex literature indicates that depression involves more than a deficiency of monoamines.

There is growing interest in understanding and therapeutically targeting the molecular machinery mediating neural plasticity as a new approach to depression (reviewed in (Pittenger and Duman, 2008). The brain has enormous ability to adapt, or be plastic, not just during development, but also throughout adulthood. The adult brain generates new neurons (neurogenesis) and both new and old neurons show alterations in their activity and connections to other cells. Synapses transmitting communication between cells rapidly form and degrade, and the receptors mediating synaptic transmission are constantly being trafficked into and out of the synapse. Each of these components of neural function and plasticity shows experience-dependent regulation and are increasingly linked to the pathophysiology and treatment of depression and anxiety (Reviewed in (Citri and Malenka, 2008, Pittenger and Duman, 2008).

An important risk factor for multiple neuropsychiatric disorders, including depression and anxiety, is exposure to psychological trauma and stress (Anisman and Zacharko, 1990, Kessler, 1997). Stress can be described as experiences that challenge the ability of an organism to cope or adapt (Lazarus and Folkman, 1984). The brain is both the control center of the stress response, as well as a target for its effects. Therefore, the ability of the brain to adapt to new situations is imperative for a healthy

stress-response, making deficits in neural plasticity a prime therapeutic target for stress-related disorders, such as depression. As with antidepressant treatments, abundant evidence points to the effects of stress on neural plasticity, while manipulations of these same targets alter the molecular and behavioral responses to stress (reviewed in (Pittenger and Duman, 2008).

We sought to further understand the role of various mediators of synaptic activity, particularly those involved in glutamatergic transmission in anxiety- and depression-related behaviors, treatment response to anxiolytics and antidepressants, and stress-induced behavioral changes. To provide a background to the studies conducted here, we will first give an overview of the mediators of synaptic activity we chose to investigate, including the ionotropic glutamate receptors as well as voltage-gated potassium channels. This is followed by a descripton of some of the preclinical rodent assays used to investigate these disorders and reactions to stress. We then describe some of the common clinical symptoms observed in depressed patients and discuss how they might relate to abnormalities in glutamatergic signaling. This will lead us into the evidence showing that stress can also alter glutamatergic transmission, and cause some of the morphological and molecular changes that are associated with anxiety and depression. Finally, we will discuss how these stress-induced changes may be blocked or reversed by manipulating glutamatergic signaling and how this could lead to novel therapies for depression.

2 MEDIATORS OF SYNAPTIC ACTIVITY

There are multiple mechanisms of neural plasticity, including alterations in neuron number and shape, and changes in synaptic transmission. As the synapse is the site of communication between neurons, transducing chemical neurotransmitter signal to electrical activity, it is key in determining the activity of neurons. Alterations in neuronal activation underlie experience-dependent changes in the brain and ultimately lead to alterations in neurogenesis, and drive morphological and molecular changes.

Synaptic transmission is regulated by multiple factors that show experience-dependent regulation and involvement in synaptic plasticity. At the presynaptic site, the amount of neurotransmitter present and its packaging into synaptic vesicles influences the amount of neurotransmitter available for release, while local calcium levels regulate vesicular release to extracellular areas. Levels of extracellular neurotransmitter are in part regulated by degradation and reuptake mechanisms, as well as by autoreceptors on the presynaptic neuron that inhibit release. The binding of these ligands to fast-activating/deactivating ionotropic receptors, or the slower metabotropic receptors, mediate the postsynaptic response, which in turn is influenced by the amount of receptor, its kinetics, as well as its location and corresponding downstream effects. These downstream effects include activation of intracellular signaling pathways regulating synaptic receptor expression, synaptogenesis, and spine growth. Synaptic transmission therefore leads to activation of systems regulating future synaptic activity, and thus synaptic plasticity. The net amount of activation at the postsynaptic site or dendrite is dependent on the summation of both excitatory and inhibitory inputs, but is also influenced by excitatory current backpropagated from a previously stimulated action potential in its own axon.

Here we will touch upon two systems involved in regulating synaptic activity and their relation to neuropsychiatric disorders. First, we briefly discuss components of the glutamatergic system that regulate synaptic activity and its relation to emotional disorders, with a focus on its ionotropic receptors. We will also briefly discuss the regulation of dendritic excitability and action potential backpropagation by local potassium channels.

2.1 GLUTAMATE PACKAGING AND UPTAKE

The widespread presence of glutamate throughout the brain lead to its first being associated with metabolic function (Krebs, 1935) and was not recognized as the brains major excitatory neurotransmitter until the last few decades (Fonnum, 1984). Glutamate can be produced *de novo* from glucose and amino acids, and it has been suggested that the majority of glucose entering the brain will eventually be transformed to glutamate (Shen et al., 1999). Its ubiquitous nature, along with its ability to cause excitotoxicity, necessitates a tightly regulated and energy intensive system controlling its release and extracellular levels. This energy intensive production and regulation of glutamate transmission has been suggested to be responsible for much of the cerebral glucose metabolism and energy use (Shulman et al., 2004). A schematic of the glutamatergic system at the synapse is presented in **Figure 1**.

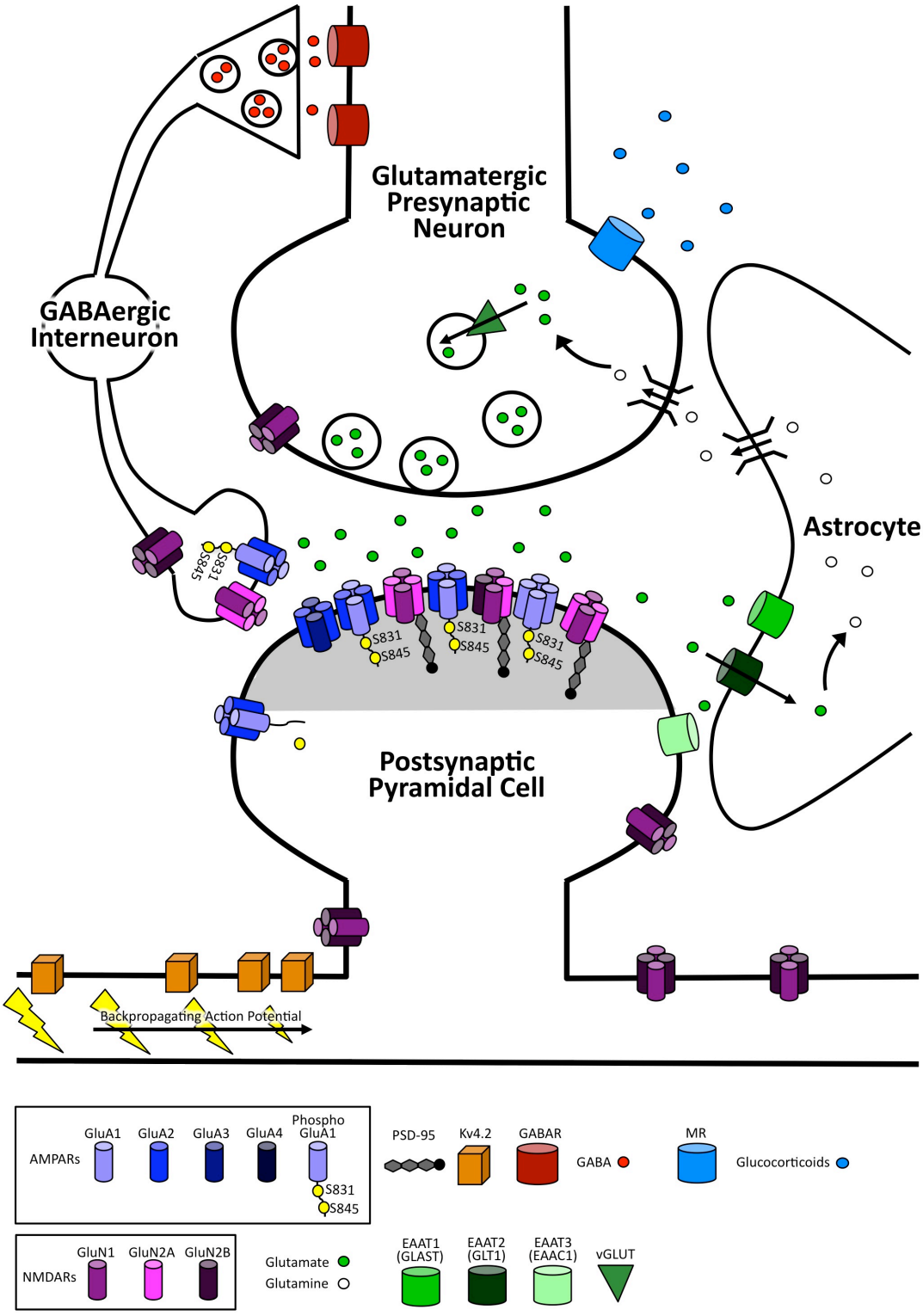

Figure 1 Schematic representation of mediators of synaptic activity at a glutamatergic synapse. Stimulation of a presynaptic glutamatergic cell by inputs such as glucorticoid receptors leads to release of glutamate. Once in the extracellular space, glutamate can be taken up by glutamate transnporters (EAATs) back into neurons or neighboring astrocytes where they are converted to glutamine. Glutamine is shuttled back to the presynaptic neuron via glutamine transporters for packaging back into vesicles by vesicular transporters (VGLUTs). Extracellular glutamate can also bind to postsynaptic receptors on GABAergic cells or pyramidal cells through the ionotropic AMPA or NMDA receptors. The activation of the postsynaptic cell can also be regulated by the amount of current backpropagated from previous axon potentials, which is inpart dampened by Kv4.2 channels.

2.1.1 Excitatory Amino Acid Transporters (EAATs)

Glutamate may also be produced via the glutamine cycle to recycle previously released glutamate (Erecinska and Silver, 1990). Extracellular glutamate is transported against its concentration gradient into neighboring glia or neurons via one of five types of excitatory amino acid transporters (EAAT1-5 in humans) (O'Shea, 2002). EAAT 1 and 2 (GLAST and GLT1 in rodents) mainly transport glutamate to astrocytes where it can be converted to glutamine, while EAAT3 (EAAC1 in rodents), transports glutamate to neurons (Arriza et al., 1994, Anderson and Swanson, 2000). EAATs and astrocytes placed near the synapse play a critical role in regulating extracellular glutamate levels and preventing spillover to extrasynaptic sites where glutamate is known to stimulate excitotoxicity (Arriza et al., 1994, Zarate et al., 2002, Shigeri et al., 2004, Zheng et al., 2008). As a single astrocyte can cover multiple synapses, the loss of individual astrocytes can have wide-reaching effects (Bushong et al., 2002).

2.1.2 Vesicular Glutamate Transporters (VGLUTs)

Glutamine transporters allow for astrocytic glutamine to re-enter neurons, where it is converted back to glutamate (Erecinska and Silver, 1990) to be packaged into vesicles by vesicular glutamate transporters (VGLUTs) (Takamori, 2006). VGLUT 1 and 2 is found in glutamatergic neurons (Fremeau et al., 2004a), as well as glial cells (Bezzi et al., 2004, Montana et al., 2004), while VGLUT 3 is located in GABAergic, cholinergic, and monoaminergic cell types (Fremeau et al., 2004b).

2.2 GLUTAMATERGIC RECEPTORS

Once released to the extracellular space, glutamate can be bound by ionotropic and metabotropic glutamate receptors. Ionotropic receptors include *N*-methyl-D-aspartate receptors (NMDARs), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), and kainate receptors, while metabotropic receptors are composed of subunits mGluR1-8. As mentioned earlier, the subunit composition, phosphorylation, kinetics and location of these receptors play important roles in their effect on the postsynaptic cell and synaptic plasticity.

2.2.1 Metabotropic Receptors

The metabotropic glutamate receptors are grouped into 3 classes of receptors composed of subunits mGluR1-8. Their actions through G-protein signaling cascades lead to a slower and longer-lasting response than the faster-acting ionotropic receptors. They are primarily located at extrasynaptic sites or on glial cells, and are therefore ideally placed for regulating extrasynaptic glutamate and GABA transmission (Pinheiro and Mulle, 2008). mGluR2/3 receptors are able to modulate presynaptic glutamate release (Takahashi et al., 1996), while mGluR5 can enhance the function of the ionotropic receptors and regulate their mRNA levels in spines (Weiler and Greenough, 1993, Weiler et al., 1997).

2.2.2 AMPARs

The AMPAR is a nonselective cation channel that mediates fast excitatory transmission (Palmer et al., 2005) and is a heteromeric assembly composed of subunits GluA1-4. Synaptic AMPAR transmission is influenced by subunit composition, phosphorylation state, and location of these receptors, making each of these an important mediator of synaptic plasticity (Takahashi et al., 2003, Whitlock et al., 2006, Matsuo et al., 2008). AMPARs can be trafficked to and from the synapse by exocytic or endocytic mechanisms, or by lateral membrane diffusion (Groc and Choquet, 2006, Newpher and Ehlers, 2008), and increased presence of AMPARs is involved in an LTP-like strengthening of the synapse (Song and Huganir, 2002, Takahashi et al., 2003, Matsuo et al., 2008). In early stages of synaptic potentiation, GluA1 homomers or GluA1/2 receptors are trafficked to the synapse, to be replaced by GluA2/3 receptors as the synapse is stabilized (Kessels and Malinow, 2009). There is some evidence that, predominantly in interneurons, GluA1 homomers can traffic Ca^{2+} into the cell and thus activate Ca^{2+}-mediated intracellular signaling pathways (Liu and Zukin, 2007). Trafficking of the GluA1 subunit is in part mediated by its phosphorylation state, with phosphorylation of the serine 831 (by PKC and CaMKII) and serine 845 (by PKA) sites on the GluA1 subunit effectively increasing synaptic AMPAR transmission (Roche et al., 1996, Barria et al., 1997, Mammen et al., 1997).

2.2.3 NMDARs

Compared to AMPARs, NMDARs have a higher affinity for glutamate and create a slower, longer-lasting postsynaptic current. In addition, NMDARs are permeable to Ca^{2+}, and therefore activate of Ca^{2+}-mediated downstream signaling pathways. The NMDAR channel requires prior dendritic excitation, or depolarization, to open as its channel is blocked by an Mg+ ion at rest (Nowak et al., 1984), as well as requiring binding by the co-agonist glycine (Kleckner and Dingledine, 1988, Dingledine et al., 1999). The multiple requirements for NMDAR transmission, as well as its long postsynaptic current, make it an ideal target to detect the summation of multiple inputs to the synapse, and therefore act as a coincidence detector that can activate downstream signaling pathways leading to long-term changes at the synapse.

NMDARs are heteromeric assemblies composed of an obligatory GluN1 subunit and one or more types of GluN2 subunits (GluN2A-D) (Rosenmund et al., 1998) or GluN3 subunits (Ciabarra et al., 1995). In forebrain regions thought to mediate the emotional and cognitive functions relevant to psychiatric disorders, GluN2A and GluN2B are the predominant receptor type in pyramidal cells (Nakanishi, 1992), while there is also evidence for GluN2C in adult interneurons (Monyer et al., 1994, Xi et al., 2009). GluN2B is replaced by GluN2A during postnatal development (Monyer et al., 1994) and after experience, resulting in a shorter postsynaptic current (Flint et al., 1997, Lau and Zukin, 2007) and therefore decreased window for coincidence detection (Tang et al., 1999, Niewoehner et al., 2007, Halene et al., 2009) and constrained plasticity (Quinlan et al., 2004) relative to GluN2B-containing receptors (Cull-Candy et al., 2001). In adulthood, the majority of NMDARs at the synapse are GluN2A/GluN1, or GluN2A/GluN2B/GluN1 receptors (Hatton and Paoletti, 2005), with GluN2B/GluN1 receptors found more predominantly at extrasynaptic sites (Martel et al., 2009), possibly due to their increased lateral diffusion (Groc et al., 2006, Hardingham and

Bading, 2010). Both the kinetics of these subunits, and their location, can lead to disparate functions as synaptic versus extrasynaptic transmission can activate opposing downstream signaling pathways (Reviewed in (Hardingham and Bading, 2010). There is also evidence for presynaptic NMDARs that act as autoreceptors, reducing presynaptic glutamate release after stimulation (MacDermott et al., 1999, Pinheiro and Mulle, 2008, Duguid and Smart, 2009).

2.2.4 Postsynaptic Density

The presence of these receptors at the synapse, as well as their downstream effects, are in part controlled by the postsynaptic density, a collection of proteins and cytoskeleton architecture that stabilizes the synapse. The postsynaptic density is composed of a multitude of different proteins, including post-synaptic density 95 (PSD-95) (Kennedy, 1998, Walikonis et al., 2000), and act to bind these glutamatergic receptors to the cytoskeleton as well as downstream signaling pathways (Kim and Sheng, 2004). PSD-95 has been specifically shown to interact with the NMDAR and is tied to the localization of NMDARs to the synapse and in NMDAR- and AMPAR-mediated synaptic plasticity (Kim and Sheng, 2004, Malenka and Bear, 2004, Beique et al., 2006, Elias and Nicoll, 2007) and is expressed in both excitatory pyramidal cells and GABAergic interneurons (Akgul and Wollmuth, 2010).

2.3 DENDRITIC EXCITABILITY AND POTASSIUM CHANNELS

Although not components of the glutamatergic system, there are various mechanisms that provide modulatory influences over the excitability and plasticity of glutamatergic neurons. Of note, potassium channels produce a hyperpolarizing current and have a number of functional roles depending on the potassium channel subtype, regional and subcellular location, post-translational modifications, and voltage- or calcium-dependency for channel opening. The voltage-dependent potassium channels (Kv), include channels producing the fast activating and deactivating A-type current (I_A), such as the Kv4 channels (Shah et al., 2010). The expression of Kv4.2 channels is mainly in dendritic regions, with expression increasing in areas distal to the cell body, and Kv4.2 mediates the I_A hyperpolarizing current in these regions (Sheng et al., 1992, Hoffman et al., 1997). This makes Kv4.2 an effective regulator of action potential backpropagation to dendritic regions, as backpropagation will induce the opening of Kv4.2 to its hyperpolarizing current selectively in regions distal to the axon hillock (Chen et al., 2006). As backpropagation can increase excitation of postsynaptic sites, it can add to the summation of excitatory inputs and augment synaptic potentiation. The presence of Kv4.2 therefore acts to dampen excessive synaptic excitability and plasticity in neurons (Chen et al., 2006, Zhao et al., 2010).

While Kv4.2 is a novel target in psychiatric disorders, each of the aforementioned components of glutamatergic transmission have been implicated in the development and treatment of depression and anxiety (Reviewed (Niciu et al., 2012, Sanacora et al. 2012, Riaza Bermudo-Soriano et al., 2012). Below we will better describe some of the preclinical tools and clinical characteristics of these disorders, and discuss how these regulators of glutamate transmission and synaptic plasticity might contribute to their symptomology.

3 PRECLINICAL AND CLINICAL STUDIES ON DEPRESSIVE DISORDERS

Depression has a lifetime prevalence of approximately 16.2% in the US population (Kessler et al., 2003), while the often comorbid condition of anxiety affects 18% of the population in a given year (Kessler et al., 2005). Major depressive disorder (MDD) is highly heterogeneous and includes diverse subtypes such as melancholic depression, atypical depression, seasonal affective disorders, among others. The core symptoms of MDD include bouts of low mood, loss of interest in normally pleasing activities (anhedonia), feelings of guilt or worthlessness, anxiety, alterations in psychomotor activity levels, energy loss, poor concentration and memory, altered sleep and appetite, recurrent thoughts of death or suicide (Zakzanis et al., 1998, Hasler et al., 2004, Harvey et al., 2005). Although considered separate disorders, there is new appreciation of the comorbidity of anxiety and depression and overlap in symptoms (Merikangas et al., 2003) and treatment, as SSRIs are effective anxiolytics as well as antidepresants (see (Kupfer et al., 2012).

The etiology of depression appears to be a complex interplay of genetic and environmental factors (reviewed in (Sullivan et al., 2000, Levinson, 2006). Depression shows a familial risk with a moderate heritability of about 40% (Bierut et al., 1999, Kendler et al., 2001). This has helped lead to the discovery of specific risk genes, including serotonin targets such as the serotonin transporter gene-linked polymorphic region (5HTTLPR) gene, as well as some evidence for COMT and BDNF (reviewed in (Levinson, 2006). Studies on individuals with the 5HTTLPR risk allele have found that this predisposing allele did not produce depressive symptoms unless combined with environmental factors such as life stress (metaanalysis in (Daniele et al., 2011). This points to an important role for gene by environment interactions in the development of depression, suggesting that predisposing biological factors alter our ability to respond to stress, leaving us vulnerable to psychiatric disorders. Exposure to stress has been shown to be associated with the development of depression and anxiety, as well as other psychiatric disorders such as addiction, PTSD, and schizophrenia (Kendler et al., 1999b, a, Caspi et al., 2003, Hammen, 2005, Schneiderman et al., 2005, Sinha, 2008, Lupien et al., 2009).

3.1 RODENT ASSAYS OF DEPRESSION- AND ANXIETY-RELATED BEHAVIORS

Rodent behavioral assays have become an essential tool for understanding the mechanisms underlying anxiety and depressive conditions and their treatments (for review see (Cryan and Holmes, 2005). Rodents display complex behaviors that are amenable to training and can be measured objectively, while also allowing for invasive neural manipulations, including manipulations of local circuits at specific time points in behavioral testing. The mouse, in particular, has become an important model species due to its receptiveness to gene targeting techniques, allowing for specificity to a target not previously possible with available pharmacological interventions.

Below we review some of the many behavioral paradigms used in the study of anxiety- or depression-related phenotypes in mice. While none of these tests cover all symptoms of these disorders, they allow measurements of some of their core symptoms

or characteristics to understand parallels in mechanisms underlying these endophenotypes. In designing and choosing different behavioral paradigms, it is important that they meet various measures of validity, such as face validity or similarity to human behavior, construct validity or similar underlying molecular mechanisms, or predictive validity or similar response to treatments (McKinney and Bunney, 1969). As different assays likely measure somewhat differing forms of behavior, studies are strengthened by performing a battery of tests with multiple assays of depression- or anxiety-related behaviors that measure overlapping, but distinct behavior.

3.1.1 Anxiety-related tasks

Multiple behavioral assays related to anxiety-like behaviors have been well characterized and allows for testing of new molecular targets in the cause or treatment of anxiety disorders. While all symptoms of anxiety are not possible to model in rodents (such as a panic attack), many are amenable to rodent assays (see review in (Cryan and Holmes, 2005). Here we made use of tests involving approach-avoidance behavior, which exploit rodents natural aversion to exposed well-lit areas that conflicts with their desire to explore novel environments (Belzung and Griebel, 2001). Preference for enclosed, versus open exposed areas, is taken as a measure of anxiety-like behavior. We used similar but non-overlapping behavioral paradigms: novel open field (OF) **(Figure 2A)** (Prut and Belzung, 2003), light/dark exploration (L/D) **(Figure 2B)** (Crawley et al., 1981) and elevated plus maze (EPM) (see **Figure 2C**) (Handley and Mithani, 1984) and these tests have been previously compared in (Lalonde and Strazielle, 2008). Anxiety-like behavior can also be measured in novelty-suppressed feeding (NSF) or novelty-induced hypophagia (NIH) where exposure to a novel cage inhibits ingestion of a food reward (Bodnoff et al., 1988). Importantly, these tests have predictive validity as they are affected by anxiolytics and anxiogenics, and they respond to drugs of diverse mechanisms, (Rodgers 1997).

However, the anxiety-like behavior in these approach-avoidance based tasks can be difficult to distinguish from hyperactivity or increased novelty seeking. Use of control measures of general activity can be included, or alternative anxiety-related assays not based on approach/avoidance such as physiological based tasks. The stress-induced hyperthermia (SIH) task takes advantage of the effect of stress to increase body temperature in the mouse, and therefore the change in body temperature can provide a physiological measurement of anxiety (Van der Heyden et al., 1997). Similarly, activation of the HPA axis can be indicative of increased stress responsivity and is not affected by general locomotor changes.

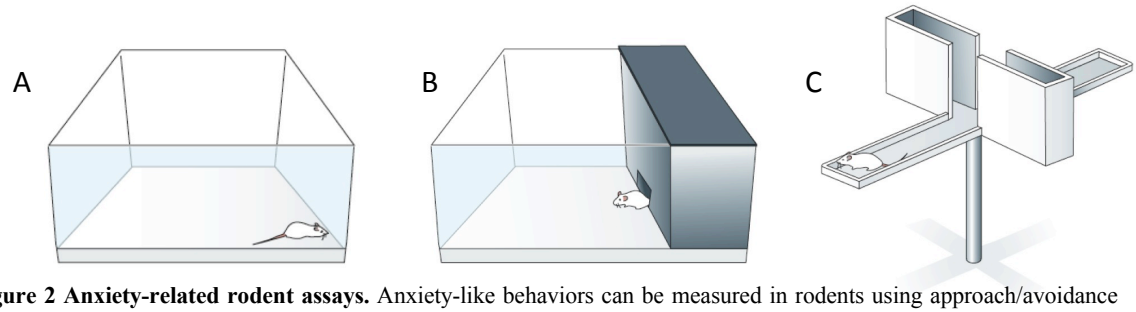

Figure 2 Anxiety-related rodent assays. Anxiety-like behaviors can be measured in rodents using approach/avoidance based tasks that take advantage of rodents natural aversion to open spaces that conflicts with their desire to explore new areas. Here we make use of the novel open field (OF) **(A),** light/dark emergence (L/D) **(B),** and elevated plus maze (EPM) **(C).** Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery 4(9):775-90,

3.1.2 Depression-related tasks

Many of the symptoms of depression have been assayed in established behavioral paradigms. While suicidal ideation or feelings of guilt cannot be measured in rodents, assays have been developed to test the anhedonic loss of interest, changes in appetite, sleep or psychomotor agitation, as well as fatigue, and cognitive deficits of depression as reviewed in (Cryan and Holmes, 2005). Additionally, behavioral responses specific to antidepressants have been measured in the forced swim test (FST) **(Figure 3A)** (Porsolt et al., 1977) and tail suspension test (TST) **(Figure 3B)** (Cryan et al., 2005a). In these tasks, mice are placed in an inescapable or stressful situation (tank of water, hanging from tail), and are observed for attempts to escape versus becoming immobile. These tasks show some face validity for depression-related behavior as immobility can be interpreted as a type of 'behavioral despair' such as seen in clinically depressed patients. Immobility in these tests is also influence by some of the same genetic predisposing factors seen in patients, as well as by risk factors associated with depression such as stress exposure, altered food intake, and sleep changes, demonstrating construct validity (Cryan et al., 2005a, Cryan et al., 2005b).

Most importantly, however, these tests gain their strength from the ability of antidepressants, but not other psychoactive drugs, to reduce immobility, thus showing predictive validity (Cryan et al., 2005a, Cryan et al., 2005b). Unfortunately, while antidepressants have a delayed onset in patients, they show immediate effects in rodent tests of antidepressant-like response in the FST and TST (Dulawa et al., 2004). Attempts have been made to find behavioral paradigms requiring chronic antidepressant treatment, especially in studies comparing traditional to fast-acting antidepressants, and some success has been found in the NIH or NSF tests (Bodnoff et al., 1988). Additionally, antidepressants are only effective in depressed patients, and antidepressants may not be expected to be effective in all genetic strains of mice. Different strains of mice allows for the use of more stress- or depression-sensitive mouse lines, however the use of genetic mutants lines typically favors the more common C57BL/6J background.

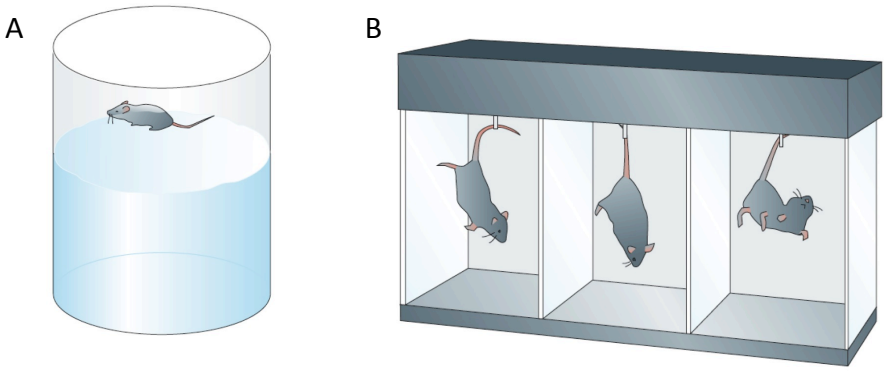

Figure 3 Depression-related rodent assays. Depression-like behaviors can be measured in rodents using assays inducing behavioural despair as measured by immobility during a stressful situation. Here we make use of the forced swim test (FST) and tail suspension test (TST). Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery 4(9):775-90, copyright 2005.

3.1.3 Stress Paradigms

As depression and anxiety are thought to arise from a complex combination of risk genes and environment, gene targeting strategies combined with simple measures of spontaneous behaviors are likely to be limited (example in (Holmes et al., 2003). A better strategy could be to measure behavioural changes after alterations to genetic targets in combination with exposure to environmental risk factors, such as stress. Also, in the case of plasticity-related targets, alterations in spontaneous behaviors are often masked by compensatory plasticity mechanisms, and phenotypic differences only become apparent after exposure to challenges such as learning paradigms or stress (Clark and Cull-Candy, 2002, Lee et al., 2003). Multiple stress paradigms have been developed to investigate the effects of stress on the brain, as well as to develop better models of depression- and anxiety-related behavior. These stress paradigms vary in their duration, with acute and chronic models often showing opposing effects, and can also vary in the stressor severity, stressor predictability, and stressor controllability (Maier, 1984, Maier and Watkins, 2005, Marin et al., 2007). Stressors in animal models include naturalistic stimuli such as predator odor, social stress or isolation, swim stress, temperature or restraint stress, but can also involved learned stressors such as those where a shock is paired with a previously neutral stimuli (Joels et al., 2007).

Common stress protocols include chronic restraint or immobilization stress (CIS), and chronic unpredictable stress (CUS), or the related chronic mild stress (CMS) paradigm (Reviewed in (Joels et al., 2007)). CIS involves restraining the animal for up to 6 hours/day, for a period that can last days to several weeks. The animal is therefore presented with a similar stressor each day and leads to a predictable form of stress, and could alternatively be consistent exposure to other stressors such as footshock, or cold, as exposed to restraint. While this stress initially leads to increases in corticosterone levels, decreased body weight and coat condition, these effects can revert to baseline as the animal adapts to the stress (Garcia et al., 2000, Marin et al., 2007). CUS, or CMS, however, involves exposure to a variety of inconsistent stressors for weeks, creating an unpredictable stressor where elevations in corticosterone and decreased body weight often remain throughout testing (Chappell et al., 1986, Marin et al., 2007).

While there are benefits and weaknesses to each of these chronic stress paradigms, we developed our own repeated predictable stress paradigm for the experients conducted here. The CIS paradigm has led to important advances in our understanding of stress-induced molecular and morphological changes, however it does not allow analysis of behavioral changes throughout chronic stress beyond alterations in body weight and coat condition. Behavioral assays conducted after CIS can give evidence of stress-induced behavioral changes, but they show strain-dependent variations, with the more common C57BL/6J mouse showing paradoxical decreases in anxiety-like behavior (Mozhui et al., 2010, Ihne et al., 2012). Thus there is a need for a chronic stress paradigm that allows for analysis of genetically modified targets in behavioral adaptations in the more common C57BL/6J mouse strain.

3.2 STRUCTURAL AND FUNCTIONAL BRAIN ABNORMALITIES IN DEPRESSION

Depression is characterized by alterations in multiple key regions mediating emotional and cognitive function (Reviewed in (Price and Drevets, 2010). Here we focus on the medial prefrontal cortex (mPFC), amygdala, and hippocampus (see **Figure 4** for illustrations of these regions in humans and mice).

The PFC is involved in executive functions such as concentration and attention, and emotional regulation and adaptive responses to stress (Goldman-Rakic, 1996, Holmes and Wellman, 2009). It is composed of multiple regions with opposing functions, including the dorsolateral prefrontal cortex (dlPFC) in humans (analogous to ventral parts of the mPFC in rodents) that is involved in cognitive tasks (Wood and Grafman, 2003), the medial prefrontal cortex (mPFC) in emotional regulation (Ressler and Mayberg, 2007), and orbital prefrontal cortex (OFC) in integrating stimuli and assessing its value (Price and Drevets, 2010). It is also adjacent to the subgenual region of the anterior cingulate cortex (sgACC), also known as Brodmann area 25, which is known to play an important role in MDD (Coryell et al., 2005).

The hippocampus is known to mediate explicit or declarative memory and helps regulate PFC activity. Posterior (dorsal in rodents) regions have been implicated in spatial memory tasks, while anterior (rodent ventral) regions are thought to be involved in emotional tasks such as contextual fear learning (reviewed in (Barkus et al., 2010). The hippocampus is mainly divided into the dentate gyrus, CA3, and CA1 region.

The amygdala is involved in memory formation of emotionally arousing stimuli such as in fear learning. The basolateral amygdala (BLA) is an important nuclei for the integration of inputs lending salience to experiences, while the neighboring central amygdala nucleus mediates the behavioral responses to these inputs (McGaugh, 2004).

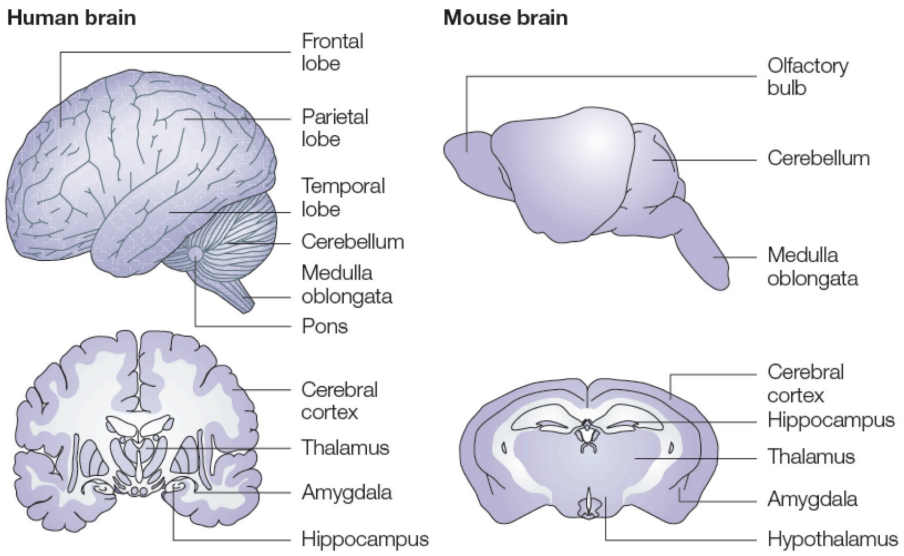

Figure 4 Key regions in human and mouse brain. While multiple brain regions are important in anxiety and depression, here we focus on the rodent medial prefrontal cortex (mPFC), basolateral amygdala (BLA), and dorsal hippocampus (DH), to test the function of parallel regions in the human brain. Reproduced by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery 4(9):775-90, copyright 2005.

3.2.1 Regional changes in brain volume

Imaging and postmortem studies have revealed morphological changes in these regions (reviewed in (Koolschijn et al., 2009). Areas of the prefrontal cortex, including the OFC and mPFC, and the neighboring sgACC, have shown reductions in gray matter volume in early-onset MDD (Reviewed in (Price and Drevets, 2010), as well as in non-symptomatic individuals with a familial risk of MDD (Drevets et al., 2004a, Boes et al., 2008), including carriers of the 5HTTLPR risk gene (Pezawas et al., 2005). Reduced volume in the sgACC is not rescued during successful treatment with some antidepressant therapies (Drevets et al., 1997), however there is evidence that it is rescued after treatment with lithium, commonly used as an add-on therapy (Drevets et al., 2008, Moore et al., 2009). These regions correspondingly show reductions in both glia and neuron size (Rajkowska et al., 1999), that have been implicated in volume loss.

Clinical studies have also shown reduced hippocampal volume in MDD (MacQueen et al., 2003), with evidence for decreased neuropil (Drevets et al., 2008) as neurons and neuropil are reduced in size, but not number (Stockmeier et al., 2004). These morphological changes are not reversed during periods of remission (Sheline et al., 1996), however antidepressant treatment did affect hippocampal volume in studies of PTSD patients (Vermetten et al., 2003) and may protect against some hippocampal volume loss (Sheline et al., 2003). Reductions in hippocampal volume become apparent after repeated occurrences of depression, and not at the first depressive episode, suggesting that structural changes are a symptom, and not a cause of depressive symptoms (MacQueen et al., 2003).

In the amygdala, there are clinical results supporting both increases (Frodl et al., 2002, Lange and Irle, 2004) and decreases in volume (reviewed in Drevets et al., 2004), and the variability is likely due to technical difficulties in measurements in this region.

These volumetric changes in grey matter could be explained by the loss of multiple elements in the brain, and have been tied to loss of glia, neuron size, synapses, and synaptic proteins (Ongur et al., 1998, Rajkowska et al., 1999, Eastwood and Harrison, 2000, Rosoklija et al., 2000, Cotter et al., 2001, Eastwood and Harrison, 2001, Cotter et al., 2002, Feyissa et al., 2009). The glial changes have been associated with loss of oligodendrocytes (Hamidi et al., 2004, Uranova et al., 2004, Aston et al., 2005), as well as loss of astrocytes (Johnston-Wilson et al., 2000, Si et al., 2004). Reductions of each of these elements have been implicated in the overall volume loss of these regions, however their causal role has not been directly tested.

3.2.2 Regional changes in metabolic activity

The morphological changes observed in clinical cases have been paralleled by functional changes in these regions. The sgACC of MDD patients demonstrate both increased (Dunn et al., 2002, Kumano et al., 2007) and decreased (Liotti et al., 2002, Kegeles et al., 2003, Pizzagalli et al., 2004) metabolic activity during depressive symptoms (as reviewed in (Price and Drevets, 2010). These discrepancies can in part be explained by the confounding volumetric loss, which, when corrected for, supports increased PFC metabolic activity ((Drevets and Price, 2005). As opposed to the volumetric changes, there is evidence that multiple diverse antidepressant therapies successfully reverse the increases in metabolism (Drevets et al., 1997, Mayberg et al., 2000, Drevets et al., 2002a).

In the amygdala, there are again mixed changes in metabolism with evidence for increased and decreased metabolism during MDD (Drevets, 2001, Conway et al., 2006) and antidepressant therapies have bee shown to increase amygdala metabolism (Drevets et al., 2002a, Drevets et al., 2004, Fu et al., 2004).

In general, regions demonstrating elevated metabolism during MDD show corresponding reductions in volume or histological changes (Price and Drevets, 2010). As the main excitatory neurotransmitter in the brain, glutamate transmission has been suggested to be responsible for much of the cerebral glucose metabolism (Shulman et al., 2004). Correspondingly, alterations in various measures of glutamate transmission are found in depressed patients (for a review see Sanacora et al., 2012). Patients showing volumetric loss also show increased glutamate transmission and cortisol secretion (Drevets et al., 2002b), and studies on the NMDARs on postmortem tissue suggests increase glutamate transmission in the PFC (Skolnick et al., 1996). Similarly, observed reductions in GABAergic transmission (Sanacora et al., 1999, Hasler et al., 2007) suggest increased glutamatergic signaling through disinhibition.

Together, this clinical data fits with a subtype of depression characterized by alterations in excitatory glutamate transmission in the PFC, hippocampus, and possibly BLA. Chronic exposure to increased glutamate transmission ultimately leads to compensatory changes in glia, as well as glutamatergic signaling and synaptic receptors, causing functional alterations in these regions and abnormal mood regulation. This subtype has recently been labeled glutamate based depression (GBD) and ties stress-induced alterations in glutamate signaling to functional changes in emotional regulation mediated by the sgACC (McCarthy et al., 2012).

Next we will discuss some of the results from both clinical and preclinical data that support a role for mediators of the glutamatergic system and synaptic activity in responses to stress, depression- and anxiety-related behaviors, and antidepressant treatment response

4 MEDIATORS OF SYNAPTIC ACTIVITY IN STRESS- AND ANTIDEPRESSANT- RESPONSE

4.1 STRESS INCREASES GLUTAMATE RELEASE AND ACTIVITY

As mentioned above, exposure to chronic or severe stress is a major predisposing factor for anxiety and depression. Through activation of the hypothalamic-pituitary-adrenal (HPA) axis, stress causes the release of stress hormones such as corticosteroids which target either glucocorticoid (GR) or mineralcorticoid receptors (MR). Treatment with glucorticoids experimentally mimics the effects of stress. In part through activation of the MRs, glucocorticoids are known to cause rapid increases in extracellular glutamate release (Stein-Behrens et al., 1994, Venero and Borrell, 1999, Groeneweg et al., 2011). Similarly, diverse types of behavioral stress increase extracellular glutamate levels in the PFC, hippocampus, and amygdala, as well as the striatum (Moghaddam, 1993, 2002, Reznikov et al., 2007, Rutherford et al., 2007, Musazzi et al., 2010), and is dependent on HPA-axis activation (Lowy et al., 1993).

Few studies have looked at glutamate levels after repeated stress, but there appear to be complicated adaptations to additional exposures to stress that vary between, and even within, brain regions. Extracellular glutamate levels remained elevated in the hippocampus, but not PFC or striatum, after repeated tail pinch in the same day (Bagley and Moghaddam, 1997, Rutherford et al., 2007). However, closer electrophysiological analysis of PFC neuron activity shows diverse responses by subsets of neurons (Jackson and Moghaddam, 2006). Unlike the hippocampus, the PFC also appears to have an additional glucocorticoid-independent mechanism for increased activation that occurs too rapidly to be induced by an initial endocrine response (Dunn, 1988, Moghaddam et al., 1994, Jackson and Moghaddam, 2006), suggesting this region responds uniquely to stress. The changes in PFC activity after repeated stress would fit with its known role in adaptive responses to stress, but it is unclear what function this adaptation would have and if it is present in both predictable and unpredictable stress, or instances that additionally lead to morphological atrophy and behavioral deficits.

4.1.1 Effects of Excessive Glutamate Release

While increases in glutamatergic transmission can lead to increased synaptic activity and synaptic potentiation, excessive glutamate release can lead to cell damage or excitotoxicity (Sapolsky, 2000b, 2003). This appears to be regulated in part by the location of glutamatergic transmission, as synaptic versus extrasynaptic transmission has opposing effects on cell growth signaling pathways such as those mediated by CREB and BDNF (Reviewed in (Hardingham and Bading, 2010) **(See Figure 5)**. These disparate effects of glutamatergic transmission explains how acute instances of stress and its concomitant glutamate release leads to synaptic potentiation and increased performance on some tasks, while chronic or excessive stress stimulating extrasynaptic sites leads to reduced LTP, cell damage and behavioral deficits (Luine et al., 1996, Kim and Diamond, 2002). This would assume that, as opposed to acute stress, chronic or excessive stress leads to either greater glutamate release or greater activation of extrasynaptic receptors, an assumption that has not been thoroughly tested, and as

discussed earlier, repeated stress can have variable effects on glutamatergic transmission in the PFC versus hippocampus.

In adult neurons, extrasynaptic transmission is typically mediated by GluN2B-, versus GluN2A-, containing NMDAR receptors. It is therefore unclear if it is the subunit-specific transmission itself, or the location of the NMDAR receptor, that leads to these disparate downstream effects on cell growth signaling (Hardingham and Bading, 2010), though some evidence suggests the former (Martel et al., 2012). However, the regional division of NMDAR subunits allows us to somewhat selectively target extrasynaptic receptors through the use of GluN2B-specific antagonists.

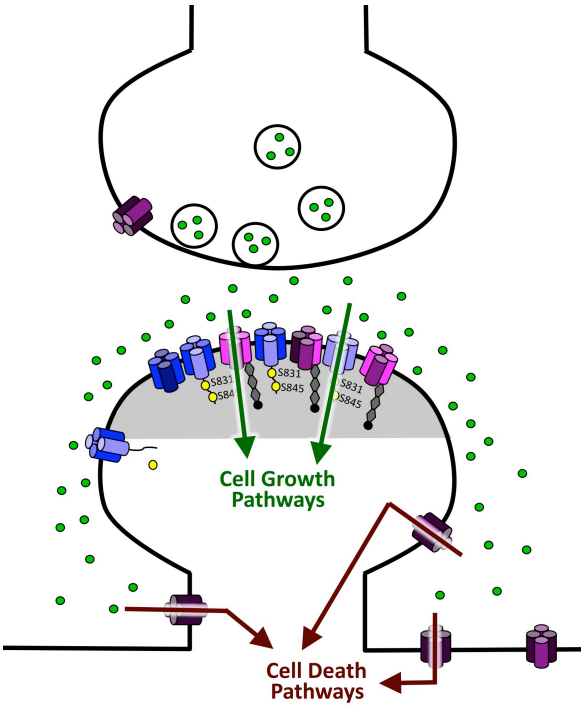

Figure 5 Synaptic versus Extrasynaptic glutamatargic transmission. Excessive glutamate release causes transmission through predominantly extrasynaptic NMDARs, leading to the acitivation of cell death versus cell growth pathways seen with selectively synaptic stimulation.

4.2 CELLULAR AND MORPHOLOGICAL EFFECTS OF CHRONIC STRESS

4.2.1 Morphological changes

Based on rhe effects of excessive glutamate release on cell damage, it is not surprising to find that chronic stress leads to atrophy of excitatory pyramidal cells in regions showing stress-induced glutamate release. In rodents, both chronic predictable (CIS) and unpredictable stress (CUS), as well as treatment with glucocorticoids, leads to dendritic atrophy and spine loss in pyramidal cells of the CA3 region of the hippocampus (Magarinos and McEwen, 1995, Sapolsky, 2000b, a), and this is blocked by drugs reducing glutamate release (Watanabe et al., 1992). This is reminiscent of the loss of neuropil observed in clinically depressed patients (Stockmeier et al., 2004).

Similar changes are observed in select regions of the PFC, where even relatively mild repeated stressors can lead to dendritic retraction and spine loss (Izquierdo et al., 2006, Li et al., 2011, Martin and Wellman, 2011). CUS also leads to a loss of synaptic proteins, such as PSD-95, GluA1, and synapsin, as would be expected with a loss of spines (Li et al., 2011). These morphological changes potentially parallel the loss of neuronal size observed observed in patient populations (Rajkowska et al., 1999), but this has not been directly tested. While patients exhibited OFC atrophy, there is evidence for hypertrophy in this region after stress in rodents (Liston et al., 2006), also demonstrating that stress can have region-dependent effects on morphology.

These inter-regional differences suggest separate regions have disparate morphological responses to stress. In patients, the amygdala shows evidence for both increases and decreases in volume (Frodl et al., 2002, Lange and Irle, 2004). In animal models, chronic stress leads to pyramidal cell hypertrophy and increased dendritic

branching and spine growth in the BLA, as well as in the BNST (Reviewed in (Roozendaal et al., 2009), which can be long-lasting from even prenatal stress (Salm et al., 2004, Vyas and Chattarji, 2004). Interneurons in the BLA instead show decreased dendritic arborization after stress, suggesting cell-type specific morphological responses to stress (Gilabert-Juan et al., 2011). Morphological changes to nonpyramidal cell types in other regions have not been well studied. Understanding these regional differences will be important in deciphering the mechanisms underlying cellular responses to stress and designing treatments that can reverse these disparate changes.

4.2.2 Alterations in glutamatergic transmission

There is some evidence that these morphological changes are tied to synaptic plasticity as LTP stimulation leads to new and larger dendritic spines (Engert and Bonhoeffer, 1999, Matsuzaki et al., 2004). While acute mild stress can enhance synaptic potentiation and LTP (Luine et al., 1996), severe stress impairs hippocampal LTP (Reviewed in (Kim and Diamond, 2002)), and enhances LTD (Xu et al., 1997). Synaptic plasticity is mediated by alterations in glutamatergic receptors, and there are corresponding changes to these receptors after stress. Acute treatment with glucocorticoids increases AMPAR transmission in the hippocampus (Karst and Joels, 2005, Groc et al., 2008, Martin et al., 2009, Krugers et al., 2010), which parallels the observed synaptic potentiation of acute stress. Similarly, acute exposure to swim stress increases surface expression of AMPARs and the NMDAR subunits GluN1, GluN2A, and GluN2B in pyramidal cells of the rat mPFC (Yuen et al., 2009, Yuen et al., 2011).

However, after chronic stress there is decreased synaptic transmission in the PFC (Li et al., 2011). Similarly, early life stress reduces levels of synaptic proteins GluN2B and GluA1 and GluA2 in the adult hippocampus (Pickering et al., 2006) and decreases GluN1 in the mPFC (Wilber et al., 2009). Exposure to chronic stress or corticosterone during adulthood led to similar decreases in the synaptic proteins in the mPFC as well as decreases in PSD-95 and synapsin (Gourley et al., 2009, Li et al., 2011). These alterations are paralleled by clinical findings showing reduced GluN2A, GluN2B, and PSD-95 levels in the PFC of postmortem samples of MDD patients (Feyissa et al., 2009) and reduced GluN1 in the hippocampus (Law and Deakin, 2001). Paralleling changes in increased spine growth, chronic restraint stress also causes upregulation of GluN1 in the amygdala of C57BL/6J, but not DBA/2J, mice, while stress augments amygdala NMDAR neuronal signaling in the DBA/2J mice that saw corresponding increases in anxiety after stress (Mozhui et al., 2010). Postmortem samples of the lateral amygdala of depressed patients revealed similarly elevated levels of GluN2A and PSD-95 (Karolewicz et al., 2009).

After stress, changes are also observed in mediators of glutamatergic uptake and cycling, indicative of adaptations to increased exposure to glutamate. After CUS, rodents show decreases in the rates of the glutamine cycle (Banasr et al., 2010), which could relate to CUS or corticosterone-induced loss of glia in the PFC (Alonso, 2000, Banasr et al., 2007, Banasr et al., 2010) that is also observed in MDD patients (Ongur et al., 1998, Rajkowska et al., 1999, Cotter et al., 2001, Cotter et al., 2002, Uranova et al., 2004, Rajkowska and Miguel-Hidalgo, 2007). Similarly, chronic stress or corticosterone exposure increases expression of GLT-1 (but not GLAST) in the PFC and hippocampus of rodents and increases in glutamate uptake (Zschocke et al., 2005, Autry et al., 2006, Zink et al., 2010). Alterations of these transporters are similarly

observed in depressed patients (McCullumsmith and Meador-Woodruff, 2002, Choudary et al., 2005, Sequeira et al., 2009, Bernard et al., 2011). Alterations are also observed in molecules responsible for the packaging of glutamate into presynaptic vesicles as the learned helplessness paradigm in rodents leads to a loss of VGLUT1 (Zink et al., 2010).

4.3 BEHAVIORAL EFFECTS OF CHRONIC STRESS

Acute and chronic stress are known to lead to behavioral alterations in rodent assays, including alterations in cognitive tasks, as well as depression- and anxiety-related behaviors (reviewed in (Willner, 2005), and some of these stress-induced changes have been correlated to the morphological and molecular changes listed above. Chronic stress or treatment with glucocorticoids disrupts hippocampal memory in rodents (Shors, 2006) and humans (reviewed in (Sapolsky, 2003), as well as PFC-mediated working memory and behavioral flexibility (Cerqueira et al., 2007, Graybeal et al., 2011, Graybeal et al., 2012). CIS also leads to alterations in tests measuring anxiety-like measures, however this appears to be species and strain dependent as anxiety-like behavior is increased in rats and some mouse strains (DBA/2J), but is decreased in other strains (C57BL/6J) (Roozendaal et al., 2009, Mozhui et al., 2010). Similarly, stress-induced increases in depression-related behavior in the FST is observed in the BALB/cByJ strain (Mozhui et al., 2010), and CUS stress in Sprague-Dawley rats leads to decreased anhedonia in sucrose preference (Li et al., 2011).

Some of these stress-induced behavioral changes have been tied to the morphological and molecular changes described earlier. Chronic stress in rats alters LTP in hippocampal-PFC connections in parallel with PFC morphological changes, and these alterations were tied to deficits in PFC-mediated working memory and behavioral flexibility (Cerqueira et al., 2007, Dias-Ferreira et al., 2009). Similarly, chronic restraint stress leads to mPFC dendritic atrophy that predicts deficits in the mPFC-mediated task of attentional set-shifting, while there was no atrophy (but instead hypertrophy) in the OFC, or signs of deficits in the OFC-mediated reversal learning task (Liston et al., 2006). In relation to depression- and anxiety-related behaviors, CUS leads to mPFC spine loss in parallel with anhedonia as measured in sucrose preference, as well as increased anxiety-like behavior in the novelty-suppressed feeding task, and these behavioral changes are rescued by interventions reversing the mPFC morphological changes (Li et al., 2011).

The mPFC is also involved in fear extinction, and 3 days of repeated stress exposure was enough to cause both mPFC dendritic atrophy as well as deficits in fear extinction (Izquierdo et al., 2006). Fear expression during fear conditioning is often increased after chronic stress procedures that lead to dendritic atrophy in the hippocampus and hypertrophy in the amygdala (Conrad et al., 1999). However, these alterations in fear expression persist even when the hippocampal morphological changes are reversed (Conrad et al., 1999). Similar hippocampal atrophy-independent trends are seen in anxiety-like behavior in approach-avoidance tasks, suggesting that morphological changes in nonhippocampal regions such as the amygdala or mPFC might mediate these behavioral effects (Conrad et al., 1999, Vyas and Chattarji, 2004). Additionally, blocking the morphological atrophy in the hippocampus after CIS did not reverse stress-induced anxiety-like behavior in the elevated plus maze, or fear memory in contextual fear memory tasks (Christian et al., 2011).

The majority of the studies conducted to date have found correlations between molecular and morphological changes and the behavioral changes after stress. Few have directly tested the consequences of preventing stress-induced morphological changes on stress-induced behavioral deficits. Later we will discuss experimental manipulations that have attempted to block stress-induced morphological and molecular changes, including those that attempted to measure the effect of these interventions on behavioral changes. Finally, we will discuss manipulations that attempt to not only block, but reverse the morphological and molecular changes after stress has ended, and how these treatments can effectively act as antidepressants.

4.4 MEDIATORS OF SYNAPTIC ACTIVITY IN ANXIETY- AND DEPRESSION-RELATED BEHAVIORS

To better understand how these stress-induced molecular changes can lead to behavioral alterations, these molecular targets can be directly altered to test their role in rodent assays of anxiety- and depression-related behaviors. We previously noted stress-induced alterations in synaptic mediators such as PSD-95, AMPARs, NMDARs, as well as other modulators of glutamatergic signaling such as the EAATS and VGLUTs. These mediators of synaptic activity can be targeted via pharmacological or genetic techniques, to which mouse models are particularly amenable. Here we review pharmacological or genetic manipulations of these targets and their resulting effects on these spontaneous anxiety- and depression-related behaviors.

4.4.1 Kv4.2

Mice with a constitutive deletion to Kv4.2 were found to have reduced anxiety-like behavior in the OF, but not in the EPM. These knockout mice also showed variations in depression-related behaviors, with reduced immobility in the FST, but not TST, and were insensitive to some, but not all antidepressants (Lockridge et al., 2010). However more recent studies found no anxiety-related alterations in these mice (Lugo et al., 2012).

4.4.2 PSD-95

Chronic stress or corticosterone exposure leads to reduced mPFC PSD-95 levels (Gourley et al., 2009, Li et al., 2011), and it is similarly reduced in the PFC of postmortem samples of MDD patients (Feyissa et al., 2009). While there is a lack of pharmacological tools directly targeting PSD-95, a PSD-95 constitutive knockout mouse has altered anxiety-like behavior in the EPM, and increased stress-responsivity as measured in the SIH (Feyder et al., 2010). The role of PSD-95 in depression-related behavior has not been studied to date.

4.4.3 AMPARs

In the case of the ionotropic glutamatergic receptors, administration of drugs blocking non-NMDARs (i.e., AMPAR and kainate receptors), such as NBQX, does not affect depression-related activity in the FST (Maeng et al., 2008). Administration of drugs selectively targeting AMPARs, such as GYKI 52466 or LY32635, have been found to cause anxiolytic-like (Matheus and Guimaraes, 1997, Kotlinska and Liljequist,

1998, Alt et al., 2006, Kapus et al., 2008), anxiogenic-like (Vekovischeva et al., 2007), or no changes (Kapus et al., 2008, Fitzgerald et al., 2010) in anxiety-related behaviors, depending on the rodent species tested or behavioral paradigm used.

The role of AMPAR receptors in these behaviors has also been studied through genetic manipulation of the GluA1-4 subunits and their phosphorylation sites. Mice with a constitutive deletion of the GluA1 subunit have increased stress-induced hyperactivity, reduced depressive-like behavior in the FST that dissipated over repeated swim exposures, and reduced anxiety-like behavior as measured by the EPM and L/D (Fitzgerald et al., 2010). GluA1 KO mice showed normal alterations in anxiety-like behavior in response to repeated stress (Wiedholz et al., 2008). However, increased risk assessment in the L/D emergence task, and elevated anxiety-like behavior in the non-approach/avoidance-based task of SIH, suggest that these mice do not display a purely anxiolytic-like phenotype, but might instead display increased manic-like, or hyper-approach behavior. The behavioral effects of GluA1 deletion may seem counter to work showing increased hippocampal and mPFC GluA1 levels after antidepressant treatment (Du et al., 2007, Li et al., 2010), and decreased levels after exposure to chronic stress (Pickering et al., 2006, Li et al., 2011). However these results could be explained by the lack of regional specificity of systemic or constitutive GluA1 manipulations, as this subunit likely exerts different behavioral contributions in the hippocampus, mPFC and amygdala.

As phosphorylation of the GluA1 subunit is thought to be involved in mechanisms of synaptic plasticity (Lee et al., 2003), these sites are likely to play a role in the stress-induced alterations in LTP and LTD (Qi et al., 2009). Mice with knockin mutations of the serine 845 and serine 831 phosphorylation sites on GluA1 were found to have normal baseline surface GluA1 levels, but deficits in LTP and LTD (Lee et al., 2003). These mice were reported to have increased anxiety-like behavior in the OF, and increased depression-related behavior in the TST (Svenningsson et al., 2007). They also failed to show normal augmentation of fear memory formation after stress-inducing exposure to a predator scent (Hu et al., 2007).

4.4.4 NMDARs

Blockade of NMDARs with systemic antagonists such as ketamine, PCP, and MK-801 disrupt the PFC-mediated cognitive tasks that are disrupted after stress (Abdul-Monim et al., 2007, Nikiforuk et al., 2010, Kos et al., 2011, Smith et al., 2011). NMDAR antagonists such as ketamine have been suggested to have antidepressant activity (Trullas and Skolnick, 1990) and reduce depression-related behavior in clinical (Berman et al., 2000, Zarate et al., 2006, 2012, Valentine et al., 2011, Diazgranados, et al. 2010) and preclinical assays, such as the FST (Maeng et al., 2008, Li et al., 2010, Autry et al., 2011), and, unlike cognitive deficits, these behavioral effects last for days after drug administration. NMDAR antagonists also have been reported to have anxiety-reducing effects (Cryan and Dev, 2008, Barkus et al., 2010).

Constitutive genetic deletion of the obligatory GluN1 subunit are lethal, however viable conditional knockouts of this subunit have been generated with postnatal deletion in specific regions and cell types. Mice with a restricted deletion of GluN1 to pyramidal cells of the CA3 region of the hippocampus displayed no differences from control mice in HPA-axis activation, anxiety-like behavior in the EPM, spatial working memory in the Y-maze, or freezing behavior in contextual fear conditioning (Cravens

et al., 2006, Christian et al., 2011). Deletion of GluN1 restricted to a subset of GABAergic, as opposed to pyramidal, cells in corticolimbic regions displayed novelty-induced hyperlocomotion, anhedonia in saccharine-preference and anxiety-like behavior in the OF and EPM, that was exacerbated by age and social isolation stress (Belforte et al., 2010). However no significant (a trend was apparent) alterations were observed in depressive-like behavior in the FST (Belforte et al., 2010). These behavioral changes were likely due to compensatory effects that reduced GAD67 expression and cortical disinhibition, as similar mice with a post-adolescent, versus postnatal deletion, did not display similar phenotypes (Belforte et al., 2010).

While there is a lack of GluN2A-specific pharmacological antagonists, mice with a constitutive deletion of this subunit display decreased anxiety- and depressive-like behavior (Boyce-Rustay and Holmes, 2006). These mice also have reduced amygdala spines, suggesting a possible link between dendritic morphology in the amygdala and the reduced anxiety-like phenotype in these mice (Mozhui et al., 2010).

Acute systemic administration of pharmacological antagonists specific to the GluN2B-subunit is sufficient to produce the antidepressant-like effects seen with non subunit-selective NMDAR antagonists such as ketamine, both clinically (Preskorn et al., 2008) and preclinically (Maeng et al., 2008, Li et al., 2010). Administration of selective GluN2B antagonists such as Ro 25-6981 produces no effect on anxiety-like behavior in the mouse EPM (Mathur et al., 2009), but anxiolytic-like in the NSF task (Li et al., 2010). Administration of another GluN2B antagonist, ifenprodil, was also anxiolytic-like in the rat EPM (Fraser et al., 1996). Genetic deletion of GluN2B is postnatal lethal, however mice with a conditional deletion selective to corticolimbic pyramidal cells displayed reduced anxiety-like behaviors (von Engelhardt et al., 2008).

4.4.5 Transporters

Mice lacking specific EAATs have been developed, including GLAST, GLT-1, and EAAC1 constitutive knockouts. GLAST KO mice display increased novelty-induced hyperactivity and abnormal sociability, indicators of a phenotype related to the positive and negative symptoms of schizophrenia (Karlsson et al., 2008, Karlsson et al., 2009). However, this knockouts role in depression- or anxiety-related behaviors, or stress response, has not been tested. Considering previous evidence showing stress-induced changes in GLT-1, but not GLAST, levels, a lack of effect in these behaviors would not be surprising (Zschocke et al., 2005, Autry et al., 2006, Zink et al., 2010).

Deletion of the vesicular glutamate transporter 2 (VGLUT2), is lethal, however conditional knockout restricted to pyramidal cells of corticolimbic regions revealed alterations in activity and anxiety-like or risk-taking behaviors (reviewed in (Wallen-Mackenzie et al., 2010).

4.5 NMDAR AND ANTIDEPRESSANT MEDIATION OF THE EFFECTS OF STRESS

4.5.1 NMDAR blockers and deletions

The role of glutamate in mediating effects of stress suggests that altering glutamatergic transmission can block stress-induced molecular, morphological and

behavioral changes. In early support for this, administration of the anti- epileptic drug phenytoin during chronic stress blocks the dendritic atrophy observed in the hippocampus (Watanabe et al., 1992). The administration of NMDAR, but not AMPAR, antagonists blocks stress-induced morphological changes in pyramidal cells in the hippocampus (Magarinos and McEwen, 1995), with similar results in the mPFC (Martin and Wellman, 2011). Administration of NMDAR-antagonists also block stress-induced alterations of hippocampal LTP (Kim et al., 1996). Hippocampal CA3 and CA1 pyramidal cell atrophy caused by restraint stress was blocked by CA3 pyramidal cell-specific conditional knockout of GluN1, suggesting that these effects are mediated specifically by pyramidal cell NMDARs (Christian et al., 2011). These findings suggest that increased NMDAR-mediated glutamate transmission has a critical role in mediating the morphological effects of stress. This would be consistent with the aforementioned work showing that excessive glutamate transmission through NMDARs, particularly, GluN2B at extrasynaptic sites, can lead to cell damage (Hardingham et al., 2010). However, the specific role of specifically GluN2B-containing receptors in these morphological effects has not yet been clarified, nor has the role of NMDARs in the behavioural effects of chronic stress.

4.5.2 Antidepressants with glutamate-modulating properties

Multiple antidepressants reduced depolarization- and stress- evoked glutamate release in the hippocampus (Bonanno et al., 2005) and PFC (Musazzi et al., 2010) (For a review see Musazzi et al., 2011). Treatment with the atypical antidepressant tianeptine, but not fluoxetine, can block stress-induced atrophy in the hippocampus (Magarinos et al., 1999, Czeh et al., 2001). Tianeptine can also block the increase of glutamate release in the BLA (Reznikov et al., 2007, McEwen et al., 2010), as well as the stress-induced amygdala hypertrophy and corresponding increases in anxiety-like behavior (McEwen et al., 2010). Similarly, anxiolytics can reduce stress-induced increases in glutamate in the hippocampus and PFC (Bagley and Moghaddam, 1997) and reduce hippocampal atrophy (Magarinos et al., 1999). Chronic treatment with some antidepressants also reduces NMDAR transmission (Reynolds and Miller, 1988, Skolnick et al., 1996, Paul and Skolnick, 2003). These same treatments appear to augment AMPAR transmission, as multiple chronic antidepressant treatments increased phosphorylation of the GluA1 subunit (McEwen et al., 2010, Svenningsson et al., 2007), and synaptic GluA1 and GluA2 levels (Du et al., 2007). AMPAR potentiators have efficacy as antidepressants (reviewed in (Witkin et al., 2007). Also of note, the antidepressant riluzole enhances glutamate transporter activity (Fumagalli et al., 2008), and the mood stabilizer valproate has been shown to increase EAAT1 but decrease EAAT2 levels in the hippocampus (Ueda and Willmore, 2000, Hassel et al., 2001).

4.5.3 Reversing stress effects

There is emerging evidence that alterations in glutamatergic transmission and antidepressant therapies not only block the development of stress-induced alterations, but can also reverse them. This is not surprising considering the U-shaped curve of glutamate transmission on cellular morphology, as glutamate transmission limited to synaptic receptors can lead to cell growth as opposed to cell death (Hardingham et al. 2010). When administering NMDAR antagonists during stress, there is essentially a

blockade of pyramidal cell extrasynaptic NMDAR receptors, while excessive glutamate release is free to target synaptic AMPARs, possibly leading to hypertrophy, as opposed to hypotrophy. Administration of tianeptine during CUS caused hippocampal hypertrophy (Czeh et al., 2001) and administration of an NMDAR antagonist throughout CIS in rats caused mPFC dendritic hypertrophy not observed in non-stressed animals (Martin and Wellman 2011). Therefore, exposure to stressors can lead to antidepressant-like actions if there is excessive glutamate release, blockade of extrasynaptic receptors, and unblocked synaptic AMPARs. This fits with the previous data showing a role for NMDAR-, but not AMPARs, in mediating stress-induced atrophy. This is not to say that dendritic hypertrophy from baseline is necessarily antidepressant-like, but could provide a mechanism by which treatments such as tianeptine and ketamine are able to reverse stress-induced atrophy and work as an antidepressant.

This suggests a scheme by which antidepressant-like effects could be produced under certain conditions of glutamate release. NMDAR antagonists such as MK-801 and ketamine not only can act to block extrasynaptic pyramidal cell sites, but are also known to induce increases in extracellular glutamate release (Moghaddam et al., 1997). The exact mechanism of this induced glutamate release is unclear, but could be due to a blockade of NMDARs on GABAergic inhibitory neurons that leads to excitatory cell disinhibition (Jackson et al., 2004, Homayoun and Moghaddam, 2007), or through blockade of presynaptic NMDAR autoreceptors that normally inhibit glutamate release.

Ketamine induces glutamate release at the same sub-anesthetic doses that have antidepressant activity (Moghaddam et al., 1997, Berman et al., 2000, Zarate et al., 2006, Maeng et al., 2008, Li et al., 2010). These antidepressant-like effects are dependent on AMPA/kainate receptor activity, indicating a requirement for increased synaptic transmission (Koike et al., 2011, Maeng et al., 2008, Autry et al., 2012) and are reproduced by GluN2B-specific antagonists (Maeng et al., 2008, Preskorn et al., 2008, Li et al., 2010), supporting a role for extrasynaptic GluN2B-containing receptors.

Treatment with these NMDAR antagonists leads to long-lasting antidepressant-like effects that are paralleled by mPFC increases in synaptic proteins such as GluA1 and PSD-95, as well as increased spine growth (Li et al., 2010). Manipulations blocking the increase in synaptic proteins, such as rapamycin-induced blockade of mTOR signaling pathways, similarly block increased antidepressant-like behaviors (Li et al., 2010). These same NMDAR antagonists can reverse CUS-induced spine loss and behavioral changes (Li et al., 2011), and are known to increase BDNF, and neurogenesis (Metsis et al., 1993, Gould and Cameron, 1997).

In total, this suggests a model where the balance between synaptic and extrasynaptic glutamatergic transmission in corticolimbic regions affects the development of depressive- versus antidepressant-like behaviors. Excessive glutamatergic stimulation during stress leads to increased extrasynaptic GluN2B activation causing a cascade of molecular and morphological changes in the mPFC, hippocampus and BLA that lead to depression-related behaviors. Blockade of these extrasynaptic GluN2B sites during excessive glutamate release of stress or NMDAR antagonism leads to predominantly synaptic transmission and activation of molecular and morphological changes that induce antidepressant-like effects **(See Figure 6).** This role of NMDARs in mediating depression-related functions has previously been suggested in Marsden et al., 2011, and reviewed in Sanacora et al., 2012.

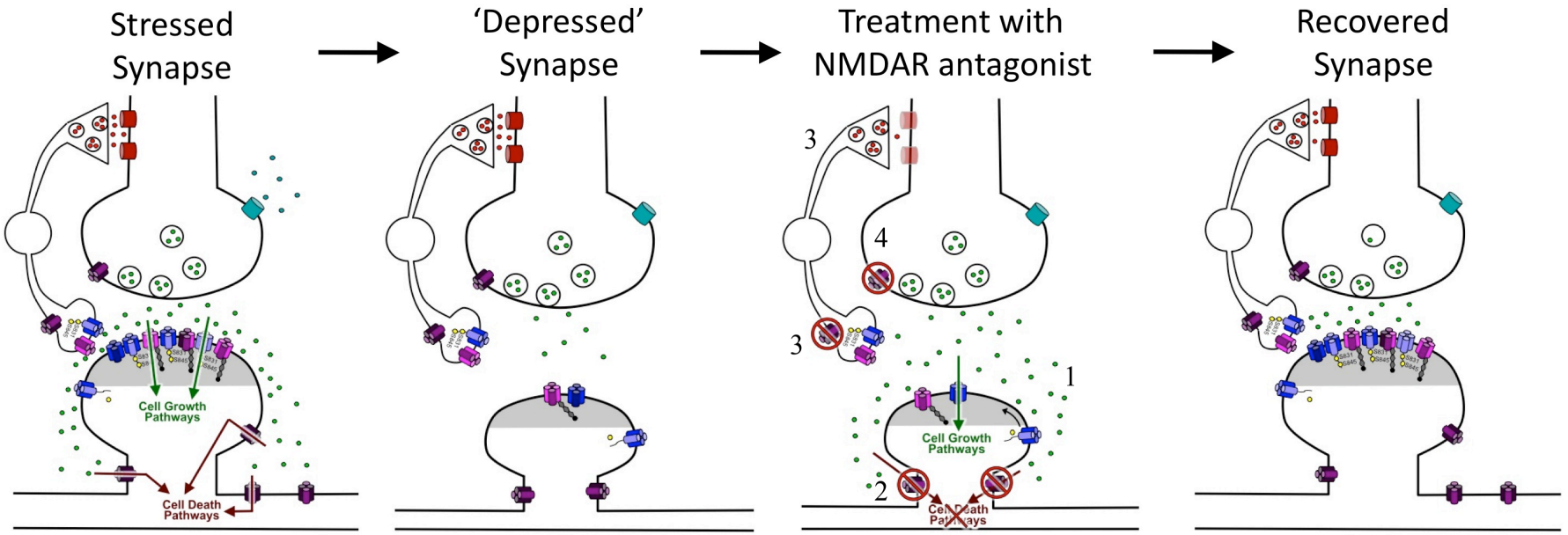

Figure 6 Changes to the synapse after stress, and reversal with NMDAR antagonists. Exposure to stress leads to excess glutamate release which can spillover to extrasynaptic GluN2B-containing receptors. Transmission through these receptors can lead to the activation of cell death pathways **(A)** that presumably leads to the dendritic atrophy, spine loss, and loss of synaptic proteins in the postsynaptic pyramidal cell **(B)**. Application of NMDAR antagonists reverse these morphological changes, potentially by their ability to both increase extraceullular glutamate release (1) while blocking postsynaptic extrasynaptic GluN2B receptors (2) **(C).** This increased glutamate release could be mediate by disinhibition of the presynaptic glutamatergic cell by blockade of GABAergic cell NMDARs (3), or alternatively by blockade of NMDAR autoreceptors on the presynatpic cell (4). The resulting selective activation of cell growth pathways leads to recovery from spine loss and dendritic atrophy **(D)**

5 AIMS

These stress- and antidepressant- induced changes involve multiple mediators of synaptic activity and plasticity including PSD-95 and receptors of the glutamatergic system, such as AMPARs and NMDARs. However, we propose that it is not alterations in these targets *per se* that lead to depression-related behaviors, but their involvement in pathways regulating plastic changes leading to morphological and therefore functional changes. For example, while blockade of NMDARs have antidepressant-like effects, we propose that this could involve the combination of NMDAR blockade and excessive glutamate release, and that extrasynaptic NMDAR deletion in the absence of excessive glutamate release will not affect depression-related behavior. Therefore, antidepressant-like effects of NMDAR antagonists will require a blockade of extrasynaptic GluN2B-containing receptors, unblocked synaptic or AMPAR transmission, and increased glutamate release, possibly through blockade of presynaptic or GABAergic cell NMDARs. Behavioral changes induced by repeated stress will conversely require increased glutamate transmission through extrasynaptic GluN2B receptors, but will not be affected by manipulations to targets at synaptic sites such as PSD-95 or GluA1. To test these hypotheses, we carried out the following aims:

I. Evaluate mediators of synaptic activity that are altered after stress for effects in spontaneous measures of anxiety- and depression-related behaviors (Papers I, II, IV, V)

II. Evaluate these synaptic proteins in the antidepressant-like effect of the GluN2B-selective antagonist Ro 25-6981 (Paper V)

III. Evaluate the corticolimbic regions mediating the antidepressant-like response to reduced GluN2B transmission (preliminary data/Appendix)

IV. Develop a novel paradigm to test behavioral adaptations to stress in the C57BL/6J mouse and evaluate the role of synaptic proteins in behavioral adaptations in this paradigm (Paper I, IV, V)

6 METHODOLOGICAL CONSIDERATIONS

6.1 BEHAVIORAL ASSAYS

In all of our behavioral testing we took great care to control for environmental influences such as changes in stress history, time of day and year, and husbandry conditions, as many of these factors can lead to inter-laboratory variability (Crabbe et al., 1999). This was a potential issue in comparing studies between laboratories at NIH and Karolinska, where the testing equipment, housing, and research staff were often different. Within laboratories, we always strived to limit variation by controlling for time of day, facility conditions such as temperature and light-cycle, and keeping the experimenter conducting all tests consistent. Mice were always given one week of adaptation to the facility, and given 1 hour in the procedure room before testing. That being said, we did encountered some inter-laboratory variation in baseline levels of anxiety in a few of the tests, such as NIH, and pilot studies in a new strain of mice or location were often helpful to re-establish ideal testing conditions.

6.1.1 Anxiety-related tasks

In phenotyping the spontaneous anxiety-related behaviors we generally made use of a battery of similar, but non-overlapping behavioral assays. As discussed earlier, we used inherent control measures in these tasks to check for any confounding hyperactivity or novelty-seeking behaviors. When possible, we tested baseline changes in locomotion in the OF. In the L/D, we used the total distance traveled in the last 5 minutes as a measure of general changes in locomotion that is less reflective of anxiety-like behavior as the novelty of the light compartment is at its minimum at the end of the task. As the recording equipment used for this task at Karolinska Institute did not allow for measurements of in shelter activity in Paper II, we made use of the novel open field to test mice in locomotion, and used measurements of risk assessment in L/D to confirm the specificity of effects on anxiety-related behavior. Similarly, assessment of entries into the closed, as opposed to open, arms of the EPM can serve as a control for increased novelty-seeking. As these L/D and EPM vary in their severity of provoking anxiety, employing both minimized the possibility of missing anxiety-like behaviors due to floor or ceiling effects.

6.1.2 Spontaneous FST

In the FST, we measured immobility in the last 4 minutes of the 6 minute test, using the first two minutes as an induction period. Behavior was handscored by the same experimenter throughout all testing. In general results in the FST were consistent and confirmed previously published findings, however we did find some inconsistencies in effects of gentoype within our own testing of the pGluA1 and fGluN2B^{CaMKII} mice in the FST. As previous work in these mice suggested altered function only after exposure to a challenge or stressor (Lee et al., 2003, Hu et al., 2007), it is likely that behavioural results are sensitive to previous stimuli such as stress exposure, and we often found inconsistencies when there were alterations in injection stress before testing. In both genetic and pharmacological studies, we were able to test

for confounding hyperactivity in the FST by performing the OF or L/D in the same cohort of mice with at least one week between these tests and the FST. As noted earlier, anxiety- and depression-related assays gain validity in part because of their sensitivity to stress, while prior experience with stress can be difficult to control and is unavoidable with the use of some experimental methods. Here we strove to reduce or keep consistent exposure to stress where possible, but acknowledge that this remains a source of variation in our studies.

6.1.3 Repeated inescapable forced swim (riFS)

To analyze the behavioral adaptations to a repeated stress in mice on a C57BL/6J background, we developed a novel repeated inescapable forced swim paradigm (riFS) that involved daily 1-minute forced swim for 10 consecutive days. This procedure allowed us to observe the development of despair-like behavior by measuring immobility during that one minute. While most forced swim procedures last between 6 and 15 minutes, we felt that the shorter duration would allow observation of greater differences between experimental groups as longer durations could lead to ceiling effects in immobility. To counter for this shorter duration and increase the stressfulness of the task, we used a slightly larger swim tank and added collapsible platform that would allow the animal to try, and fail, to escape the tank at the end of the first minute. Though not used here, this design also allowed us the option of creating an escapable stress condition (reFS), where the platform could instead be fixed at the surface of the water under an escape hole, allowing the animal to escape to its homecage **(Figure 7).**

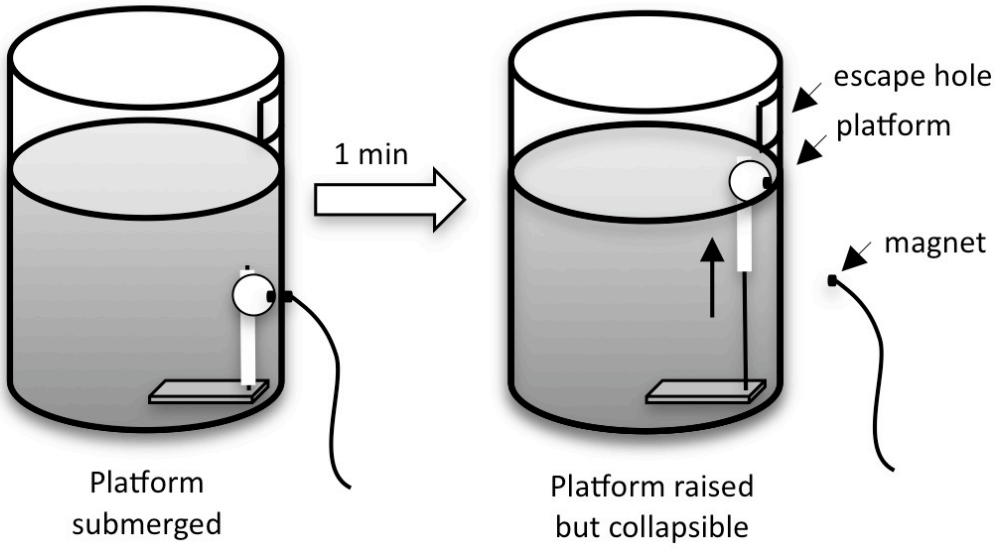

Figure 7 Repeated inescapable forced swim (riFS). We designed a putatively more stressful swim tank design involving a swim tank with a larger diameter than the traditional spontaneous FST. Throughout the first minute of testing, a platform was kept submerged out of the reach of the mouse by a magnet on the outside of the tank. After the minute was over, the platform was release and allowed to float to the surface of the water underneath an escape hole. If left unfixed, the platform would collapse under the weight of a mouse trying to reach the escape hole (riFS). Alternatively, the platform could be fixed to the side of the tank with an additional magnet, allowing the mouse to escape through the exit hole and to its home cage (reFS).

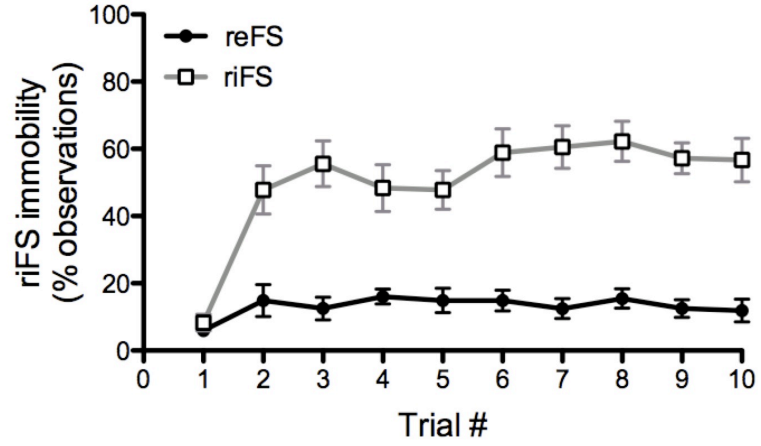

Figure 8 Immobility over 10 trials of the repeated stress. Mice were placed in the swim tank for 1 minute per day over 10 days. Upon the first presentation, mice showed little or no immobility. The percent time immobile, however, increased quickly in the inescapable riFS condition. This same increase in immobility was not observed in the escapable reFS condition

We found that mice generally swam during the entire first trial, and immobility consistently increased across the 10 days, but this was not the case in the escapable condition **(Figure 8)**. Analysis of corticolimbic regions via western blotting after the 10 days of stress revealed significant alterations in the synaptic proteins GluN2B and GluA1 in the inescapable, but not escapable, stress groups as compared to homecage controls **(Figure 9**). These alterations in synaptic proteins followed patterns expected from morphological changes normally observed after chronic stress (decreased in hippocampus, increased in amygdala), however the morphological effects of this stress regime have not been directly tested. This despair-like behavior in the inescapable condition was found to be mediated by the vmPFC as excitoxic lesions to this region significantly altered immobility **(Figure 10).** We checked for any confounding locomotor or acute FST effects by testing the effects of experimental manipulations in the OF and FST. However, there were a number of examples in which alterations in immobility in the spontaneous FST failed to affect immobility in riFS, suggesting that effects in the spontaneous FST are dissociable from effects in the riFS test.

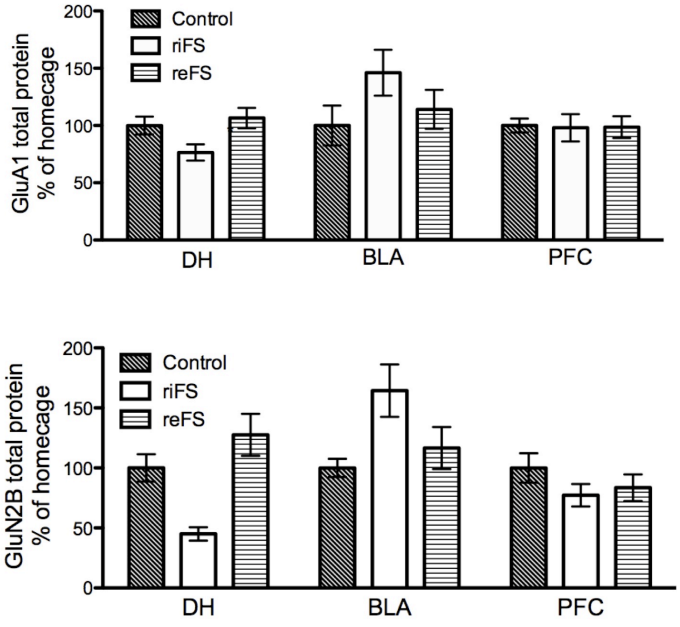

Figure 9 Alterations in corticolimbic synaptic proteins after repeated stress. Tissue punch of fresh frozen brains collected after the 10 day repeated stress revealed decreased GluN2B and GluA1 in the DH in the inescapable, but not escapable condition. Contrastingly, a trend for increased expression of these two synaptic proteins was observed in the BLA after inescapable stress. No significant changes were observed in the mPFC. These alterations in two synaptic protein parallels the hypotrophy and hypertrophy normally observed in the DH and BLA, respectively, after chronic stress and appears to be blocked by the option to escape the stressor.

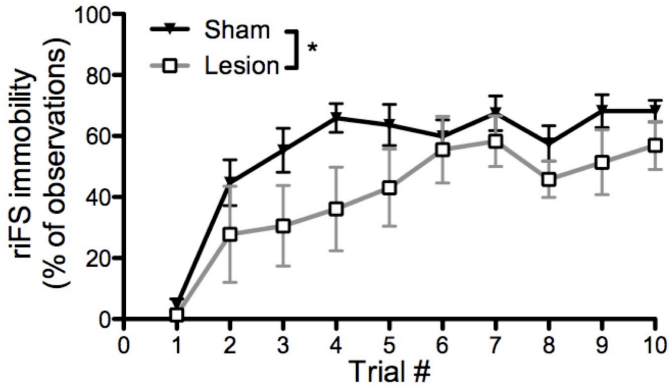

Figure 10 Despair-like behavior in riFS is mediated by the mPFC. Excitotoxic lesions of the mPFC significantly altered despair-like behavior in riFS over the 10 days, suggesting that this region is involved in the behavioural responses to repeated stress.

6.2 CONVERGENT PHARMACOLOGICAL AND GENETIC TECHNIQUES

6.2.1 Pharmacological techniques

When available, we made use of pharmacological manipulations to target mediators of synaptic activity. Pharmacological tools allow for both temporal and regional specificity, as treatments can be administered at any age or stage of behavioral testing, as well as regional specificity, as treatments can be infused into discrete regions of the brain. They also allow for better comparisons to human clinical trials as most treatments involve systemic pharmaceuticals. However, these treatments often have off-target effects that may not be well characterized (Keiser et al., 2009). Additionally, pharmacological tools are not available for some targets such as GluN2A or PSD-95. While pharmacological interventions allow for temporal, and regional specificity, they do not offer cell-type selectivity or permanent effects throughout long-term testing. Therefore, we also made use of genetically altered mouse lines to analyze our targets of interest and the role of cell-type in their behavioral effects.

6.2.2 Constitutive knockouts

For the synaptic proteins lacking specific pharmacological agents, such as GluN2A, PSD-95, and GluA1, we made use of constitutive knockouts to analyze broadly the role of these targets in spontaneous behaviors, as well as responses to stress and treatments. However, as constitutive deletions are expressed throughout development and in all regions of the body, these knockouts often display developmental and compensatory effects (see (Zhou et al., 2009) for example of changes in GluA1 KO), and behavioral alterations can be difficult to interpret. The alterations in anxiety- and depression-related behaviors observed here would be best followed up with more specific target deletion, such as with conditional knockouts.

6.2.3 Conditional knockouts

We did make use of conditional knockouts in the NMDAR-related targets in order to explore beyond the pharmacological treatments available. While constitutive GluN1 and GluN2B deletions are lethal, postnatal conditional knockouts allow for viable

progeny. Cre/lox conditional knockouts cause a tagged gene of interest ('floxed' gene) to be deleted only in regions and time points that parallel expression of Cre recombinase. The expression pattern of Cre is controlled by a promoter and multiple promoters exist to choose from that vary in the regions and timepoints of Cre expression. Here we made use of two different promoter-driven Cre lines. The CaMKII-driven Cre line expressed Cre (and therefore deleted floxed genes), in only pyramidal cells of the cortex and CA1 postnatally. The Ppp1r2-driven Cre line expressed Cre in 40-50% of GABAergic neurons, mainly parvalbumin positive fast-spiking neurons, in corticolimbic regions. Each of these Cre lines could then be crossed with mouse lines carrying floxed GluN1 or GluN2B in order to cause a deletion of these genes in these Cre-expressing areas. This conditional knockout strategy also allowed us to have some regional specificity. However, this specificity is limited by the available patterns of Cre recombinase expression driven by the promoters available. In the $fGluN2B^{CaMKII}$ mouse line, we had a GluN2B deletion specific to pyramidal cells in the cortex, but the pattern of Cre expression necessitated CA1 deletion as well, making interpretations of purely cortically-driven behavior difficult. Also, Cre expression patterns can change with age and the $fGluN2B^{CaMKII}$ saw the spreading of Cre expression to the striatum after 20 weeks of age (Brigman et al., 2010), necessitating younger ages for testing. This age-dependent Cre expression can make comparison between genetic lines difficult as each line may require a different age of testing. Here we also made use of a GluN1 deletion in GABAergic neurons that had a postadolescent expression to avoid developmental effects. This line was therefore not tested until after 20 weeks of age, making comparison to the $fGluN2B^{CaMKII}$ line difficult. Similarly, the Ppp1r2 driven Cre lines had deletion in only 40-50% of GABAergic neurons, whereas a complete deletion could have led to clearer interpretations of behavioral data.

6.2.4 Virally-mediated knockdown

The lack of regional and temporal specificity of conditional knockouts can in part be ameliorated with the use of lentiviral-mediated knockdown of the targeted gene. Here, instead of breeding floxed GluN2B mice to different Cre-expressing strains, we used stereotaxic local infusions of a Cre-expressing lentivirus to the mPFC 3 weeks before behavioral testing. This allowed for a mPFC specific manipulation, and hopefully reduced the amount of compensatory and developmental effects normally seen. However, there can be variations in the amount of successful infection, resulting in an incomplete knockdown. It is also unclear what functional effects this genetic knockdown has as it will depend on the amount of turnover of already existing GluN2B receptors, and this will be tested using western blotting to measure GluN2B protein levels. Additionally, with the virus used here, we loose cell-type specificity, though we are hoping in the future to make use of cell-type specific vectors.

Each of these techniques has benefits and challenges. While pharmacological tools such as NMDAR antagonists often have off-target effects, we cannot ignore these tools as the clinical effect of these drugs may, in part, be due to these unintended targets. Below we'll show that the more target-specific genetic manipulations to NMDARs do not replicate the pharmacological effects of antagonists blocking these same receptors. However, in trying to determine the molecular mechanisms of behavioural responses to these drugs and of stress, genetic tools allow more specific analysis of the exact targets involved.

7 RESULTS AND DISCUSSION

7.1 AIM 1: SYNAPTIC MEDIATORS IN SPONTANEOUS BEHAVIORS

Using the mouse, we were able to utilize genetic and pharmacological manipulations to test the role of mediators of synaptic activity in behaviors related to anxiety and depression. This allowed us to understand the role of these molecules in the etiology of these disorders, as well as to understand the baseline behavioral changes that could influence future studies on treatment and stress response. Many of the manipulations of these molecules were analyzed in a battery of complementary behavioral paradigms; however, here we will focus on the effects in the spontaneous 6 minute FST for depression-related behavior and antidepressant-like effects. We found that constitutive deletions of synaptic proteins (PSD-95, GluA1, GluN2A), led to antidepressant-like phenotypes in the FST though with some corresponding hyperactivity, and these results did not reconcile readily with previously found roles of these targets in depression etiology and treatment. The role of NMDARs, especially the GluN2B subunit, in these spontaneous behaviors, appeared to depend on the method of deletion as we saw different results in pharmacological versus genetic manipulations.

7.1.1 Kv4.2 (Paper I)

In Paper I, we analyze the phenotype of mice with a constitutive deletion of Kv4.2. As this was a novel mutant not previously characterized in our laboratory, we first tested these mice on a basic neurological battery to analyze baseline behaviors that might affect future testing. Previously tied to alterations in dendritic excitability, Kv4.2 deletion resulted in behaviors indicative of heightened reactivity to novel stimuli, including in the OF, fear response to auditory tone, and increased corticosterone response to stress. While there was some evidence for decreased anxiety-like behavior in the EPM, the lack of effect in the L/D, along with indications of a novelty-induced hyperactivity, suggests further testing is needed determine whether these mice show a true anxiety-related phenotype.

The battery of tests employed indicated a lack of depression-related effects as measured in the FST **(Figure 11).** This mouse, previously found to have augmented synaptic plasticity, increased dendritic excitability, and altered NMDAR levels (Jung et al., 2008) did not show pro or antidepressant-like behavior in the FST. This lack of phenotype could be due to alternative mechanisms that can compensate for the loss of Kv4.2 (Hu et al., 2006, Andrasfalvy et al., 2008). In mice with a deletion of Kv4.2, and subsequent changes in synaptic plasticity, we might expect to see a stronger phenotype in behaviors inducing behavioral adaptations, such as after repeated stress. Here we tested these mice in the riFS proceduce and found no difference between the genotypes. In sum

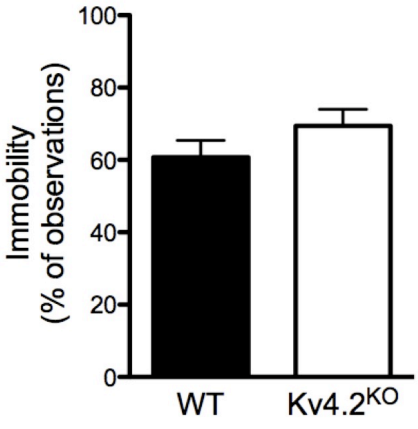

Figure 11 Kv4.2 in depression-related behavior. Mice with a constitutive deletion of Kv4.2 displayed no alterations in depressin-related behavior as measured in the FST

however, the findings of this paper did not support a contribution of Kv4.2, and the associated alteration in dendritic excitability and synaptic plasticity, in mediating an antidepressant-like response in the FST.

7.1.2 PSD-95 (Paper V)

Continuing our interest in dendritic or postsynaptic activity, we investigated the depression-related behavior of mice lacking functional PSD-95. PSD-95 is a key component of the postsynaptic density known to be involved in the anchoring of NMDARs, as well as transducing glutamate receptor transmission to intracellular signaling pathways (Kim and Sheng, 2004, Malenka and Bear, 2004, Beique et al., 2006, Elias and Nicoll, 2007). These mice were previously described, and, despite PSD-95s direct interaction with NMDARs, found to have no alterations in NMDAR levels, but altered AMPARs in some, but not all synapses. Behaviorally, these mice displayed altered anxiety-like behavior and stress responsivity, as well as subtle changes in amygdala spine morphology (Feyder et al., 2010, Camp et al., 2011). However, to our knowledge, these mice have not been tested in FST. We found an antidepressant-like phenotype as measured by the FST **(Figure 12A)** in Paper V, with no associated change in locomotion as measured in the OF. This antidepressant-like effect of the deletion is unexpected given evidence that depressed patients show reduced PSD-95 levels, and NMDAR antagonist treatments that have antidepressant-like responses in the FST increased PFC PSD-95 levels. Manipulations of PSD-95 with more regional and time specificity would be useful in exploring its role in antidepressant-like behaviors.

7.1.3 GluA1 (Paper III and V)

In Paper III, we showed that deletion of the GluA1 subunit of the AMPARs similarly leads to altered social interaction in a novel, but not familiar environment, but does not lead to changes in anhedonia as measured by sucrose preference. Compared to the lack of depression-related behavior as measured by anhendonia, Paper V revealed a reduction in depression-related behavior in the FST as published previously (Fitzgerald et al., 2010) and confirmed here in Paper V **(Figure 12B).** Similar to the PSD-95 KO, this in part conflicts with previous data to the extent that AMPARs have been shown to be upregulated after ketamine (Li et al., 2010) and chronic antidepressant treatment (Du et al., 2007). In addition, AMPAkines have antidepressant-like activity (Witkin et al., 2007), and AMPARs are necessary for NMDAR antagonists antidepressant-like response (Maeng et al., 2008). Unlike the PSD-95 mice, this reduction in immobility in the FST could be due to the confounding hyperactivity observed in the OF, as well as compensatory changes due to this deletion revealed by genome scan of these mice (Zhou et al., 2009). Again, region or time specific manipulations of this subunit would help clarify the conflicting data of this subunit on depression-related behaviors

7.1.4 GluA1 phosphorylation sites (Paper II and V)

We investigated the function of the GluA1 subunit further in Paper II and V with mice designed with a knock-in mutation on two key phosphorylation sites on this

subunit (S845 and S831), known to be involved in trafficking of GluA1 to the synapse. These mice were previously found to have normal surface levels of GluA1, suggesting compensatory trafficking levels were able to maintain GluA1 levels when these phosphorylation sites are absent (Lee et al., 2003). However, upon stimulation, these phosphomutant mice displayed altered synaptic plasticity as measured by LTP and LTD, and showed learning deficits in spatial learning tasks (Lee et al., 2003). Similar to the GluA1 and Kv4.2 knockout mice, a phenotypic battery of these mice revealed some changes in anxiety-like behavior that could also be interpreted as alterations in response to novelty. While these mice did not show the same OF hyperactivity as the GluA1 KO, they did show evidence of hyper-approach behavior in the EPM as they approached both open and closed arms more than wildtype mice.

The fact that these mice require stimulation to show abnormalities in synaptic plasticity suggests that behavioural phenotypes might similarly not become apparent until challenged with stress, learning paradigms, or even exposure to previous behavioural testing. This could explain in part the variation in behavioural measures in depression-related tasks as previous work in our laboratory found a pro-depressive phenotype in the TST (Svenningsson et al., 2007), but we found decreased depressive-like activity in a FST conducted at the end of a test battery, and no change in FST experiments involving a saline injection **(Figure 12C).**

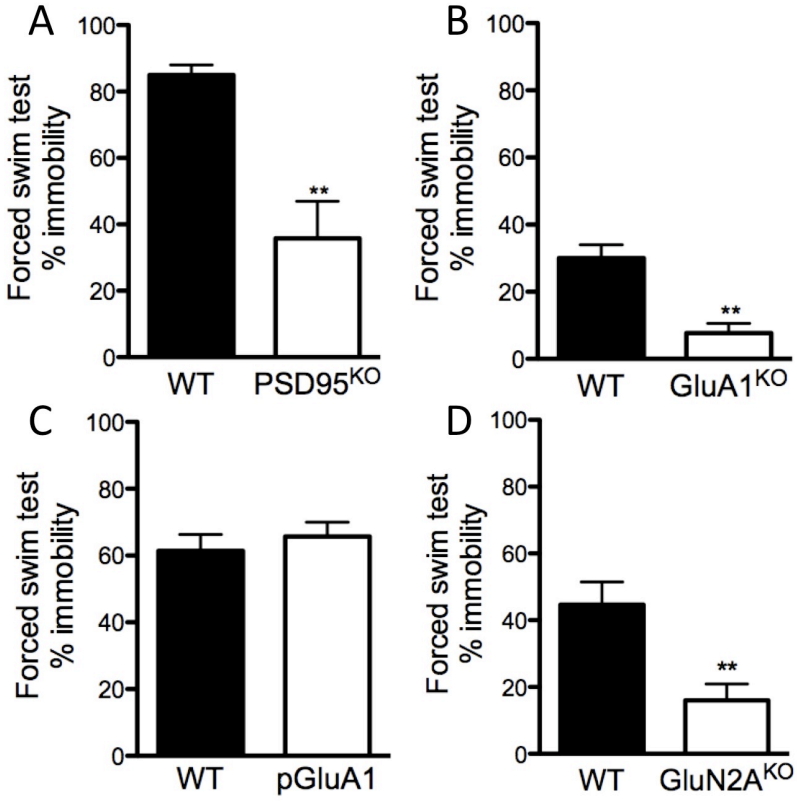

Figure 12 Mediators of Synaptic Activity in depression-related behavior. Mice with deletions to **(A)** PSD-95 showed reduced depression-related behavior in the FST. A similar phenotype was observed in GluA1 KO mice **(B),** but not in phosphomutant GluA1 mice **(C).** GluN2A KO mice showed reduced depression-related behaviors **(D)**

7.1.5 NMDARs (Papers IV, V)

NMDAR antagonists reduce depression-related behavior acutely both in clinic and preclinical assays including the FST (Berman et al., 2000, Zarate et al., 2006, Maeng et al., 2008, Li et al., 2010). Here, we genetically targeted subunits of NMDAR in mouse lines and analyzed their behavior in anxiety- and depression-related tasks.

NMDARs containing the GluN2A, versus GluN2B subunit, are thought to be the predominant NMDAR at the synapse in adulthood, and its shorter postsynaptic current leads to a decreased window for coincidence detection and constrained plasticity. While there is a lack of GluN2A-specific antagonists, constitutive GluN2A KO mice are viable. Previous testing revealed a decreased anxiety and depressive-like phenotype (Boyce-Rustay and Holmes, 2006). Here, we confirm the antidepressant-like effect of this deletion in the FST **(Figure 12D),** however we also observed potentially confounding hyperactivity in the OF. This FST phenotype suggests that the antidepressant effects of NMDAR antagonists can in part be reproduced by a selective blockade of the GluN2A-containing NMDARs. However, as with the other constitutive mutants, these results should be interpreted with caution, as these behavioral results can be confounded by hyperactivity, and seizure propensity, as well as compensatory changes. A selective pharmacological agent, or conditional knockout mice will be useful for direct comparison to GluN2B-containing receptors in the future.

Unlike the GluN2A subunit, selective antagonists, such as Ro 25-6981, are available for the GluN2B-containing receptors and are shown to reduce depression-related behaviors in both clinical and preclinical settings including the FST **(Figure 13A)**. As the predominant forebrain NMDAR subunit during early development, deletion of the GluN2B subunit is lethal. However, conditional postnatal knockouts are available and viable, and furthermore allow analysis of the circuits and cell-types involved in GluN2B-mediated behaviors. Here, we tested anxiety- and depression-related behavior in mice with a postnatal deletion of GluN2B specific to the pyramidal cells of the cortex, as well as CA1 region of the hippocampus as previously described (Brigman et al., 2010) and found no alterations in the FST **(Figure 13B).**

To parse the role of GluN2B on specific cell-types, we also tested depression-related behavior with a postnatal deletion of GluN2B in 40-50% of GABAergic interneurons in corticolimbic regions. Mutant mice with a similar GABAergic deletion of GluN1 were previously found to have compensatory/developmental changes leading to disinhibition of excitatory cells, while these developmental changes were not observed in a post-adolescent knockout (Belforte et al., 2010). We therefore made use of postadolescent GluN1, and postnatal GluN2B interneuronal knockout mice in testing for depression-related behavior. In both mouse lines, we did not find antidepressant-like effects of the deletions in the FST, and we were unable to recapitulate the effects of systemic treatment with NMDAR antagonists **(Figure 13C,D).** This could be due to the incomplete deletion of the targets, particularly in the GABAergic type neurons. Alternatively, it could suggest that the antidepressant-like effects of NMDAR antagonists are not due to GluN2B blockade *per se*. The studies described below attempted to delineate the mechanisms underyling the antidepressant-like effect of NMDAR antagonists.

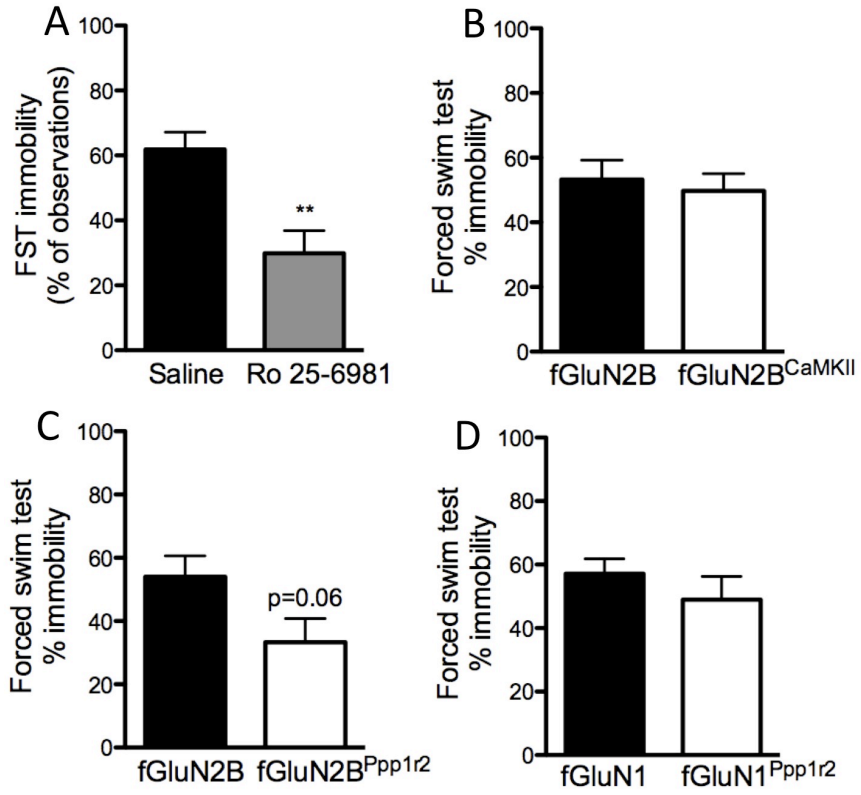

Figure 13 NMDAR subunits in depression-related behaviors. While pharmacological antagonism of GluN2B by Ro 25-6981 reduced depression-like behavior in the FST **(A),** similar effects were not reproduced in genetic deletions of GluN2B in pyramidal cells **(B)**, or in an interneuron specific deletion of GluN2B **(C),** or GluN1 **(D).**

7.2 AIM II: SYNAPTIC PROTEINS IN ANTIDEPRESSANT-LIKE RESPONSE TO RO 25-6981 (PAPER V)

To uncover what synaptic proteins might be mediating the antidepressant-like effect of Ro 25-6981, we tested the antidepressant-like response of this drug in the FST in our various mutant mice. In the mutants previously showing baseline reductions of depression-related behavior, analysis of their effect on Ro 25-6981 was occluded by a 'floor effect' in FST immobility. Also, while the antidepressant-like effect of Ro 25-6981 was generally robust in C57BL/6J mice, the drug failed to reduce immobility in two of the strains (fGluN2B^{Ppp1r2}, and pGluA1). This could be due to differences in husbandry or test history. It should be noted that in the pGluA1 mice, even though the drug failed in the WT mice, it showed augmented antidepressant-like effect in the mutants and the mechanisms behind this are unclear. However, the fGluN1^{Ppp1r2} mice showed no baseline alterations in the FST and demonstrated that GABAergic GluN1 deletion did not block the antidepressant-like effect **(Figure 14)**. It should be noted that this is only a partial deletion of GABAergic cell GluN1, occurring mainly on parvalbumin positive fast-spiking neurons. Compared to other interneurons in the cortex, these cells show significant adulthood reductions in NMDAR expression and a relatively low NMDAR/AMPAR ratio, suggesting other classes of interneurons may play a more important role in NMDAR-mediated activity (Wang and Gao, 2010). While other GABAergic cell types may be still playing a role, it suggests that NMDA antagonists may not be in part exerting their antidepressant-like effect by inducing

glutamate release through blockade of interneuronal NMDARs and subsequent disinhibition of pyramidal cells. An alternative target for glutamate release could be blockade of presynaptic NMDAR autoreceptors that normally act to inhibit presynaptic glutamate release. Studies of effective glutamate release in response to Ro 25-6981 in each of these mutants would help clarify the mechanisms involved.

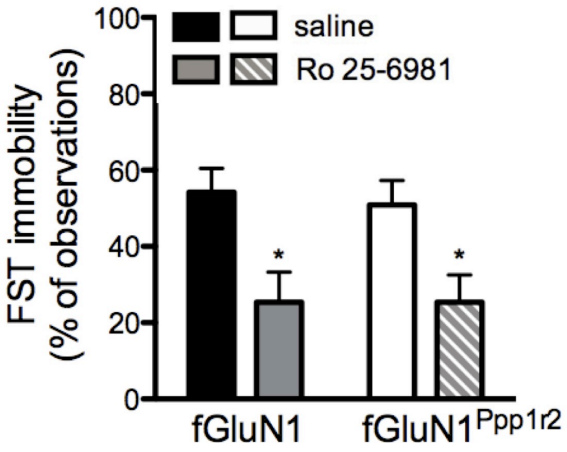

Figure 14 Interneuronal NMDAR subunits in antidepressant-like response to GluN2B antagonism. Mice with a postadolescent deletion of the GluN1 subunit of NMDAR on a subset of GABAergic interneurons display normal antidepressant-like response to Ro 25-6981.

7.3 AIM III: REGIONS INVOLVED IN RESPONSE TO RO 25-6981

7.3.1 Local infusions of Ro 25-6981 (Appendix)

The mPFC, BLA, and DH have been implicated in the pathophysiology of depression, while both the mPFC and DH have been implicated in the behavioral response to ketamine and other NMDAR antagonists (Li et al., 2010, Autry et al., 2011). To directly test the regions involved in the antidepressant-like response to Ro 25-6981, we infused the drug into these regions and then tested the resulting anxiety- and depression-related phenotypes in the L/D and FST. As this represented preliminary data not reported in the attached manuscripts, the methods and results are described in detail in Appendix. In the L/D, there were no significant alterations in anxiety-like behavior following drug infusion into any of these regions, although there was some locomotor hyperactivity. In the FST, only mPFC, but not BLA or DH, infusions resulted in a significant decrease in FST immobility and reproduced the effects seen in systemic administration **(Figure 15A-C).** Together this points to a key role in the mPFC in antidepressant-like response to Ro 25-6981, corresponding to previous evidence showing that activation of intracellular signaling pathways in the mPFC are necessary for antidepressant-like response (Li et al., 2010).

7.3.2 mPFC lentiviral knockdown of GluN2B (Appendix)

As noted, GluN2B cell-type specific mutants did not replicate the antidepressant-like effect of Ro 25-6981, and we proposed that this could be due to the restriction of the deletion to a specific cell-type or to compensatory changes. To circumvent some of these issues, we performed a lentiviral mediated knockdown of GluN2B by infusing a Cre-expressing virus or GFP-control, into the mPFC of GluN2B floxed mice, putatively causing knockdown in all cell types, and tested mice in the L/D and FST 3 weeks later. Similar to the phenotype of the GluN2B mutant mice, we were unable to reproduce the AD-like effect of Ro 25-6981 **(Figure 15D).** Again, this points away from a GluN2B knockdown being sufficient to reproduce the antidepressant-like effect of Ro 25-6981.

NMDAR antagonists increase glutamate release in the PFC, possibly leading to increased synaptic transmission that mediates an antidepressant-like effect through subsequent spine growth. Therefore an antidepressant-like effect of genetic NMDAR deletion might be induced with addition of glutamate release. Exposure to the stress of the FST can supply this increase in extracellular glutamate. Therefore, antidepressant-like effect of NMDAR deletion might become apparent after additional exposures to stress and suggests that testing depression-related behaviors after repeated exposures to stress can be advantageous in understanding the mechanism of NMDAR-mediated antidepressant-like responses

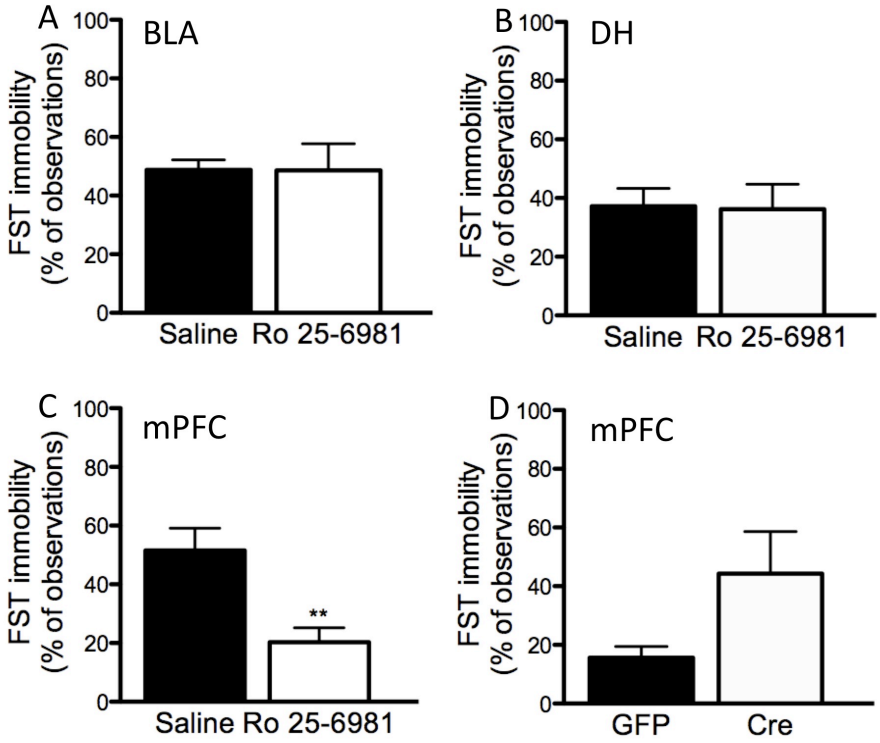

Figure 8 Regional analysis of GluN2B reduction in depression-related behavior. Local infusion of the GluN2B antagonist Ro 25-6981 to the mPFC **(C),** but not BLA **(A)**, or DH **(B),** significantly reduced depression-like behaivor in the FST. This effect was not reproduced by lentiviral-mediated knockdown of GluN2B **(D).**

7.4 AIM IV: SYNAPTIC MEDIATORS IN REPEATED STRESS (PAPER IV, V)

To investigate the effects of exposure to repeated swim stress, we developed the repeated inescapable forced swim paradigm (riFS). While synaptic transmission can lead to cell growth, glutamate release through typically GluN2B-containing extrasynaptic NMDARs typically leads to cell death pathways. In contrast to antidepressants, stress causes elevations in glutamate transmission without a concomitant postsynaptic NMDAR blockade, leading to the atrophy and spine loss as seen in PFC pyramidal cells after stress. Behavioral changes associated with these stress-induced effects may be reduced by deletion or blockade of NMDAR on pyramidal cells. Loss of GluA1, on the other hand, may not have the same protective effects, due to its predominantly synaptic localization and prior finding that AMPAR receptor blockers do not prevent the atrophy caused by stress (Magarinos and McEwen,

1995). Here we tested multiple mouse strains in our riFS procedure in order to analyze their role in behavioral responses to stress.

Systemic administration of Ro 25-6981 was successful in reducing riFS immobility in C57BL/6J mice **(Figure 16C)**. This effect was mimicked by fGluN2B^{CaMKII} mutation **(Figure 16D).** However, a similar effect was not found in either the fGluN2B^{Ppp1r2}, or fGluN1^{Ppp1r2} mice, suggesting that the effects of NMDAR loss were specific to pyramidal cells **(Figure 16E, F)**. This behavioural effect of GluN2B deletion would be in line with previous reports on the role of NMDARs in mediating pyramidal cell atrophy after chronic stress. We also found that GluN2A or GluA1 deletions did not affect riFS behavior (**Figure 16A, B).** This contrasted with the immobility-reducing phenotype of both mutants in the FST, suggesting dissociation between the two assays.

To isolate the contribution of GluN2B in the mPFC to the riFS behavior, we used a lentiviral knockdown of GluN2B. We focused on the mPFC based on our finding that Ro 25-6981 infused into this region was sufficient to produce a FST antidepressant-like effects, as well as a significant effect of mPFC lesions in the riFS paradigm. Counter to our prediction, mPFC GluN2B knockdown did not reduce the development of despair like behavior in the riFS procedure **(Figure 16F).** Successful knockdown of GluN2B still awaits confirmation via in situ hybridization and Western blotting. Assuming knockdown is confirmed, this lack of behavioral effect could indicate a genuine lack of critical involvement of this region. Future testing targeting other regions will be useful to explore this issue further.

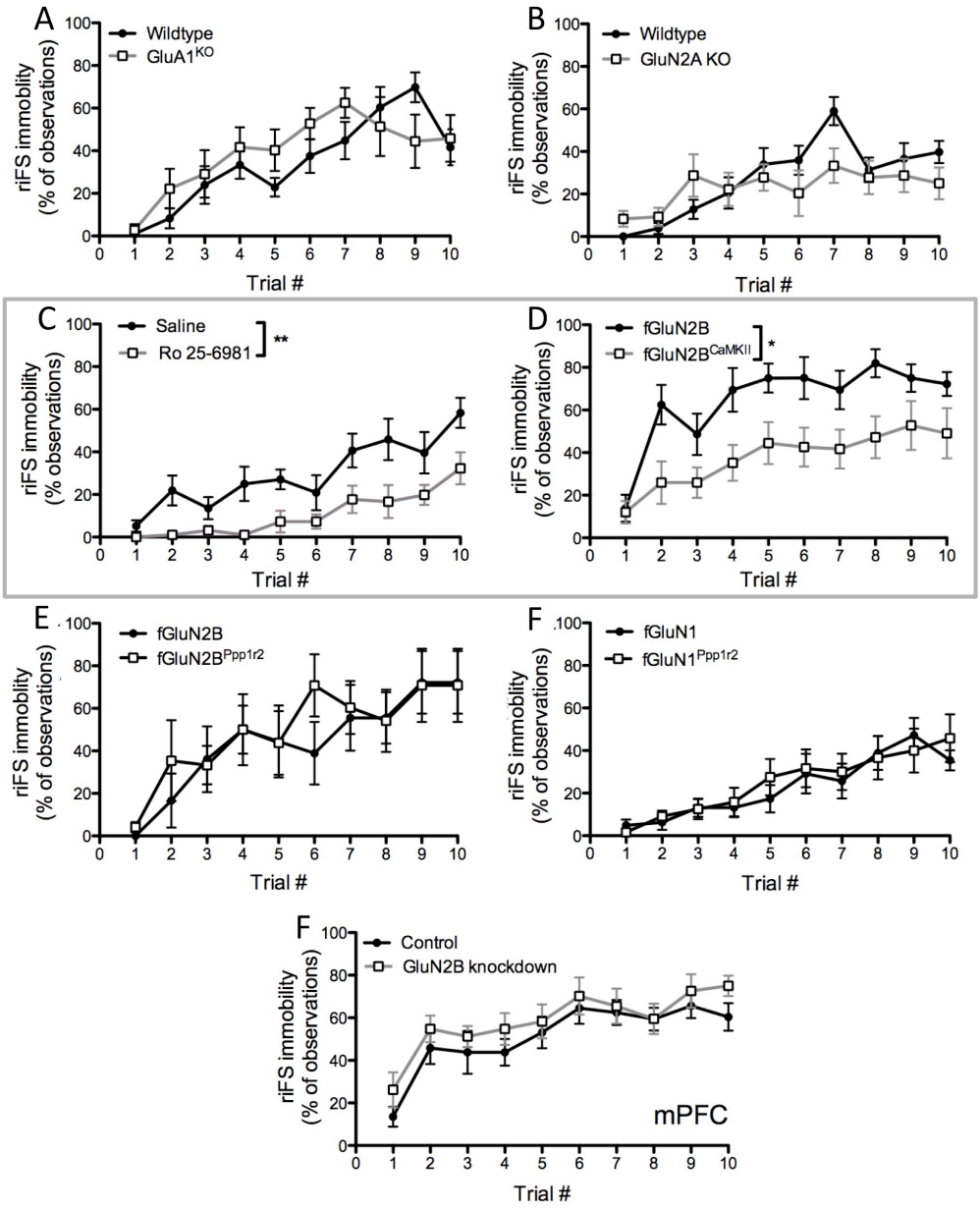

Figure 16 Mediators of synaptic activity in behavioural respsones to repeated stress (riFS). Constitutive deletions of GluA1 **(A)** and GluN2A **(B)** did not significantly alter despair-like behavior in repeated stress, though they had previously shown reduced immobility in the spontaneous FST. NMDAR angtaonism specific to the GluN2B subunit **(C)** significantly reduced despair-like behavior. This effect was reproduced in a genetic deletion of GluN2B specific to pyramidal cells of the cortex and CA1 **(D),** but not in GluN2B or GluN1 deletions restricted to corticolimbic interneurons **(E, F).** A lentiviral mediated knockdown of GluN2B specific to the mPFC also did not effect riFS behavior, suggesting that the effect seen in **C, and D** is mediated by regions other than the mPFC or requires cell-type specificity.

8 CONCLUSIONS

The aims of the current thesis were to first **1)** evaluate various mediators of synaptic activity in anxiety- and depression-related behaviors. We then investigated the mechanism underlying GluN2B antagonists ability to reduce depression-related behaviors by **II)** evaluating the role of various mediators of synaptic activity in this antidepressant-like response and **III)** analyzing the brain regions mediating this effect. Finally, **IV)** we sought to understand how these molecules are involved in behavioral responses to repeated stress using a novel repeated inescapable forced swim paradigm.

For **Aim 1**, we found that some of the mediators of synaptic activity analyzed here (GluA1, GluN2A, PSD-95) altered spontaneous measures of anxiety- and depression-related behaviors as previously reported. These reductions in depression-related behaviors do not support previous data showing that antidepressants increased expression of these molecules (Du et al., 2007, Li et al., 2010), or that stress correspondingly reduced them (Pickering et al., 2006, Gourley et al., 2009, Wilber et al., 2009, Li et al., 2011). The potential for compensatory changes inherent with constitutive knockouts is noted (Zhou et al., 2009), and analysis with conditional knockouts would better analyze the these molecules in depression-related behaviors.

We made use of conditional knockouts for analysis of NMDAR subunits in these behaviors and found no alterations in anxiety- or depression-related tasks, supporting the notion that these synaptic mediators are activated only after challenges such as stress exposure or learning paradigms (Clark and Cull-Candy, 2002, Lee et al., 2003). Such selective recruitment could also fit with an extrasynaptic location of GluN2B that is only activated during glutamate spillover (Hardingham and Bading, 2010). The lack of baseline FST phenotype also allowed us to use these strains in analysis of their altered response to NMDAR antagonist treatment or exposure to stress, as described in Aims 2 and 4. In regards to **Aim II** and the antidepressant-like response to Ro 25-6981, we found that deletion of GluN1 on GABAergic cells did not block the antidepressant-like effect, contrary to our original hypothesis. This casts doubt on the proposed relationship between disinhibition of pyramidal cells and increased glutamate release in antidepressant-like activity. However, incomplete GABAergic cell knockout in these mutans remains an important caveat in this conclusion.

We expanded our investigation of the antidepressant-like response to Ro 25-6981 in the context of **Aim III.** Previous studies have supported both the mPFC and the hippocampus as key regions mediating the antidepressant-like effects of NMDAR antagonism, however no studies have directly infused these drugs into these regions. Here we found that only infusion of Ro 25-6981 into the mPFC, but not BLA or DH, induced an antidepressant-like behavioral response, suggesting that antagonism in the mPFC is sufficient for this response. However, similar to our earlier studies on the GluN2B conditional knockout mice, we were unable to replicate this effect of GluN2B antagonism with a lentiviral-mediated GluN2B knockdown in the mPFC.

Finally, we investigated mechanisms involved in behavioral responses to repeated stress as listed in **Aim IV**, and found that pharmacological GluN2B antagonism reduced the development of despair-like behavior. Reductions in this despair-like behavior were also observed in the pyramidal cell selective deletion of GluN2B, but not with deletions selective to GABAergic cells. Moreover, while mice with a constitutive

deletion of GluA1 and GluN2A showed reduced depressive-like behavior in the FST, they did not show similar reductions during a repeated swim stress.

In total, our results support the following:

I. The antidepressant-like effect of systemic GluN2B antagonism was recapitulated by selective infusion into the mPFC, but not DH or BLA, demonstrating the importance of the mPFC in mediating the antidepressant-like response to GluN2B antagonists.

II. Pyramidal or interneuronal genetic deletion, or mPFC-knockdown, of GluN2B did not mimic the reduction of depression-related behavior of pharmacological GluN2B antagonism, showing that gene-driven loss of GluN2B transmission in this region and/or cell-types is not sufficient to produce an antidepressant-like effect in the FST.

III. Deletion of NMDAR subunits on a subset of GABAergic interneurons did not prevent the antidepressant-like response to systemic GluN2B antagonism, suggesting that disinhibition of pyramidal cells via GABAergic NMDAR antagonism may not be not necessary for this antidepressant-like response.

IV. The novel riFS paradigm revealed an important role for pyramidal cell, but not interneuronal cell, GluN2B-containing receptors in behavioral adaptations to a repeated stress.

Together this suggests that reduction of GluN2B receptors does not in and of itself lead to changes in depression-related behaviors, and that the antidepressant-like response to NMDAR antagonists must depend on mechanisms beyond reduction of GluN2B-containing receptors. Here we suggest that it is the NMDAR-antagonist induced rise in extracellular glutamate that may be missing in genetic deletions of GluN2B. The resulting increase in synaptic, versus extrasynaptic, transmission activates cell-growth pathways (potentially in the mPFC) leading to an antidepressant-like response. While one proposed mechanism for NMDAR antagonist-induced glutamate release is blockade of GABAergic NMDARs, we did not find support for that in our findings. Nonetheless, glutamate release could still be induced through other subtypes of GABAergic neurons or blockade of NMDAR autoreceptors.

Alternatively, excessive extrasynaptic, versus synaptic, transmission can lead to the activation of cell death pathways, and lead to the morphological and functional alterations observed after chronic stress. Our evidence suggests that it is GluN2B transmission on pyramidal cells of the cortex or CA1 that leads to the behavioural effects of chronic stress. As the morphological changes after stress are previously observed on these same cell types, and blockade of pyramidal cell GluN1 blocks morphological changes, it suggests that pyramidal cell NMDAR transmission might mediate both the morphological and behavioural alterations to stress. This suggests a model where repeated stress leads to increased glutamate transmission through extrasynaptic pyramidal cell GluN2B receptors, leading to the activation of cell death pathways in this cell type. Blockade of GluN2B on pyramidal, but not GABAergic cells, will block the morphological, and behavioural effects of stress **(Figure 17).** Previous work did not find a behavioural effect of pyramidal cell GluN1 deletion in the hippocampus, suggesting a selective role for the mPFC in mediating these behavioural

effects. Unfortunately, our mPFC-specific GluN2B deletion did not confirm this and requires further testing.

As we suggest that excessive glutamate release paired with postsynaptic GluN2B blockade leads to antidepressant effects, a blockade of extrasynaptic GluN2B during stress can not only prevent the negative effects of stress, but actually cause antidepressant effects. Additional studies would be needed to investigate the possible beneficial effects of stress exposure.

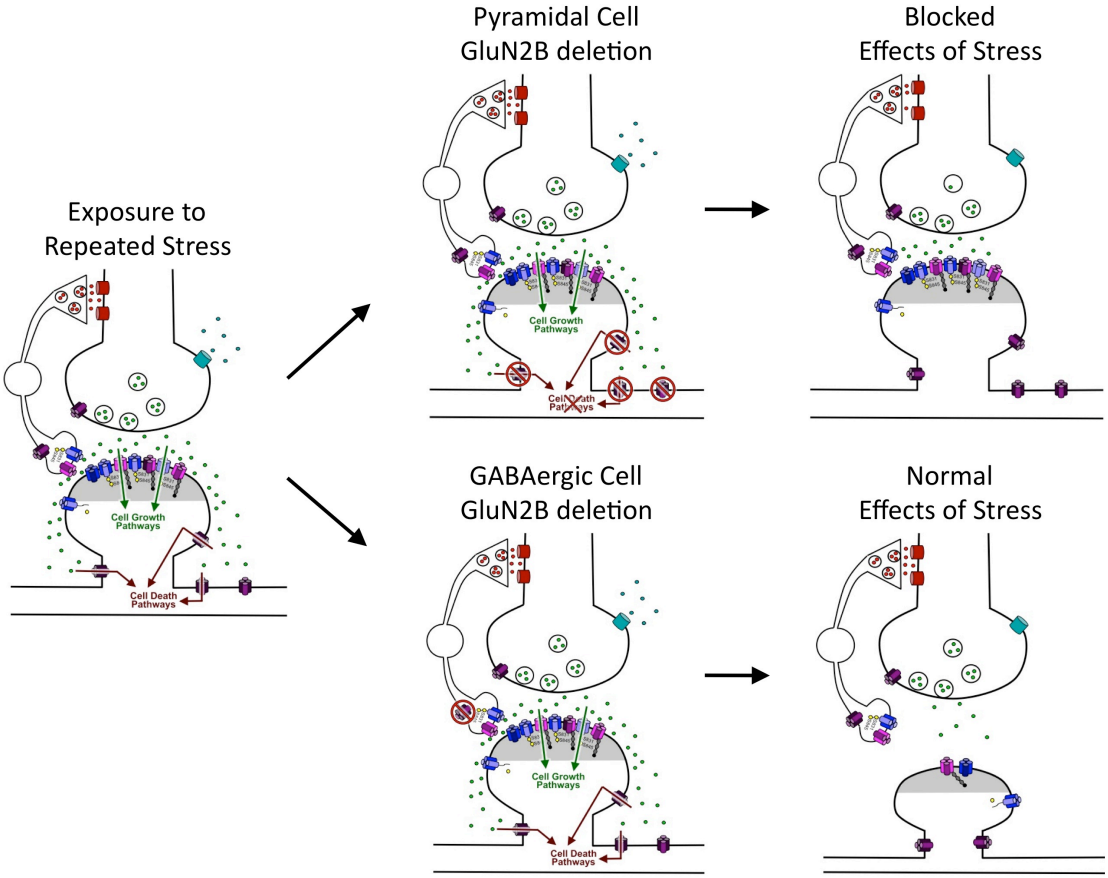

Figure 17 The behavioural effects of repeated stress is mediated by GluN2B on pyramidal cells, but not on interneurons. Mice lacking GluN2B selectively on pyramidal cells showed reduced despair-like activity over the course of our riFS repeated stress. This reduction in the behavioural responses to stress was not observed in mice with a deletion of GluN2B on a subset of interneurons. This suggests that stress-induced glutamatergic transmission through pyramidal cell extrasynaptic GluN2B receptors leads to the morphological changes previously observed in these same cell types, and ultimately leads to the behavioural effects of stress

9 FUTURE DIRECTIONS

In our measures of spontaneous anxiety- and depression-related behaviors, we noted that the behaviors observed in constitutive knockout mice were often complicated by compensatory effects. Ideally, studies in the future would use conditional knockout mice similar to our NMDAR-related mouse strains to better analyze the role of these targets in these behaviors, and to more directly compare to the behavioral effects of NMDAR deletion. These studies could similarly benefit from better pharmacological tools directly targeting these receptors and allowing for regional specificity, or alternatively, viral-mediated approaches. For example, studies using these approaches to investigate the GluN2A subunit could be very informative, and new GluN2A-specific antagonists are being developed (Kocsis, 2012).

In our studies of repeated stress, we originally proposed that behavioral changes in the mPFC would be linked to the dendritic atrophy previously seen in this region after chronic stress paradigms. While we have measured changes to synaptic proteins after this task, we have not directly tied such morphological changes to this behavior by studying riFS-induced morphological changes. These morphological studies could be followed by analysis of NMDAR blockade on the morphological, as well as behavioral, effects of riFS stress. As morphological changes are generally observed in pyramidal cells, a useful approach would be to use a cell-type specific Cre-virus to selectively knockdown pyramidal GluN2B in the mPFC and measure the morphological and behavioral consequences. This could be compared to manipulations in other regions or cell types to confirm the role of the mPFC morphology in these behaviors.

We have also proposed that it is predominantly the extrasynaptic location of GluN2B that leads to its important role in stress-induced behavioral changes. However, it is difficult to directly test for subunit location *in vivo*. One NMDAR antagonist, memantine, may have selectivity for extrasynaptic NMDAR receptors (Xia et al., 2010). As memantine is not known to increase glutamate release, it may be expected to block the effects of stress via extrasynaptic blockade without having antidepressant-like effects in the FST. Treatment strategies utilizing drugs such as memantine could lead to selective blockade of extrasynaptic NMDARs and therefore block cell death pathways in only regions and instances of stress, allowing for selectivity not available with current pharmacological treatments.

These results have helped propose a role for regulating synaptic transmission in treatment strategies for depression. While current antidepressant therapies such as monoaminergic-based treatments have been tied to mediators of synaptic activity, their exact mechanism of action remains unclear. Traditional antidepressant treatments leading to increased AMPAR levels may increase synaptic transmission, but not selectively in regions or celltypes activated in depression. Additionally, mechanisms reducing glutamate release could protect against the negative consequences of stress, but also block the increased synaptic transmission possibly needed for recovery. In total, a lack of understanding of the mechanisms behind current therapies could explain their lack of consistent effects, that ultimately leads to the large gap between the number of patients prescribed antidepressants and those successfully treated. Instead, the converging evidence on novel glutamatergic and plasticity-related therapeutic targets suppots a new generation of mechanistically-based treatments that can more directly and consistently address the numerous challenges of treating depression.

10 ACKNOWLEDGEMENTS

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