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Cai X*, Kallarackal AJ*, Lee H-K, Huganir RL, Thompson SM. Selective potentiation of TA-CA1 excitatory synapses by serotonin and its dysregulation in depression. (In revision)

Kallarackal AJ, Simard JM, Bailey AM. The effective of apamin, a small conductance calcium activated potassium (SK) channel blocker, on a mouse model of Neurofibromatosis 1. (Submitted)

Abstracts

Kallarackal AJ, Gaylor K, Bailey A, Thompson S, Cai X (2011, June) *Serotonin mediated potentiation of glutamatergic transmission in depression and requirement for antidepressant efficacy*. Gordon Research Conference: Excitatory synapses and brain function.

Kallarackal AJ, Gaylor K, Bailey A, Thompson S, Cai X (2010, November) *Opposing effects of depression and antidepressants on serotonin mediated potentiation in area CA1*. Society for Neuroscience.

Kallarackal AJ, Cai X, Gaylor K, Bailey A, Thompson SM (2010, April) *Serotonin mediated potentiation in area CA1: Altered in depression and critical for antidepressant action*. Serotonin Club

Cai X, Kallarackal AJ, Lee HK, Huganir RL, Thompson SM (2010, July) *Local potentiation of excitatory synapses by serotonin and antidepressants and its dysregulation in depression*. Serotonin Club

Cai X, Yang K, Kallarackal AJ, Thompson SM (2009, October) *SK channels regulate synaptic signaling, dendritic integration and induction of LTP at temporoammonic-CA1 cell synapses*. Society for Neuroscience

Kallarackal AJ, Cai X, Thompson SM (2009, April) *Opposing effects of depression and antidepressants on serotonin mediated potentiation in area CA1*. Cold Spring Harbor Laboratories.

Kallarackal AJ, Thompson SM, Cai X (2008, November) *Effect of chronic unpredictable stress and antidepressant treatment on 5-HT1b receptor mediated potentiation of glutamate responses in CA1 distal dendrites*. Society for Neuroscience.

Thompson SM, Kallarackal A, Mok L-P, Cai X (2008, November) *Second messenger signaling pathways coupling 5-HT1B receptors to postsynaptic AMPA receptor insertion in CA1 pyramidal cells distal dendrites*. Society for Neuroscience.

Cai X, Kallarackal A, Thompson SM (2008, November) *Selective potentiation of temporo-ammonic - CA1 cell excitatory synaptic transmission by activation of postsynaptic serotonin 5HT1B receptors*. Society for Neuroscience.

Cai X, Kallarackal A, Thompson SM. (2007, November). *Blocking SK-type Ca2+-activated potassium channels facilitates forward- propagated action potentials in hippocampal CA1 pyramidal neurons*. Society for Neuroscience.

Bailey AM, Kallarackal AJ, Chen M, Simard JM. (2006, October). *Apamin significantly improves spatial cognition in a mouse model of Neurofibromatosis 1*. Society for Neuroscience.

Bailey AM, Kallarackal AJ. (2006, March) *Neurofibromatosis 1 (Nf1$^{+/-}$) Spatial Learning Deficits in the Barnes Maze.* Eastern Psychological Association.

Kallarackal AJ, Simard JM, Bailey AM. (2005, November) *Olfactory discrimination learning in a mouse model of Neurofibromatosis*. Society for Neuroscience.

Chen M, Kallarackal A, Li Z, Dugger N, Hoffman P, Simard JM. (2005, November) *Small-conductance calcium-activated potassium (SK) channels in Neurofibromatosis-1 mouse brains*. Society for Neuroscience.

Kallarackal AJ., Bailey AM. (2005, March). *Olfactory discrimination learning in a mouse model of Neurofibromatosis.* Eastern Psychological Association.

ABSTRACT

Title of Dissertation: Serotonergic Modulation of Glutamatergic Transmission: Bridging Two Theories of Depression

Name of Candidate: Angy J. Kallarackal

Doctor of Philosophy, 2011

Dissertation Directed by: Scott M. Thompson, Ph.D.

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Major Depression is a debilitating disease affecting close to 10% of the world population. Although there are many antidepressant drugs available, 30% of patients are unresponsive and the rest must wait 3-4 weeks for therapeutic efficacy. The serendipitous discovery of these drugs in the 1950s initiated the monoamine theory of depression. This theory postulates that depression is caused by a depletion of synaptic serotonin and that effective therapies work by restoring this imbalance. However, the many gaps in this theory together with inefficient therapies have led to the search for a new explanation of the pathology of depression. Recent data has implicated a dysfunction of the glutamate system to be at fault for the symptoms of depression.

Both of these theories have been developed independently, however I propose that they are not in fact distinct but may actually complement each other. Therefore I hypothesized that glutamatergic dysfunction underlies the etiology of depression, but that serotonin is capable of modulating glutamatergic transmission in a manner that rescues this defect. I investigated the effect of serotonin elevation on glutamate transmission and how this phenomenon may be altered in an animal model of depression. I found that elevation of endogenous serotonin activates 5-HT_{1B}Rs which in turn signal to phosphorylate glutamatergic AMPA receptors. This potentiation of the glutamatergic response is enhanced in animals subjected to chronic unpredictable stress and absent in naïve animals chronically treated with antidepressants. This finding pointed to a decrease in basal glutamatergic transmission in depressed animals, which I confirmed by measuring AMPAR/NMDAR ratios. Finally, I found that activation of 5-HT_{1B}Rs and subsequent phosphorylation of the GluA1 subunit of the AMPAR is necessary for the therapeutic effects of antidepressants, and that phosphorylation of S831 is necessary for normal basal affective state in a number of behavior measures.

Together my data present a novel pathway through which 5-HT_{1B}R activation can specifically enhance AMPAR function in the hippocampus and provide a connection between two previously disparate theories of depression. These findings provide insight into the locus of dysfunction in depression and also point to new potential targets in the treatment of this disease.

SEROTONERGIC MODULATION OF GLUTAMATE TRANSMISSION: BRIDGING TWO THEORIES OF DEPRESSION

By

Angy J. Kallarackal

Dissertation submitted to the Faculty of the Graduate School of the

University of Maryland, Baltimore in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

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DEDICATION

To my parents

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Next, I would like to thank the many collaborators who contributed to the completion of this project. Dr. Xiang Cai began this project before I joined the lab and graciously allowed me to work with him to efficiently produce the set of experiments that became my thesis. I would also like to thank Dr. Aileen Bailey, my undergraduate mentor, who helped take my project to the next level with her behavioral expertise. Working in her lab paved the path to graduate school, and it has been a pleasure to

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CHAPTER ONE

INTRODUCTION

Major Depression

Major Depressive Disorder (MDD) is one of the leading causes of mortality and morbidity worldwide, and is a leading reason for suicide. Approximately 7% of the world population suffers from some form of mood disorder, with 2-5% of Americans suffering from severe forms of the disease (Murray & Lopez, 2006). According to the World Health Organization 850,000 individuals die through self-inflicted methods each year. The prevalence of suicide among the other devastating symptoms of depression, make it a disease desperately in need of a cure.

Symptoms. Depression is characterized by a wide variety of symptoms. According to the DSM IV depression symptoms include depressed mood, reduced interest in activities that used to be enjoyed, sleep disturbances, loss of energy or a significant reduction in energy, difficulty concentrating, holding a conversation, paying attention, or making decisions that used to be made fairly easily, and suicidal thoughts or intentions (American Psychiatric Association, 2000). Patients may present with any of these symptoms and often, no two patients exhibit the same exact combination. For this reason, psychiatrists have developed a number of scales for evaluating the severity of depression in patients. Common screens used in adults include the Beck Depression Inventory Scales, the Center for Epidemiologic Studies Depression Scale, and Zung Self Rating Depression Scale (Sharp & Lipsky, 2002). Currently, there are limited biological hallmarks of depression; therefore physicians must rely on the presentation of symptoms to diagnose patients. The scales have helped in the standardization of diagnosis and aided in evaluating the effectiveness of various treatments.

Risk factors. Depression is probably caused by a combination of genetic and environmental risk factors. Twin studies show that depression has a moderate level of

heritability with a higher incidence in females compared to males (Kendler et al., 2006). The heritability to liability of depression is 30% greater in females than in males (Kendler et al., 2001), suggesting that while there may be different environmental factors involved, there is a biological basis underlying the difference in susceptibility. Many linkage studies have compared depressed patients to healthy volunteers, providing a multitude of candidate genes that may be responsible for the onset of depression. Unfortunately, no single gene has been identified as having complete penetrance. Aspects such as age of onset and recurrence of depression appear to be the most heritable factors (Levinson, 2006).

In the identification of risk factors for depression, environmental influences have been easier to identify. There are many environmental factors associated with depression, mainly involving some form of stress. Depression is rare in children, with approximately 2% of elementary aged children reporting depression, though this number increases steeply to 8% by the time of adolescence (Birmaher, Brent, & Benson, 1998). This is probably due to an inability of younger children to explain their emotions, but also due to an increase in stressful experiences with age. Regardless, stressful life events have a causal relationship with the onset of depression in patients (Kendler et al., 1999). Depressed patients report more stressful life events than non-depressed subjects including physical illness, family relationships and poor work situations (Billings, Cronkite, & Moos, 1983). In addition to distinct stressful events, depressed patients tend to have fewer resources for support compared to non-depressed people (Billings et al., 1983), compounding the risk for depression. Interestingly, there are varying views as to how genetics and environment interact within depressed patients. It is possible that genetically

susceptible people are more sensitive to environmental triggers of depression; however there is some evidence that genetic factors may result in high-risk behaviors in individuals, which in turn leads to the creation of environmental stressors and consequently, to depression (Kendler & Karkowski-Shuman, 1997).

Comorbidities. Another complication of studying depression in patient samples is the prevalence of comorbid disorders. Most patients with depression have at least one comorbid psychiatric disorder at some point in their lives (Rohde, Lewinsohn, & Seeley, 1991). Among the comorbid disorders are anxiety disorders (Pini et al., 1997), substance abuse disorders (Compton, Thomas, Stinson, & Grant, 2007), obsessive compulsive disorders (Perugi et al., 1997) and eating disorders (Hudson et al., 2007). Patients that experience both depressive and manic episodes are often diagnosed with bipolar disorder, which shares common symptoms and treatments with unipolar depression, but is categorized as a distinct disease (Ghaemi, Boiman, & Goodwin, 2000). Much progress has been made in terms of identifying and categorizing patients with mood disorders, however as our level of understanding has not increased at the rate of disease incidence in patients.

Impact. According to the WHO, depression is the leading cause of disability in the United States. It is projected that depression will becomes the second leading cause of health burden in the United States by the year 2030 (Mathers & Loncar, 2006). Despite the high incidence and socioeconomic burden, the etiology of depression remains poorly understood. As it stands, researchers have been unable to identify neither cause nor cure for this debilitating disease. While there are some antidepressant treatments available,

less than 25% of those affected have access to these treatments (World Health Organization, 2011).

Antidepressants

There are numerous antidepressant (AD) drugs on the market to treat depression, though about 30% of patients are unresponsive to currently available drug treatments (Doris, Ebmeier, & Shajahan, 1999). In general, patients with mild to moderate depression do not display great responsiveness to ADs but the severely depressed patients show significant responses compared to placebo (Fournier et al., 2010). The basis for current AD drug development dates back to the 1950s with the development of the monoamine oxidase inhibitor (MAOI) iproniazid and the tricyclic antidepressant (TCA) imipramine, which exerted beneficial effects on mood, though at the time they were being used in the treatment of various other disorders including schizophrenia and diabetes (Slattery, Hudson, & Nutt, 2004).

Electroconvulsive shock therapy (ECT). Before the development of antidepressant drugs, the main method of treating depressed patients was electroconvulsive shock therapy (ECT). Surprisingly, it remains the most effective form of AD therapy to date, working in up to 75% of patients after a short course of therapy (Husain et al., 2004). The treatment remains controversial based on public perception of the severity of the treatment and the history of misuse (Hirshbein & Sarvananda, 2008). It remains unknown why ECT is so effective in patients, especially when there is no specific neurotransmitter system it works on. Theories include a regulation of the neurotrophic system (Altar et al., 2004), alteration in glutamate receptor population (Watkins, Pei, & Newberry, 1998) and modulation of the GABAergic system (Sanacora et al., 2003). Not surprisingly, despite is

effectiveness, the advent of psychiatric drug development made this form of therapy seem less desirable.

Tricyclic antidepressants (TCAs). The first tricyclic antidepressant (TCA) drug, imipramine hydrochloride, was developed in 1956 to treat patients with schizophrenia; however, it was found that the drug made the symptoms worse (López-Muñoz & Alamo, 2009). The first placebo-controlled imipramine study in depressed patients was conducted in 1959 and found the drug to be effective in 74% of patients (Ball & Kiloh, 1959), and inspired the generation of other tricyclics for antidepressant use. Tricyclic ADs are three-ring chemical compounds that block both serotonin and norepinephrine transporters, therefore increasing the concentration of both neurotransmitters at the synaptic cleft. Though different TCAs have different affinities for these transporters and may increase the concentration of one monoamine more than the other. Additionally, TCAs have affinity for cholinergic, histaminergic and adrenergic receptors. Their actions on serotonin and norepinephrine transporters are believed to be responsible for therapeutic effects of these drugs; however non-specific targets contribute to the many side-effects exhibited when TCAs are given (Peretti, Judge, & Hindmarch, 2000). Side effects include dry mouth, drowsiness, sexual dysfunction, urinary retention and cognitive impairment. Importantly, high doses can result in serious cardiovascular problems and even death (Jefferson, 1975). TCAs gradually became replaced by selective serotonin reuptake inhibitors (SSRIs) due to the large number of side effects associated with this class of drugs, however TCAs are still used today in treatment-resistant patients (Gervasoni, Aubry, Gex-Fabry, Bertschy, & Bondolfi, 2009). SSRIs are blockers of serotonin transporters and thereby increase the amount of serotonin available at synapses.

Selective serotonin reuptake inhibitors (SSRIs). Following the serendipitous discovery of the AD actions of MAOIs and TCAs, scientists launched an effort to develop psychoactive drugs synthesized through rational design. Fluoxetine, the first SSRI was developed by Eli Lilly in the 1970s (Wong et al., 1975) and then approved for AD use by the FDA in 1987. SSRIs remain the most popular class of AD drugs and include fluoxetine (Prozac), sertraline (Zoloft), and escitalopram (Lexapro). Although these drugs were developed for use in depressed patients, they have other effects that are either beneficial (treatment of obsessive compulsive disorder, anxiety disorders and bulimia) as well as negative side effects such as nausea, insomnia, and sexual dysfunction (Stahl, 1998). More concerning however is the increased associated risk of suicide in adolescent patients given SSRIs (Fergusson et al., 2005). Outside of negative side effects, the two the biggest complaints of both SSRIs and TCAs are that they take weeks to become effective. While these monoaminergic ADs have aided millions of people in the treatment of their symptoms, there is an urgent need for novel ADs with a faster time course and fewer side effects.

The discovery of AD drugs, in addition to the standardizing of diagnosis has greatly aided in the studying of depression. Unfortunately, human studies are rather limited, especially in regard to a disease such as depression. The self-reporting of symptoms makes it difficult for clinical studies to be consistent. Fortunately, the study of depression has been greatly advanced by the development of imaging techniques which provide more objective data allowing researchers to further investigate the underlying etiology of the disease. These studies have helped to pinpoint specific brain regions that are altered in depressed patients, including the hippocampus (Bremner et al., 2000) and

the prefrontal cortex (PFC) (Hasler et al., 2007). It remains difficult however to control for the combination of genetic factors and environmental factors in any population of patients. One of the more significant advances in the study of depression has been the development of animal models. The combination of human studies and animal modeling complement each other and is the key to finding the cause and cure for this debilitating disease.

Animal models of depression

The idea that humans and animals share common mental processing from instinctual behavior to higher processes such as emotion is as old as Charles Darwin's 1871 theories from *Descent of Man*. Although it is generally accepted that animals are capable of feeling emotion, the task of scientifically studying this phenomenon is a constantly evolving process. There are a number of depression animal models that have been developed to identify mechanisms involved in the disease and to provide screening methods for AD drugs. Animal models of depression generally use behavioral or genetic manipulations and are evaluated using tasks that measure symptoms similar to those observed in human depression. While it is impossible to model all aspects of depression in an animal, such as suicidal thoughts and ideation, traits such as anhedonia (diminished interest or pleasure), weight loss/gain, sleep disturbances and cognitive problems can be measured (Cryan & Holmes, 2005)

Tests for depression. A number of tests have been developed to assay endophenotypes related to depression. Endophenotypes are phenotypes that fill the gap between available descriptors and the underlying cause of the disease. These endophenotypes come from clusters of genes that code for phenotypes commonly seen in

patients but are not necessarily involved in the negative symptomology of the disease. Often these genes can help to point to the pathway involved in the manifestation of the disease. In identifying endophenotypes it is important that they co-segregate with genes known to be involved in the disease and have a higher correlation with depression than with any other disease (Hasler, Drevets, Manji, & Charney, 2004). The two most popular tests are the forced swim test (FST) and the tail suspension test (TST). Both tests are a measure of behavioral despair. The FST involves putting a rodent into a pool of water, while the TST involves suspending a rodent by its tail. Both tests measure the amount of time the animal is spent immobile and correlates immobility to behavioral despair. Thus, an animal that spends more time swimming/struggling, after an acute injection of an AD drug, is less "depressed." While these tests are popular for their ease of use and reliability, they lack some validity in that researchers can detect changes in these tasks following an acute injection of AD (Detke, Johnson, & Lucki, 1997; Petit-Demouliere, Chenu, & Bourin, 2005) even though humans only respond to ADs after chronic use.

Measures that are responsive to chronic and not acute application of ADs include the sucrose preference test (SPT) and the novelty suppressed feeding (NSF) task (Rygula et al., 2006; Dulawa & Hen, 2005). The SPT is a measure of anhedonia, or the inability to feel pleasure. In this test, rodents are presented with a two- bottle choice test with one bottle containing normal water and one bottle containing a sucrose solution. Most strains of rats and mice exhibit a natural preference for sucrose which is then diminished in depression models and recovered by chronic AD treatment (Pothion, Bizot, Trovero, & Belzung, 2004). The NSF task is a measure of hyponeophagia, or an inhibition of feeding based on novelty. In this test, animals are food deprived for 24 hours or more and then

placed in a dark novel arena with food pellets present in the center on a pedestal illuminated by a bright light. This task can also be conducted without food deprivation, but rather the use of a highly palatable food in the test arena. In both scenarios, the animal is placed in a corner of the box and the latency to eat the food in the center is measured. Animals exhibiting a depressed or anxious phenotype take longer to eat, which is reversed with chronic but not acute AD treatment (Dulawa & Hen, 2005).

Validation of models. The tasks described above have been crucial for assessing the validity of animal models of depression. As with the development of any animal model, it is important to assess face, construct and predictive validity. A model has face validity if the modeled symptoms resemble those exhibited in humans. A model has construct validity if the principles behind the development of the model are in congruence with the human disease. Finally, a model has predictive validity if treatments that work in humans also work similarly in the model (Willner & Mitchell, 2002). Therefore, appropriate animal models of depression 1) will exhibit changes in emotion, cognition and motivation, 2) will be sensitive to chronic stress or genetic factors implicated in human depression, and 3) will respond to AD treatments. These criteria have led to the development of a number of reliable models of depression which have provided much insight into the disease and allowed for the development of new ADs (Figure 1.1).

Genetic Models. A number of potential genetic models of depression have been identified and generated using both forward and reverse genetics. Some models have created rodent lines by using forward genetic screens and selectively breeding animals that exhibit depressed phenotypes, such as the H/Rouen mice, Flinders Sensitive Line

rats, and congenital Learned Helplessness rats (Yacoubi & Vaugeois, 2007). Congenital Learned Helplessness (cLH) rats exhibit anhedonia, (Vollmayr et al., 2004), display changes in stress responses (Edwards, King & Fray, 1999) and respond to ADs (Shumake, Colorado, Barrett, & Gonzalez-Lima, 2010) giving the model face, construct, and predictive validity. Congenital learned helplessness rats are selected for based on their vulnerability to learned helplessness (LH). LH is established by subjecting a rodent to repeated inescapable foot shocks. However when the shock becomes escapable some animals do not escape, and have learned to become helpless. Animals that have cLH have been bred for generations so that they exhibit helplessness without the prior inescapable training trials. Studies involving these animals have provided insight into multiple potential mechanisms of depression. Among the changes observed in these animals are an increase in synaptic transmission onto VTA-projecting lateral habenula neurons (Li et al., 2011), an elevation of dorsal raphe 5-HT_{1B}Rs (Neumaier et al., 2002) and reduced hippocampal cAMP response element binding (CREB) mRNA (Kohen, Neumaier, Hamblin, & Edwards, 2003).

Stress Models. More prevalent than genetic models are behavioral models of depression. Chronic stress has long been associated with incidence of depression in humans (Hammen, 2005). Therefore, a number of animal models of depression have been developed based on the notion that repeated stressful events can produce behaviors that simulate depressive symptoms in patients. Two of the most widely used behavioral models of depression are the chronic unpredictable stress (CUS), social defeat stress (SDS) paradigms. CUS and SDS are ideal models of depression in that they involve relatively mild stressors and respond to chronic AD treatment in a time course similar to

human disease (Rygula et al. 2006; Willner, Towell, Sampson, Sophokleous, & Muscat, 1987). CUS is a broadly defined method that varies from laboratory to laboratory. Most CUS paradigms are based on the work of Wilner et al., (1987) though some will add/subtract certain stressors. Briefly, CUS involves stressing rats or mice for three weeks using two to three stressors per day, which include but are not limited to: restraint, social isolation, food/water deprivation, forced swim, cage rotation, strobe light (Wilner et al,. 1987). SDS, also referred to as chronic social defeat (CSD), consists of an experimental animal being placed into the cage of a retired breeder mouse, the resident. The intruder/experimental mouse is allowed to be attacked by the resident/retired breeder mouse for 5 minutes after which they are separated by a wire mesh divider but kept in the same cage for an additional hour (Berton et al., 2006). This model does not rely solely on physiological stress and was developed to focus on psychological stress that may be more relevant to the genesis of depression in humans. Importantly, both models exhibit anhedonia in the SPT that is responsive to chronic AD treatment (Rygula et al., 2006; Willner et al., 1987), giving them both face and predictive validity.

These models in concert with human studies have been crucial to the understanding of depression. They have played a major role the discovery and understanding of current AD treatments and have led to multiple theories of depression. Although it is the serendipitous discoveries of iproniazid and imipramine that has mostly influenced these theories of depression, animal studies have helped to fill gaps and provide new direction for the development of effective ADs.

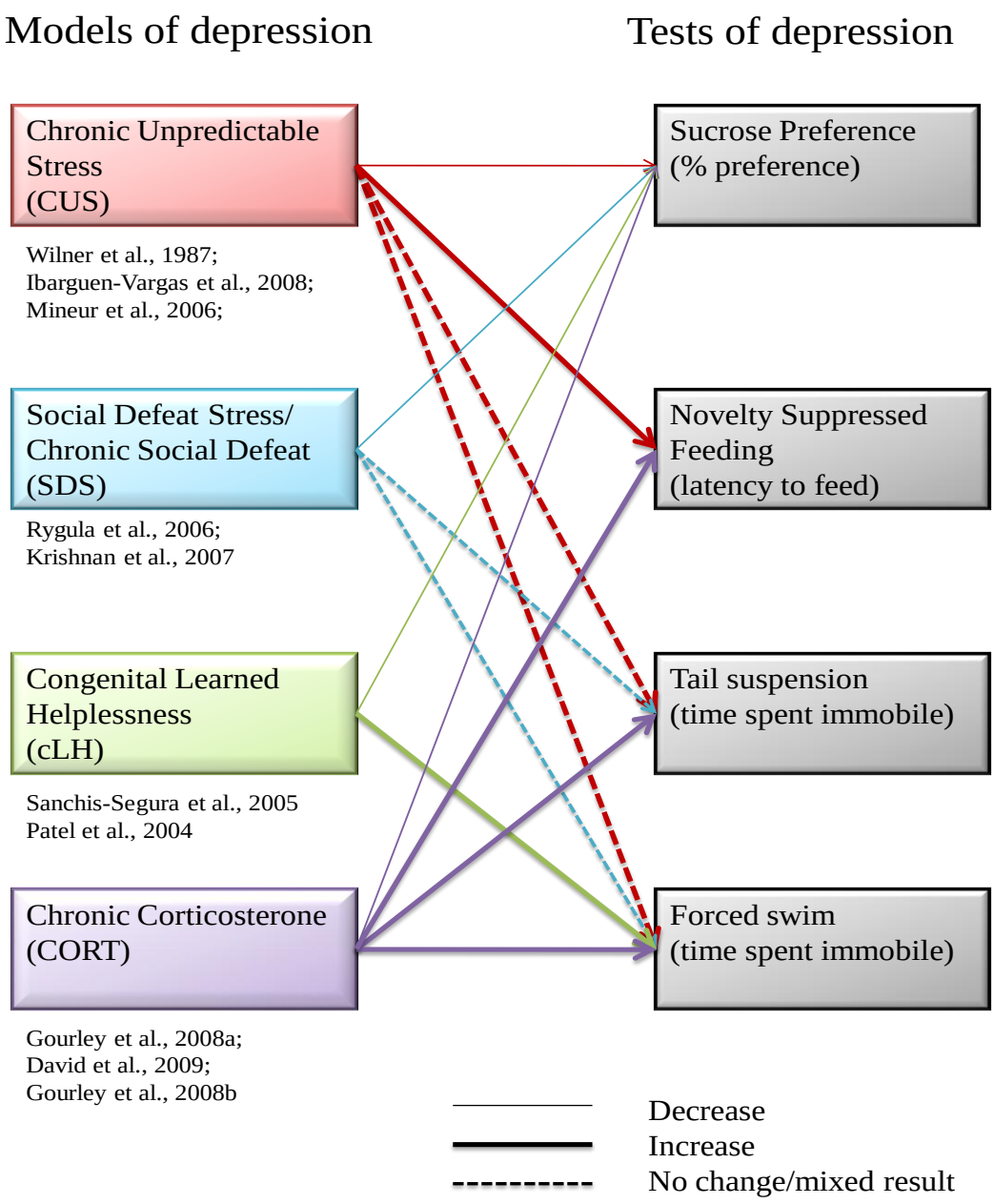


Figure 1.1. Various methods used to model depressive-like behaviors in rats and mice. Methods for modeling depression include behavioral stress paradigms (CUS and SDS), genetic manipulation (cLH) and chemical induction (CORT). Measures of depression include anhedonia (sucrose preference), hyponeophagia (novelty suppressed feeding) and behavioral despair (forced swim and tail suspension).

Monoaminergic theory of depression.

One of the original proponents of the catecholamine theory of depression, Joseph Schildkraut, stated that "some, if not all depressions are associated with an absolute or relative deficiency of catecholamines," based on the evidence that drugs such as monoamine inhibitors "produce affective changes in man" (Schildkraut, 1965). This theory has become the basis of most research in the field of depression and is one of the most cited articles in the American Journal of Psychiatry. At the time Schildkraut himself acknowledged that the ability to test the theory with current techniques was limited; however more than 50 years later, it is difficult to determine if this theory is valid.

Serotonin physiology. Serotonin is a monoamine neurotransmitter that can modulate physiological processes ranging from body temperature to appetite to mood. Serotonin is synthesized from the amino acid L-tryptophan. The main source of serotonin in the brain comes from the raphe nuclei. The raphe nuclei are located along the length of the brainstem and send projections throughout the brain, including to the frontal cortex, striatum, substantia nigra, nucleus accumbens and hippocampus. Following its release, serotonin is either metabolized by MAOIs or recycled back to the presynaptic terminal through the serotonin transporter, SERT, both of which are targets for ADs. Serotonin can act on any one of 14 receptor subtypes. Serotonin receptors can be ionotropic or metabotropic, excitatory or inhibitory, and can act as either self-regulatory autoreceptors or heteroreceptors (Barnes & Sharp, 1999), (Table 1.1). The net result of increasing serotonin in the synaptic cleft, whether it is through endogenous means or AD drugs, varies depending on the population of receptors present. It has therefore been difficult to pinpoint which receptors are important in the therapeutic actions of ADs.

Receptor Family	Type	Localization	Generalized Function	References
5-HT$_{1(A-F)}$	g-protein coupled	hippocampus, lateral septum, frontal cortex, caudate, globus pallidus, substantia nigra	Inhibition of adenyl cyclase	Verge et al., 1985; Lanfumey & Hamon, 2004
5-HT$_{2\ (A-C)}$	g-protein coupled	olfactory bulb, cortex, caudate, globus pallidus, choroid plexus	Activation of phospholipase C	Appel et al., 1990; Baxter et al., 1995; Leysen, 2004
5-HT$_3$	ligand gated ion channel	cortex, hippocampus, amygdala, olfactory bulb	Conductance of Na^+, K^+, Ca^{2+}	Maricq et al., 1991; Tecott, Maricq, & Julius, 1993
5-HT$_{4\ (A-E)}$	g-protein coupled	olfactory tubercule, hippocampus, striatum, inferior colliculus, substantia nigra, cortex	Activation of adenyl cyclase	Eglen et al., 1995; Vilaró, Cortés, & Mengod, 2005)
5-HT$_{5(A-B)}$	g-protein coupled	unknown due to lack of specific pharmacological agents	Inhibition of adenyl cyclase	Nelson, 2004; Glennon, 2003
5-HT$_6$	g-protein coupled	cortex, nucleus accumbens, cerebellum, caudate, hippocampus, olfactory tubercule	Activation of adenyl cyclase	Hamon et al., 1999; Woolley, Marsden, & Fone, 2004
5-HT$_7$	g-protein coupled	cortex, septum, globus pallidus, thalamus, hypothalamus, amygdala, superior colliculus	Activation of adenyl cyclase	Ruat et al., 1993; Gustafson et al., 1996

Table 1.1 Serotonin receptor families each have a distinct distribution and function in the central nervous system.

Mixed evidence. The strongest evidence for the monoamine theory of depression is still the finding that drugs such as imipramine and fluoxetine, which increase synaptic levels of monoamines, have worked in a number of depressed patients. These early studies were supported by evidence that administration of both of these drugs enhance serotonin in blood platelets (Marshall, Stirling, & Tait, 1960). Coinciding with these studies were reports that the anti-hypertensive drug reserpine, known to deplete catecholamines, caused patients to become depressed (Quetsch et al., 1959; Lemieux, Davignon, & Genest, 1956). Further analysis of these studies has revealed that only 10% of all patients in these studies exhibited depression symptoms, similar to the prevalence in the general public (Baumeister, Hawkins, & Uzelac, 2003).

More recent research has provided mixed support for the theory; including some that contradict the hypothesis that depression is caused by a hypofunctioning serotonin system. One prediction of the monoamine hypothesis would be that decreasing serotonin by depleting the precursor for synthesis, tryptophan, would cause a depressed mood in patients. It appears that tryptophan depletion exerts a negative effect on mood in patients that are already being treated for depression (Moreno et al., 1999) but not in healthy control patients (Delgado et al. 1994; Salomon et al. 1997). Additionally, a prediction of the serotonin hypothesis would be that enhanced serotonin synthesis through a tryptophan enhanced diet would elevate mood, however this is not the case (Mendels et al., 1975). On the other hand, recent studies have provided support for monoamine dysfunction in depression. Specifically, one study found that a polymorphism in the tryptophan hydroxylase-2 gene, coding for the enzyme responsible for the synthesis of serotonin, has an association with major depression (Zhang et al., 2005). Another study found

significantly higher amounts of monoamine oxidase in depressed individuals (Meyer et al., 2006), suggesting that patients may suffer from lower amounts of monoamines because they are broken down more frequently. In the end, it is likely that the inconsistencies in these studies are hampered by an inability to truly control for all the variables present in patient populations.

Although they are better controlled, animal studies have also provided mixed support for the monoamine hypothesis of depression. Decreasing serotonin in rats through tryptophan depletion results in a depressed phenotype in the forced swim test, but does not alter sucrose preference (Blokland, Lieben, & Deutz, 2002). Also, genetic depletion of serotonin in the SERT knockout mouse results in a depressed phenotype in the forced swim test (Holmes et al., 2002). Interestingly, this occurs only on 129/sv background and not the C57Bl6 background, suggesting that the effect may be related to the background and not the mutation. This model may be somewhat difficult to interpret however due to the many changes in 5-HT receptor populations observed in the adult animal (Urani, Chourbaji, & Gass, 2005). In fact, it may be alterations in a single population, or subpopulation of serotonin receptors that is responsible for the symptoms of depression, rather than a global dysfunction of the neurotransmitter system. There is evidence that chronic stress may increase densities of serotonin receptors such as 5-HT_{1A} (Grippo et al., 2005) and 5-HT_{1B} (Neumaier et al., 2002) in rodent brains. Both of these receptors can be either autoreceptors or heteroreceptors, and it is unclear whether one or both sets are altered. Additionally, animals with a global 5-HT_{1A} knockout mutation exhibit AD-like responses in the tail suspension test, but also exhibit anxiety symptoms (Heisler et al., 1998). As it stands, the animal data and human data connecting

serotonergic dysfunction and depression are confusing at best. It is concerning that researchers have been unable to develop a reliable animal model based on this theory. This may be due to a lack of focus in the search for a culprit. Therefore it is important to try to identify specific serotonergic system components that are disturbed in depression, rather than assuming a global imbalance. Among the more promising candidates are 5-HT_{1B}Rs (Moret & Briley, 2000).

5-HT_{1B}Rs. The human 5-HT_{1D}R can be subdivided into 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$. The latter has 93% amino acid homology to the rodent 5-HT_{1B}R, and is referred to as the h5-HT_{1B} receptor (Adham, Romanienko, Hartig, Weinshank, & Branchek, 1992). 5-HT_{1B}Rs are metabotropic $G_{i/o}$ coupled receptors. As with other receptors, 5-HT_{1B}Rs are dynamically regulated. The surface expression of 5-HT_{1B}Rs appears to depend on the S100 protein, p11 (Svenningsson et al., 2006). They are found presynaptically on serotonergic terminals (Sari et al., 1997) and postsynaptically on the dendrites of non-serotonergic neurons (Stean et al., 2005). Presynaptically, they control release of serotonin by negatively coupling to cyclic AMP, though there is evidence that activation can cause increases in intracellular calcium as well (Giles, Lansdell, Bolofo, Wilson, & Martin, 1996). It remains unknown whether these signaling cascades are specific to pre- versus postsynaptic 5-HT_{1B}Rs.

The evidence for the role of 5-HT_{1B}Rs in depression and AD treatment is conflicting, probably due to its presence on both cell dendrites and serotonergic nerve terminals. Multiple human studies have found evidence for dysfunction of 5-HT_{1B}Rs in depression. 5-HT_{1B} hippocampal mRNA is enhanced in post-mortem tissue from patients with psychiatric disorders, including bipolar disorder and schizophrenia (López-Figueroa

et al., 2004). Post-mortem tissue from depressed patients also exhibits a significant decrease in p11 protein in the anterior cingulate cortex (Svenningsson et al., 2006), which would correspond to a decrease in surface h5-HT_{1B}Rs. Additionally, researchers have found that a polymorphism in the h5-HT_{1B}R at the G861C locus that is associated with major depression but not bipolar disorder or schizophrenia (Huang et al., 2003). Although it is difficult to discern from these studies how and where 5-HT_{1B}Rs are altered in depressed patients, there is some evidence that they are in fact different from healthy subjects.

Data from animal studies have also provided evidence for a role of 5-HT_{1B}Rs in depression. Both pharmacological and genetic manipulation of the receptor results in interesting depression-related phenotypes. As autoreceptors, 5-HT_{1B}Rs limit the effects of SSRIs on serotonin concentration in the cleft (Malagié et al., 2008). As expected, both the 5-HT_{1B}R antagonist SB-224289 and the 5-HT_{1B}R knockout animal exhibit enhanced effects of fluoxetine on serotonin concentration in the hippocampus (Hervas et al., 2000; Knobelman et al., 2001). These data suggest that modulation of 5-HT_{1B}Rs is important in AD efficacy, because normally they restrict the amount of serotonin available by shutting off release through autoreceptor activation. It has been postulated that the time delay in AD efficacy is related to the amount of time it takes for 5-HT_{1B} autoreceptors to desensitize with chronic treatment, and an ideal AD would increase serotonin while blocking 5-HT_{1B} autoreceptors (Matzen et al., 2000). However it appears that blocking or removing all 5-HT_{1B} receptors is not the answer. Behaviorally, 5-HT_{1B}R-KO mice have provided mixed results in regards to depression tasks. These animals display enhanced aggressive behavior (Saudou et al., 1994) and do not respond to ADs in the forced swim

test (Chenu et al. 2008; Gardier et al. 2001). Additionally, the 5-HT_{1B}R agonist anpirtoline, produces AD-like effects in normal animals (Schlicker, Werner, Nickel, & Gothert, 1992). On the other hand, 5-HT_{1B}R-KO mice exhibit *increased* sensitivity to fluoxetine in the tail suspension test (Jones & Lucki, 2005; Mayorga et al., 2001). This discrepancy may be due to differences in presynaptic versus postsynaptic receptor control of these behaviors. Indeed, it appears that heterosynaptic 5-HT_{1B}R activation may be necessary for the effects of ADs. Chenu and colleagues found that animals treated with either 5,7 dihydroxytryptamine (5,7 DHT), which lesions serotonergic neurons, or paracholorphenylanine methyl-ester (p-CPA) which depletes serotonin, exhibited AD-like responses to anpirtoline in the forced swim test (Chenu et al., 2008). This study implies that the antidepressant effect of 5-HT_{1B}R activation is not mediated through alterations in serotonin release but rather heterosynaptically, through postsynaptic activation, or changes in the release of another neurotransmitter.

While the 5-HT_{1B}R data is interesting, it still does not answer many questions regarding the monoamine theory of depression. Importantly, why does it take weeks for ADs to become effective therapeutically (Katz et al., 2004), when they increase serotonin in the cleft immediately? If the antidepressant effect of 5-HT_{1B}R activation is heterosynaptic, the explanation of autoreceptor desensitization no longer holds true. While serotonergic balance may be important for some cases of depression, it appears that there is more to the story, and that there is another culprit responsible for the maintenance of affective state. It is becoming increasingly evident that antidepressants must do more than just increase serotonin in order to be truly effective.

Alternative theories of depression: beyond monoamines

While it is difficult to deny that monoamines play a role in the treatment of depression, the support for this theory is inconclusive. Since the original observations of the 1950s that led to the development of this theory, a number of tools and models have been developed to allow us to better test it. The development and validation of animal models of depression and tests of antidepressant efficacy have brought forth a number of new candidates that may be responsible for the symptoms of depression and have provided new targets for better medications. These tools have also helped to narrow down the affected brain regions in depressed patients and animal models leading to more focused research that can provide insight into the biological basis of depression.

Hippocampal dysfunction. While depression is likely caused by dysfunction of a variety of brain regions and cell types, there is increasing evidence that most patients exhibit some type of hippocampal dysfunction. The hippocampus is part of the limbic system which also includes the amygdala, limbic cortex and fornix. This system is believed to control emotion as well as learning and memory (Richardson, Strange, & Dolan 2004). Additionally, the hippocampus is highly plastic and sensitive to stress (Magarinos, McEwen, Flugge, & Fuchs, 1996) making it vulnerable to environmental insults that lead to depression.

The advent of high resolution imaging techniques has allowed researchers to measure hippocampal volume and activity in living patients. MRI scans have shown that patients diagnosed with their first episode of depression exhibit less hippocampal white matter than healthy subjects, with no other significant changes in overall brain volume (Frodl et al., 2002). However, MacQueen and colleagues did not see a change in

hippocampal volume after the first episode of depression, but did find changes in both left and right hippocampal volume after multiple depression episodes (MacQueen et al., 2003). Additionally, depressed patients exhibit changes in hippocampal metabolic activity even when hippocampal volume is controlled for (Saxena et al., 2001). These imaging results are further supported by the evidence of alterations in hippocampal dependent behavioral deficits exhibited in depressed patients. Specifically, depressed patients tested on a virtual reality based task of visuospatial navigation exhibited deficits compared to healthy controls (Gould et al., 2007). This finding sheds light onto some of the cognitive deficits exhibited in depressed patients and may provide insight into the underlying causes of depression.

Data from animal models have provided further support for hippocampal dysfunction in depression. Animals subjected to chronic stress exhibit glucocorticoid-dependent insults to neuronal structure and function in the hippocampus (Krugers et al., 2010). Among the functional changes observed in the hippocampi of stressed animals are decreases in synaptic currents in the dentate gyrus (Karst & Joëls, 2003) and deficits in synaptic plasticity (Alfarez, Joels & Krugers, 2003.) Additionally, one group found a significant correlation between dentate gyrus-CA1 relative activity and performance on the FST (Airan et al., 2007). This relative activity was decreased in animals subjected to chronic stress and recovered with chronic fluoxetine treatment. Alterations in hippocampal brain function are one the strongest biological hallmarks found in depression. Knowing this, we can test new models of depression using both behavioral and physiological markers.

Neurotrophic theory of depression. The most popular theory of depression to

follow the monoamine hypothesis is the neurotrophic theory of depression. This theory postulates that chronic stress results in neuronal atrophy of the brain, particularly in the hippocampus, and that enhancing neurotrophic factors, most notably brain derived neurotrophic factor (BDNF), can reverse or block this effect. BDNF promotes growth of new neurons and survival of existing neurons by binding to the trkB receptor tyrosine kinase. The generation of newborn neurons is generally restricted to the developmental period in the brain, however the hippocampus is an exception to this rule (Eriksson et al., 1998). It is unclear whether it is generation of new neurons, or the other growth promoting effects of BDNF release that are important in terms of depression.

There is evidence for a role of BDNF in human depression though it is somewhat limited. First, depressed patients exhibit a decrease in BDNF serum levels (Karege et al., 2002) which can be recovered with antidepressant treatment (Shimizu et al., 2003). Additionally, post-mortem tissue from suicide patients exhibits decreases in both BDNF and trkB mRNA in the prefrontal cortex and hippocampus (Dwivedi et al., 2003) while patients treated with antidepressant drugs exhibit a higher level of brain derived neurotrophic factor (BDNF) than untreated patients, specifically in the dentate gyrus, hillus and supergranular regions of the hippocampus (Chen et al., 2001). These studies indicate that patients suffering from depression may have decreased neurogenesis or cell viability and that antidepressants act by rescuing this dysfunction. Unfortunately, these studies are purely correlational, and we must turn to animal models for a more stringent test.

Strong evidence for a neurotrophic theory comes from studies showing that both chronic mild stress (Grønli et al., 2006) and social defeat stress (Pizarro et al., 2004)

decrease BDNF expression in the brains of rodents. Additionally, a number of antidepressant treatments, including electroconvulsive shock, sertaline and desipramine increase BDNF and trkB mRNA expression in the rat brain (Nibuya, Morinobu & Duman, 1995). Interestingly, BDNF injection itself is capable of producing antidepressant effects in the learned helplessness and forced swim tests (Shirayama, Chen, Nakagawa, Russell, & Duman, 2002; Siuciak, Lewis, Wiegand, & Lindsay, 1997). Animal studies have also been able to better distinguish between the neuroprotective effects and neurogenesis effects of BDNF, though the results are still inconclusive. X-ray irradiation of progenitor cells in the hippocampus which blocks only neurogenesis and not gene transcription effects of BDNF, also blocks the effect of antidepressants in the forced swim test (Airan et al., 2007). Conversely, one group found that fluoxetine could exert antidepressant effects in the forced swim test and novelty suppressed feeding test even with the ablation of progenitor cells (Holick, Lee, Hen, & Dulawa, 2008), suggesting that antidepressants use a neurogenesis-independent mechanism for their therapeutic effects.

While these data are convincing, there are still some gaps in the story, and multiple contradictory reports. One group found that chronic social defeat actually enhanced BDNF mRNA in the ventral tegmental area, and that viral knockdown of BDNF blocked social aversion observed in these mice (Berton et al., 2006), complicating the role of BDNF in depression. However, it is certainly possibly that BDNF may be regulated in different directions depending on the brain region. Genetically mutated mice have only further complicated the story. Mice with a heterozygous mutation of the BDNF gene do not exhibit any differences from wild type mice in anxiety and behavioral despair

measures (Chourbaji et al., 2004). On the other hand female mice with a conditional BDNF knockout in the forebrain exhibit depressed phenotypes in the sucrose preference and forced swim tests, while male mice do not. Altogether, it appears that the neurotrophic theory of depression is an incomplete story. In addition to the conflicting data in the literature, there is no established link between serotonin activation and BDNF transcription, and therefore no explanation as to why antidepressants such as SSRIs work in some patients. The theory has however brought to light the possibility that changes in neuronal strength and plasticity may underlie depression and the action of antidepressants.

Glutamatergic theory of depression. A newly developing theory of depression proposes that dysfunctional excitatory synapses are to blame for depression. Glutamate is the most abundant excitatory neurotransmitter in the brain. Following release, glutamate can be recycled through the glutamate/glutamine cycle via vesicular glutamate transporters (vGLUTs) (Shigeri, Seal, & Shimamoto, 2004). Glutamate can excite the cell through activation of ionotropic receptors such as alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), *N*-methyl-D-aspartic acid receptors (NMDARs) and kainite receptors (KARs), as well as metabotropic receptors (mGluAs). Whole cell viability can be affected by glutamatergic transmission. Too much glutamate can lead to NMDAR dependent apoptosis (Choi, Koh, & Peters, 1988), however AMPAR potentiators can cause cell proliferation (Bai, Bergeron, & Nelson, 2003). Additionally, individual synapses can either undergo long-term potentiation (LTP) and be strengthened or long-term depression (LTD) and be weakened depending on the patterning and strength of presynaptic stimulation (for review see: Malenka & Bear, 2004). Increases in

plasticity and synaptic strength are thought to be controlled by phosphorylation and subsequent increases in conductance and surface levels of AMPA receptors (Malenka & Bear, 2004). Changes in receptor population are mediated by a number of downstream signaling molecules including kinases, phosphatases and transcription factors. Specifically, phosphorylation of serine residues at positions 845 and 831 on the GluA1 subunit of the AMPA receptor have been implicated in controlling synaptic plasticity (Lee et al., 2000).

Glutamatergic dysfunction has been tied to the pathology of depression through both human and animal studies. In general the literature on the direction of the change in depressed patients is mixed (Table 1.1). There is evidence of increased plasma levels of glutamate that positively correlate to severity of depression in patients (Mitani et al., 2006). However, a few studies have found a decrease the glutamine/glutamate level in depressed patients (Auer et al., 2000; Pfleiderer et al., 2003). Additionally, post-mortem studies using in situ hybridization have found decreases in multiple glutamate receptor subunits in depressed patients including the GluA1 subunit (Beneyto, Kristiansen, Oni-Orisan, McCullumsmith, & Meador-Woodruff, 2007; Choudary et al., 2005). Conversely, patients treated with fluoxetine exhibit decreased serum levels of glutamate (Maes, Verkerk, Vandoolaeghe, & Scharpe, 1998), whereas glutamate is increased in patients treated with electroconvulsive shock therapy for depression (Auer et al., 2000). These discrepancies likely come from the inability to control for all variables, including medication history, in human studies. Additionally, it is difficult to tell from these studies which effects are causing the symptoms of depression and which may be a compensatory mechanism.

Study	Measurement	Finding
Maes et al., 1998	serum HPLC	decreased glutamate in antidepressant treated patients
Levine et al., 2000	^{1}H MRS	17% increase in CSF glutamine in depressed patients
Auer et al., 2000	^{1}H MRS	14% decrease of glx and glutamate in anterior cingulate cortex in depressed patients
Pfleiderer et al., 2002	^{1}H MRS	Decreased glx level in cingulate cortex in depressed patients; normal levels following effective ECT
McCullumsmith & Meador-Woodruff, 2002	in situ hybridization	Decreased EAAT4 mRNA in post mortem tissue from depressed patients
Michael et al., 2003	STEAM spectroscopy	Decreased glx level in left amygdalar region in depressed patients
Sanacora et al., 2004	^{1}H MRS	Increased glutamate in occipital cortex of depressed patients
Choudary et al., 2005	in situ hybridization	Increased SLC1 and decreased GluA1 mRNA in post mortem tissue from depressed patients
Mitani et al., 2006	HPLC	Positive correlation between plasma glutamate levels and severity of depression
Hashimoto et al., 2007	HPLC	Increased glutamate in frontal cortex in post mortem tissue for depressed patients
Hasler et al., 2007	^{1}H MRS	Decreased glx level in prefrontal cortex in depressed patients
Beneyto et al., 2007	in situ hybridization	Decrease GluA1, GluA3, NR2A, NR2B subunits in depressed post mortem tissue

Table 1.2 Clinical studies investigating glutamatergic dysfunction in Major Depression. ^{1}H MRS= Proton Magnetic Resonance Spectroscopy; HPLC= High performance liquid chromotography; Glx= glutamine/glutamate; SLC1= High affinity glutamate transporter family; EAAT4= Excitatory amino acid transporter

Animal models have aided in clarifying the role of glutamatergic transmission in depression. Generally they have supported the theory that AMPARs are downregulated in depression. One study found that animals subjected to immobilization stress exhibit decreases in AMPAR and increases in NMDAR mRNA in the CA1 and CA3 regions of the hippocampus (Bartanusz et al., 1995) (Figure 1.2). A more recent study examined the difference between rats that were vulnerable to chronic social stress and those that were resilient. Interestingly, the vulnerable rats exhibited a significant decrease in GluA1 and increase in GluA2 subunits of the AMPAR in the hippocampus compared to resilient rats (Schmidt et al., 2010). This finding is important in that it supports the idea that glutamatergic function is related to the manifestation of symptoms in depression and not an artifact of the stress itself. Additionally, this study examined genetic polymorphisms in the rats and found a single nucleotide polymorphism (SNP) in the GluA1 subunit that correlated with vulnerability to stress. These data are further supported by work of Chourbaji and colleagues, who found that knocking out the GluA1 gene can result in a depressed phenotype in the learned helplessness test (Chourbaji et al., 2008). Together these data make a strong argument that disruption of glutamatergic transmission, specifically AMPAR function, can result in depression.

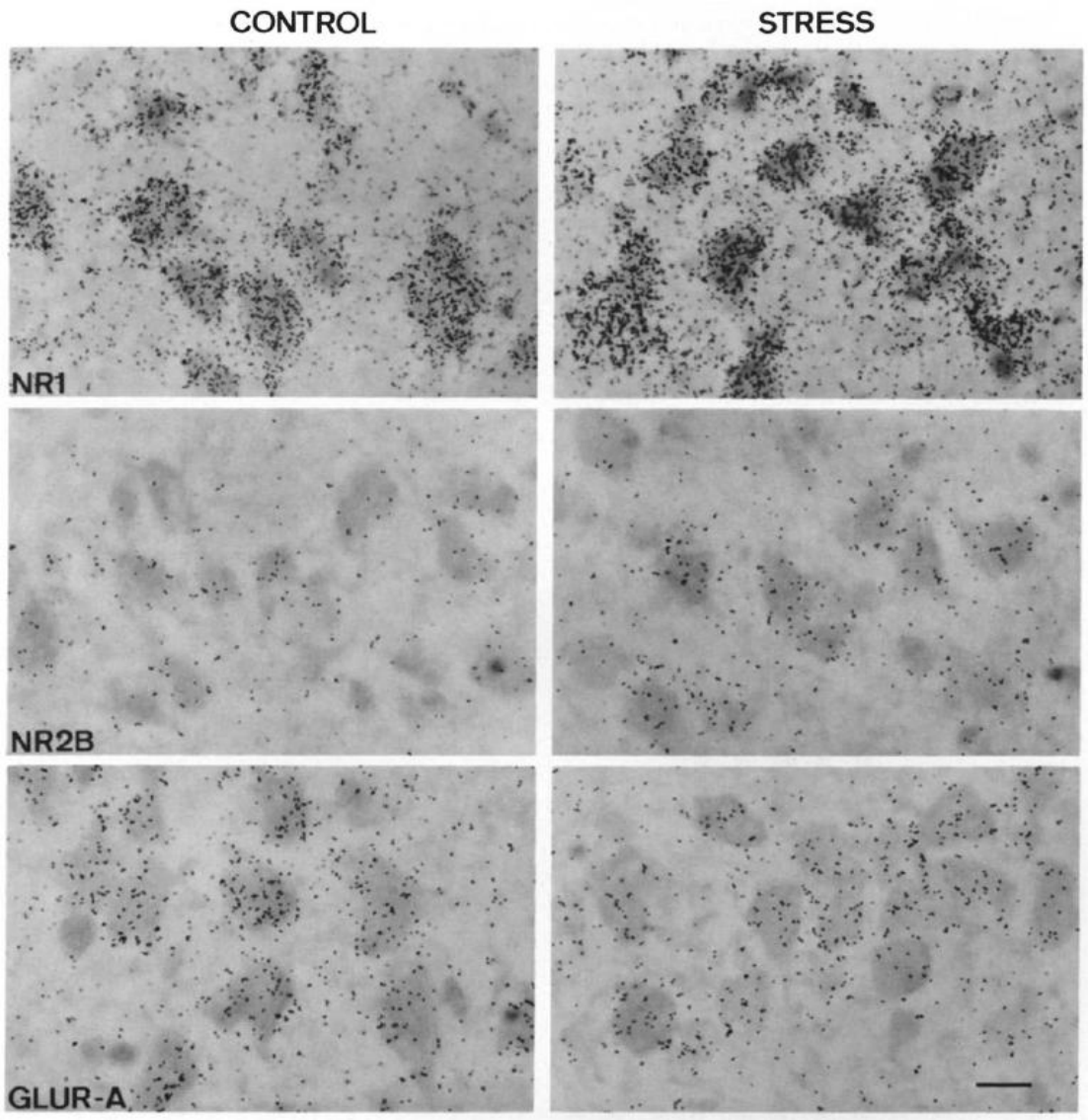


Figure 1.2 *In situ* hybridization of NR1, NR2B, and GluA1 in the CA3 region of the hippocampus in control rats, and rats subjected to immobilization stress. Hippocampal sections were made from control animals and animals subjected to acute immobilization stress for 150min. Slices were made 24hr after stress. mRNA of NMDAR subunits NR1 and NR2B were increased following immobilization stress, whereas mRNA of the GluA1 subunit of the AMPAR is decreased following stress. Scale = 13µm. (from Bartanusz et al., 1995).

The glutamatergic theory of depression has become increasingly promising with the discovery that glutamatergic agents can act as antidepressants. The drug LY39208 which acts as positive allosteric modulator of AMPA receptors can decrease immobility in both the tail suspension and forced swim tests (Li et al., 2001). Interestingly, this compound can also increase the efficacy of traditional antidepressants such as fluoxetine and imipramine, when co-administered at sub-therapeutic doses (Li et al., 2003). These results are further supported by the finding that the antidepressant actions of lithium in the forced swim and tail suspension tests are blocked by AMPAR blockers (Gould et al., 2008). Additionally, AMPA receptor potentiators can mimic cellular responses produced by traditional antidepressants. For example, both types of drugs are capable of increasing BDNF mRNA in the hippocampus (Bai et al., 2003).

Recently there has been much focus on targeting NMDA receptors in the therapy of depressed patients. Multiple groups have shown that blocking NMDARs can result in a rapid, long-lasting antidepressant effect. Rodents treated acutely with the NMDAR antagonist ketamine exhibit an antidepressant-like phenotype on the forced swim test (Garcia et al., 2008) novelty suppressed feeding test, and learned helplessness test (Li et al., 2010). These data are supported by the double blind placebo controlled study in which treatment-resistant patients were treated with an acute injection of ketamine. Patients exhibited an antidepressant response within two hours that was maintained for 72 hours (Berman et al., 2000). Later it was shown that this effect could last up to seven days (Zarate et al., 2006). Interestingly, blocking NMDARs no longer has an antidepressant effect when AMPARs are simultaneously blocked by NBQX (Maeng et al., 2008), suggesting that it is important to balance the ratio of AMPA to NMDA

receptor activation for antidepressant efficacy. This idea is supported by the fact that ketamine while blocking NMDARs, also enhances glutamatergic release (Moghaddam, Adams, Verma, & Daly, 1997), thereby enhancing AMPAR activation in relation to NMDAR activation.

While there is much excitement centered on the novel idea that glutamate may be a target for fast and effective antidepressant therapy, this theory remains incomplete. It has not yet been explained why traditional antidepressants work in a large number of patients even though they have no known affinity for glutamate receptors. Additionally, although it has been shown that AMPAR function may be involved in antidepressant efficacy, it remains unknown whether it is necessary. It is important to consider findings from all branches of depression research to develop a model that can account for most of the studies. Although to date, these theories have stood as independent models of depression, there is indirect evidence that they may all be connected.

How can we resolve these theories?

Increasing evidence points to an overlap between the monoamine, neurotrophic, and glutamatergic theories of depression. The questions remaining within each can be answered when looking to evidence from the others. It appears that the newly emerging glutamatergic theory may be able to bridge the gap between the previously distinct serotonin and neurotrophic models of depression.

Serotonergic modulation of glutamate. The long delay between serotonin receptor activation and antidepressant effectiveness in patients points to the involvement of a downstream signaling mechanism. Among the many actions of serotonin is the ability to modulate glutamate transmission. In the hippocampus, iontophoretic application of 5-HT

can either hyperpolarize (Andrade & Nicoll, 1987) or depolarize CA1 pyramidal cells (Andrade & Chaput, 1991). Also, hippocampal slices treated with 5-HT exhibit an increase in phosphorylation of the GluA1 subunit of the AMPAR at serine 831 and serine 845 (Svenningsson et al., 2002). These findings can be extended to include antidepressant drugs as well. Chronic administration of the antidepressants paroxetine and desmipramine causes an increase in the membrane bound fraction of AMPA receptors in the rat hippocampus (Martinez-Turrillas et al., 2002). One explanation for this finding is that serotonin enhances surface trafficking of receptors. In fact, rats chronically treated with the SSRI fluoxetine, exhibited increases in the phosphorylation of AMPA receptors at sites important for AMPAR conductance and trafficking, serines 831 and 845 (Svenningsson et al., 2002). A recent study by Barbon and colleagues found that rats chronically treated with fluoxetine exhibited a time-dependent increase in GluA1-4 mRNA and protein in both the hippocampus and cortex (Barbon et al., 2011). The levels of mRNA peaked at 2 weeks of treatment, whereas the protein levels increased in this time period and remained high. This time course nicely coincides with the time period in which antidepressants become effective in patients. Therefore it is possible that the delay in the efficacy of antidepressants in patients is due to a time lag in the amount of time it takes for serotonin receptor activation to increase AMPAR synthesis and transport.

Although it is evident that enhancing serotonin can enhance glutamatergic transmission, it is unclear what the components of this signaling pathway are. Activation of one or more serotonin receptor must be involved in phosphorylation and/or transcription of glutamatergic signaling components. Morphologically, 5-HT_{1B}Rs represent a good candidate in that mRNA for this receptor is found in CA1 pyramidal

neurons in the hippocampus (Svenningsson et al., 2006), and protein is found in the stratum lacunosum moleculare layer of the hippocampus (Ait et al., 1995; Ihara et al., 1988; Sari et al., 1999), the site of densest serotonergic innervation in the hippocampus (Bjarkam, Sørensen, & Geneser, 2003). Additionally, 5-HT_{1B}R activation has been shown to increase intracellular calcium (Giles et al., 1996) and result in phosphorylation of extracellular signal-related kinase-2 (ERK2) (Mendez, Kadia, Somayazula, El-Badawi, & Cowen, 2002), a member if the mitogen activated protein kinase (MAPK) pathway. Intracellular calcium is necessary for the phosphorylation of calmodulin kinase (CamK) which in turn can phosphorylate serine 831 of the GluA1 subunit of the AMPAR (Barria, Derkach, & Soderling, 1997). Phosphorylation of this site by CamK results in increased conductance of the AMPA receptor (Derkach, Barria, & Soderling, 1999). CamK phosphorylation is both necessary and sufficient for the induction and maintenance of electrically induced LTP (Lisman, Schulman, & Cline, 2002), a process that is characterized by increased synthesis and function of AMPARs. ERK phosphorylation may also be involved in this process. ERK phosphorylation is decreased in the dentate gyrus of corticosterone treated animals, and increased in both the dentate gyrus and CA1/CA3 following antidepressant treatment (Gourley et al., 2008). Interestingly, AMPAR synthesis and trafficking is also enhanced through ERK1/2 activation (Hall & Ghosh, 2008). This provides evidence that 5-HT_{1B}R activation can potentially enhance glutamatergic function through a calcium- and/or ERK-mediated signaling cascade. Enhancement of glutamatergic transmission and subsequent increases in intracellular calcium and phosphorylation of ERK are believed to be important for individual synapse strength in processes such as electrically induced LTP. Together, this sets up a potential

pathway through which antidepressants like SSRIs can increase serotonin and strengthen excitatory synapses.

Glutamatergic modulation of BDNF. BDNF activation of trkB receptors is also intimately tied to synapse strengthening processes such as LTP. Activation of the trkB receptor results in a signaling cascade that includes phosphorylation of ERK as well as activation of phosphatidylinositol 3 (PI3) kinase (Yoshii & Constatine-Paton, 2010). Application of BDNF also results in morphological and functional changes that parallel LTP, including increased spine formation and higher amplitudes and frequency of miniature EPSCs in CA1 neurons in the hippocampus (Tyler & Pozzo-Miller, 2003). One hypothesis is that early LTP is modulated by an increase in AMPAR conductance and insertion, but that late, persistent LTP is caused by a MAPK and CREB dependent pathway that stimulates further increase in BDNF mRNA (Yoshii & Constatine-Paton, 2010). Supporting this theory is evidence that blocking trkB receptors after the induction of LTP blocks late phase LTP (Kang, Welcher, Shelton, & Schuman, 1997). Therefore, serotonin-mediated changes in glutamate may be the link to changes in neurotrophic factors observed following antidepressant treatment.

While enhancement of glutamatergic transmission appears to share common endpoints with neurotrophic stimulation, more glutamate does not necessarily translate to more neuronal growth. In fact, excessive glutamate release is associated with NMDAR dependent excitotoxicity of the cell (Mody & MacDonald, 1995). Therefore, it may be that the balance of AMPAR to NMDAR activation determines whether a cell survival or cell death pathway is triggered in the presence of large amounts of glutamate. Supporting this theory is evidence that specific activation of AMPARs results in an increase in

BDNF and protection from glutamate induced neurotoxicity. Drugs that positively modulate AMPARs increase hippocampal BDNF mRNA both in cultured cortical cells (Legutko, Li & Skolnick, 2001) and *in vivo* (Mackowiak, O'Neill, Hicks, Bleakman, & Skolnick, 2002). Additionally, cultured neurons pre-treated with the agonist AMPA and aniracetam, a drug that blocks AMPAR desensitization, increases trkB phosphorylation and activation of the PI3-K pathway, and also protects the cells from glutamate induced cell death (Wu et al., 2004).

Together, these studies lead to a hypothesis which may unify two previously disparate theories of depression. I hypothesize that dysfunction of glutamatergic synapses underlies the etiology of depression and serotonin is capable of modulating glutamatergic transmission in a manner that rescues this defect (Figure 1.3). The increased glutamate present in depressed brains may result in overactivation of NMDARs leading to cell death in the hippocampus. Conversely, antidepressants may exert their beneficial effects by specifically enhancing AMPAR activity which in turn stimulates production of neurotrophic factors such as BDNF and rescues the hippocampus in depressed patients. One way antidepressants may activate this pathway is through 5-HT_{1B}Rs which are capable of increasing calcium and may induce phosphorylation and subsequent changes of AMPAR function. This model is also supported by the evidence that AMPAR potentiators and NMDAR blockers can both act as antidepressants in animal models of depression. The unifying hypothesis that I have put forward makes a series of testable predictions that I propose to investigate as my thesis project.

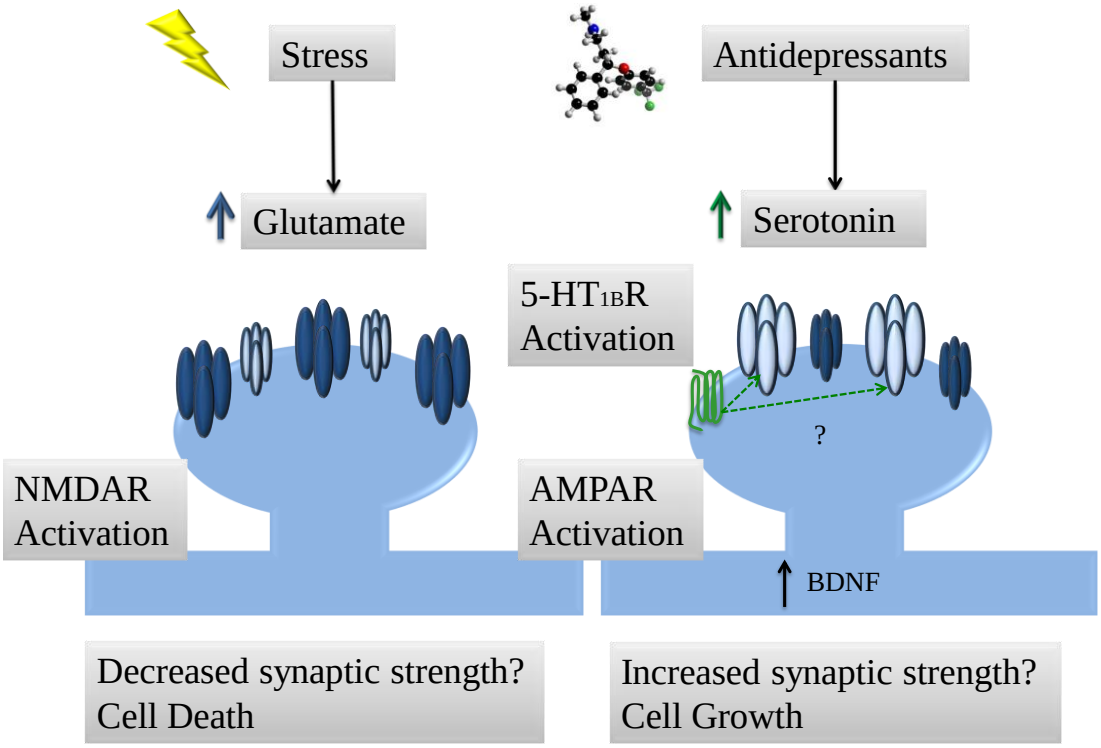


Figure 1.3 Schematic of proposed integrated model of depression. Chronic stress results in an increase in glutamate concentration in the cleft which binds to both AMPA-type and NMDA-type receptors. Overactivation of NMDARs results in programmed death of the cell (left). Chronic antidepressant treatment enhances serotonin concentration, leading to the activation of 5-HT_{1B}Rs. 5-HT_{1B}Rs may signal to AMPARs through an influx in intracellular calcium. Enhanced AMPAR activation leads to transcription of BDNF mRNA which promotes cell survival and neurogenesis.

Summary and predictions of the hypothesis.

Prediction I: Serotonin activates 5-HT_{1B}Rs and enhances glutamatergic transmission in the hippocampus.

The effect of serotonin on hippocampal neurons has been studied using various methods. It has been established that serotonin can cause both hyperpolarization and depolarization of CA1 pyramidal neurons (Andrade & Nicoll, 1987; Andrade & Chaput, 1991); however it remains unclear how serotonin affects synaptic responses. It is particularly surprising that no study has investigated the effect of serotonin on temporoammonic (TA)-CA1 synapses in the stratum lacunosum moleculare layer of the hippocampus, the region of densest serotonin fiber innervation in this part of the brain (Bjarkam et al., 2003). Therefore, I propose to test the prediction that endogenous serotonin can enhance glutamatergic transmission at TA-CA1 synapses via activation of 5-HT_{1B}Rs. I predict that elevating endogenous serotonin using acute antidepressant application will increase TA-CA1 field excitatory post synaptic potential (fEPSP) slope and that this will be blocked by a 5-HT_{1B}R antagonist. Additionally, I predict that a 5-HT_{1B}R agonist will mimic the effect of endogenous serotonin. Data from these experiments will be presented in Chapter 3.

Prediction II: 5-HT_{1B}R activation shares a common signaling pathway with electrically induced potentiation.

Serotonin, possibly through 5-HT_{1B}R activation, activates a signaling cascade that is also implicated in conventional LTP. Serotonin is capable of enhancing AMPA receptor phosphorylation at both serine 831 and serine 845 (Svenningsson et al., 2002),

sites that are linked to enhanced conductance and insertion of the receptor. These sites are phosphorylated by CamKII and PKA, respectively (Barria, Derkach, & Soderling, 1997; Roche et al., 1996). 5-HT_{1B}R activation raises intracellular calcium (Giles et al., 1996), which is necessary for the activation of CamKII and decreases cAMP accumulation, a precursor to PKA phosphorylation. Therefore, these receptors are a good candidate for mediating the effects of serotonin on these AMPAR residues. Phosphorylation of both sites is necessary for normal LTP and the induction of LTD (Lee et al., 2003). Additionally, 5-HT_{1B}R activation enhances ERK phosphorylation which is also necessary for normal induction of LTP (English & Sweatt, 1997). While 5-HT_{1B}Rs appear to mediate signaling cascades relevant to synaptic plasticity, these studies were conducted in heterologous cells. Therefore, I plan to test the prediction that 5-HT_{1B}R activation and conventional LTP share a common expression mechanism. Specifically, I predict that CaMKII, ERK and AMPAR phosphorylation will be enhanced in SLM of hippocampal area CA1, the site of TA-CA1 synapses, following treatment with a 5-HT_{1B}R agonist and are necessary for 5-HT_{1B}R induced potentiation. Additionally, I predict that both forms of potentiation will mutually occlude each other. I will present the data from these experiments in Chapter 4.

Prediction III: Basal glutamatergic strength will be diminished in an animal model of depression and strengthened with chronic antidepressant treatment.

Data from human and animal studies indicate that AMPAR levels are diminished in depressed patients (Beneyto et al., 2007) and in animal models of depression (Chourbaji et al., 2008; Schmidt et al., 2010). Most of these studies have focused on receptor mRNA and protein levels of the different AMPAR subunits. While some of

these studies examined the hippocampus, few have investigated the function of these receptors, and none have specifically measured AMPAR levels at TA-CA1 synapses. Therefore, I propose to test the hypothesis that basal AMPAR function is decreased at TA-CA1 synapses in animals subjected to chronic stress and can be restored by chronic antidepressant treatment. Specifically, I predict that AMPAR-mediated synaptic currents at TA-CA1 synapses in CUS animals are smaller than those in control or antidepressant-treated animals. This experiment will help to determine whether the receptor mRNA and protein changes observed by others translate to functional changes at the synapse. I also predict that a basal change in synaptic strength results in an enhancement of LTP at this synapse in CUS animals. These results will also be important in providing insight regarding the cognitive symptoms exhibited by depressed patients. I will present the data from these experiments in Chapter 5.

Prediction IV: Activation of the 5-HT$_{1B}$Rs and downstream signaling components are necessary for the therapeutic effects of antidepressants.

Finally, I plan to test the prediction that components of this proposed 5-HT$_{1B}$R signaling pathway are required for antidepressant efficacy. 5-HT$_{1B}$Rs have already been implicated in depression through both human (Huang et al., 2003; Svenningsson et al., 2006) and animal model studies (Jones & Lucki, 2005; O'Neill & Conway, 2001). Unfortunately, the animal studies have relied solely on the forced swim test and tail suspension test to decipher the role of 5-HT$_{1B}$Rs in depression. While these tasks are popular they are sensitive to acute antidepressant treatment even though patients are not, and are therefore not appropriate for inferring therapeutic efficacy. Surprisingly, no one has investigated the role of 5-HT$_{1B}$Rs in the sucrose preference test, a task sensitive to

both chronic stress and chronic antidepressant treatment. I predict that 5-HT_{1B}RKO mice will exhibit normal sucrose preference at baseline which is decreased with chronic stress but unable to be recovered by chronic antidepressant treatment. Additionally, I plan to investigate whether downstream activation of glutamate receptors is also necessary for antidepressant efficacy. There is already evidence GluA1 knockout animals are depressed in the learned helplessness test (Chourbaji et al., 2008), however this study did not examine whether SSRI drugs could rescue this deficit. I therefore predict that GluA1 phosphomutant animals will exhibit anhedonia in the sucrose preference test which is unable to be recovered with chronic antidepressant treatment. The results of these experiments will determine whether antidepressants must induce phosphorylation of GluA in order to be effective or if they exert their effects through a glutamate independent pathway. The data from these experiments will be presented in Chapter 6.

Conclusion:

My hypothesis makes novel and direct predictions regarding the outcome of these tests. If these predictions are not met then, my model will have to be modified or rejected. If the results of the tests are in agreement with my hypothesis, they will provide tentative support for the model in Fig. 1.3. This in turn will have implications for the development of new therapeutic approaches to the treatment of the serious human disorders.

CHAPTER TWO

METHODS

Animals

All animals were group housed on a 12:12 light dark cycle with food and water available *ad libitum* (except where noted). Rats were ordered from Harlan laboratories post-weaning and were used for experiments at 4-8 weeks in age.

Homozygous S831A mice were acquired from Richard Huganir's lab (Johns Hopkins University). Mice were generated by introducing a mutation at the mouse GluA1 gene using homologous recombination (knock-in technique). A targeting vector encoding an alanine substitution at S831 in exon 17 was constructed with a lox-P flanked neomycin resistant marker in intron 16. Correctly targeted embryonic stem cells were injected into C57BL/6 blastocysts. Chimer mice carrying the mutant allele were bred to C57BL/6 mice to generate heterozygous mice. Heterozygous mice were bred to CMV-Cre mice to delete the neomycin cassette from the germ line via the cre-loxP system then intercrossed to produce phospho-mutant homozygous mice (Lee et al., 2010). We then crossed homozygous S831A (N13) mice to C57BL6/J mice (Jackson) to produce heterozygotes. These heterozygotes were crossed to obtain homozygous S831A and homozygous wild type mice used for behavioral experiments.

Homozygous 5-HT_{1B}KO mice were acquired from Rene Hen's lab (Columbia University). Mice were generated by homologous recombination in embryonic stem cells. 3.5 day C57BL/6 mouse blastocysts were injected with positive clones. The highly chimeric mice were bred with C57BL/6 females. The positive chimeras were bred with females from the 129/Sv-ter inbred strain to obtain heterozygotes on the 129/Sv-ter genetic background (Saudou et al., 1994). Homozygous animals were obtained through

heterozygous crossings. We then crossed homozygous 129/Sv-ter 5-HT_{1B}KO mice with Sv129ImJ mice (Jackson) to produce heterozygous animals. These heterozygotes were crossed and the resulting homozygous wild types and KOs were used for behavioral tests.

Genotyping

All animals produced from heterozygous crosses were genotyped by Transnetyx Tail clips harvested from young mice were processed and genotyped using PCR. Homozygous S831A mutant animals had two copies of the 540bp mutant gene, identified by the primer sequence: F: CCCAGGTGGTAATGATTGC, R: AATGAGATAACACGGGGCTTGGTTCCTAAC. Homozygous wild type animals had two copies of the 390bp gene. Homozygous 5-HT_{1B}R-KO mice were positive only for the neomycin cassette identified by the primer sequence F: GACTTGGTTCACGTACACAG, R:CCCATCAGCACCATGTACAC. Homozygous wild type mice were only positive for the wild type gene, R: CTTCTATCGCCTTCTTGACG.

Acute hippocampal slice preparation.

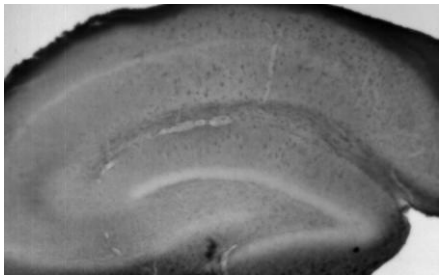
All electrophysiology and western blot data used acute hippocampal slices. I prepared slices from 4-8 week old rats, wild type C57BL6J mice or mutant S831A and 5-HT_{1B}KO mice. First the animal was deeply anesthetized using 1mL/kg Nembutal. Next the animal was quickly decapitated and the hippocampus was removed and placed into ice cold oxygenated artificial cerebrospinal fluid (ACSF) which consisted of (in mM): 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1.5 $MgCl_2$, 2.5 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose. Brain slices (400µm) were cut on a vibratome and kept in a holding chamber at room

temperature at the interface of physiological medium and humidified 95%O_2/5%CO_2 for >1 hr (Figure 2.1A).

Acute slice electrophysiology.

To isolate TA-CA1 responses, the dentate gyrus and CA3 region of the hippocampal slice were removed while the slices were in the holding chamber (Figure 2.1B). Hippocampal slices were transferred to a submersion-type recording chamber and perfused at room temperature with ACSF (flow rate= 1ml/min). ACSF was continuously bubbled with 95%O_2/5%CO_2. Picrotoxin (100µM) and CGP52432 (2µM) were included to block $GABA_A$ and $GABA_B$ receptors, respectively. Concentric bipolar tungsten electrodes placed either in SLM were used to stimulate TA afferents or SR to stimulate SC afferents (Figure 2.1B). Recording pipettes were filled with ACSF (3-5MΩ) and placed >500µm from the stimulating electrodes. Stimuli (100µs in duration) were delivered at 0.05 Hz. The stimulus intensity was set at 150% of threshold intensity, resulting in a fEPSP of 0.1-0.2mV. All compounds were applied by perfusion. Field EPSPs were recorded using a digidata amplifier, filtered at 10kHz, and amplified 1000x prior to digitization. All experiments were performed at room temperature.

A



B

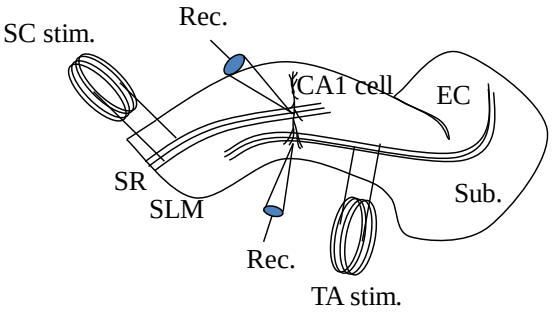


Figure 2.1 A) Light microscope image of an acute hippocampal slice. B) Schematic of hippocampal slice after CA3 and dentate gyrus regions are removed. Positions of recording and stimulating electrodes for SC-CA1 and TA-CA1 recordings are indicated.

Drugs.

A number of drugs were tested in the acute electrophysiology experiments. All drugs were applied into the perfusion tube and allowed to flow into the slice chamber with no change in flow rate. The selective serotonin reuptake inhibitor (SSRI) citalopram (Tocris) was used at 9.6µM. The SSRI fluoxetine (NIH) was used at a concentration of 10µM. These two drugs have a K_i of 20nM and 23nM respectively (Nagdir & Malviya, 2008). The tricyclic antidepressant (TCA) imipramine (Sigma) was used at 2µM and has a K_i of 17nM (Talvenheim, Nelson & Rudnick, 1979). Anpirtoline (Tocris), a specific 5-HT_{1B}R agonist, was used at 50µM. The K_i of anpirtoline for 5-HT_{1B}Rs is 2nM, significantly higher than the K_i for 5-HT_{1A}Rs (150nM) and 5-HT_2Rs (1.49µM) (Schlicker et al., 1992). A previous study using anpirtoline in brain slices found effects at 10-50µM (Svenningsson et al., 2006). Isamoltane (Tocris) a specific 5-HT_{1B}R antagonist was used

at 10μM. Isamoltane has a 27 fold higher affinity for 5-HT_{1B}Rs (IC50= 39nmol/L) compared to 5-HT_{1A}Rs (IC50= 1070nmol/L), (Waldmeier et al., 1988). It has previously been shown that 10μM isomoltane can block the effect of a 5-HT1BR agonist on extracellular 5-HT concentration (O'Connor & Kruk, 1992). The CamKII blocker KN-62 (Tocris) has an IC_{50} of 0.06μM and was used at 10μM. The MAPK blocker U0126 (Tocris) has an IC_{50} of 0.9μM and was used at 20μM

Western blotting.

The expression of various proteins and their phosphorylation state was quantified using Western blotting. Hippocampal slices were incubated in oxygenated control ACSF or drug treated ACSF for various time points at room temperature. Areas SLM or SR tissue sections were dissected from control and drug-treated hippocampal slices after freezing on dry ice, using a 1mm micropuncher. Punches were pooled and homogenized in ice cold lysis solution containing a phosphatase and protease inhibitor cocktail (PPI, Sigma, Saint Louis, MO). Homogenates were either frozen at -80 degrees (less than 1 week) or used immediately for processing. Sample protein concentration was determined using a Bradford assay. 10ug of protein from each sample was mixed with sample buffer (Laemmli, Sigma), boiled, and loaded into 4-12% Bis-Tris gel (Invitrogen). Multiple control lanes were included in most experiments. After running in 1X NuPage MOPS SDS running buffer, the gel was transferred onto polyvinylidene difluororide membranes in 1X Nupage transfer buffer (in 10% methanol). The membrane was blocked with 5% nonfat dry milk in buffer containing 1M Tris-buffered saline and 0.05% Tween, and probed with antibodies against Ser845-phosphorylated (1:1000; Chemicon), Ser831-phosphorylated GluA1 (1:1000; Chemicon), and Thr286-phosphorylated CaMKII

(1:5000; Promega) at 4°C overnight. After rinses in TBS-Tween, the membrane was incubated for 1 hr at room temperature in horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000, Cell Signaling Technology). The immunoblot was developed with enhanced chemiluminescence (Amersham). Membranes were then stripped, blocked, and reprobed with antibodies against GluA1 (0.5 μg/ml; Chemicon), CaMKII (1:2000; Cell Signaling Technology), or β-actin (1:2000; Cell Signaling Technology). Levels of phosphorylation, expressed as the ratio of phospho-specific intensity divided by total protein intensity, were used for statistical analysis.

Chronic unpredictable stress (CUS) procedure.

Adult Sprague-Dawley rats (3-4 weeks old at start) were randomly divided into a control group and a mild CUS group. In the CUS group, animals were treated as following: Day 1, cage rotation (3 h), forced swim (5 min), food deprivation (16h). Day 2, strobe light (30 min), restraint (30 min), food and water deprivation (16 h). Day 3, strobe light (30 min), social isolation (16 h). Day 4, strobe light (30 min), restraint (30 min). Day 5, cage rotation (3 h), water deprivation (16 h). Day 6, restraint (3 h), social isolation (16 h). Day 7, cage rotation (3 h), restraint (30 min). The cycle was repeated 3 times over 3 weeks. Electrophysiological experiments were then performed and analyzed with the experimenter blinded to the experimental condition of the animals.

Sucrose preference.

All animals were group housed for habituation to sucrose in which two bottles were placed in the cage, one with 1% sucrose and one with normal drinking water. All animals were individually housed during sucrose preference tests. Rats were given a

choice between two bottles for 3-4 hours, one with 1% sucrose solution and another with normal drinking water. To prevent the possible effects of side preference in drinking, the position of the bottles was reversed after 1 hour. The consumption of water and sucrose were measured by weighing the bottles. Mice were tested for 18 hours with 1% sucrose in one bottle and normal drinking water in a second bottle. Bottles were constructed using 50mL centrifuge tube, fixed with a sipper spout in the cap. A hole was drilled at the bottom of the tube to allow for pressure equilibration. Bottles were switched in position 6hrs into the test. Preference for sucrose was calculated as a percentage of consumed sucose-containing solution relative to the total amount of liquid intake. 50% means that they drank equally from both bottles, i.e. they had no preference for sucrose.

Novelty supressed feeding.

Novelty suppressed feeding tests were performed as described previously (Santarelli et al., 2003)**.** The test apparatus was a brightly lit arena (60 x 60 x 35 cm) with a solid floor placed in a dimly lit room**.** The floor of the box was covered with a layer of bedding. Two laboratory chow pellets were placed on a white paper circle platform positioned in the center of the box. Rats that had been food deprived for 24 hours or mice that had been food deprived for 18 hours were gently placed in a corner of the arena. The latency to begin eating, defined as active chewing of the pellet, was recorded. A maximum time allowance was set at 400 s. Immediately after the test, animals were returned to their home cage and allowed to feed for 5 min. Food pellets were weighed before and after the 5 min, and the amount of food consumed was calculated. Animals that ate less than 0.3 g of food within this 5-minute period were removed from all analyses, in order to ensure that only sufficiently hungry animals were included.

Tail Suspension Test

Mice were intraperitoneally injected with saline or 30mg/kg imipramine 30min prior to testing. Each mouse was taped to a wooden horizontal bar 2 inches from the base of its tail. A blinded experimenter recorded the amount of time spent immobile for a 6min period.

Open Field Test

Mice were placed in a 60 x 60x 35cm Plexiglas box for 5min. The box was divided into 12 squares using tape on the bottom of the box. Mice were video recorded and a blinded experimenter calculated the number of wall touches, time spent in center two squares and number of line crossings for each animal.

Antidepressant treatment.

Animals were given antidepressants via their drinking water in order to minimize stress. The concentrations of antidepressants were: imipramine, 100 mg/liter; fluoxetine, 80 mg/liter. Animals were given antidepressants continually for 3 to 4 weeks. Control animals received water only. Experiments were then performed and analyzed with the experimenter blinded to the experimental condition of the animals.

CHAPTER THREE

5-HT_{1B} RECEPTOR MEDIATED POTENTIATION OF TA-CA1 SYNAPSES

INTRODUCTION

Serotonin (coming from the words “serum” and “tonic”) was first described in 1868 and then eventually characterized as a vasoconstrictor in 1948 (Rapport et al., 1948). Serotonin is a monoamine neurotransmitter that when secreted can act on a number of receptors in the central nervous system and throughout the entire body. Its wide distribution allows it have a hand in multiple physiological functions. However, due to its ubiquitous nature, yet neuromodulatory action, it has been described as being “involved in everything but responsible for nothing.”

Serotonin is synthesized from the essential amino acid L-tryptophan which is converted to 5-hydroxy-L-tryptophan by the enzyme tryptophan hydroxylase. Serotonin is then produced by the enzyme amino acid decarboxylase. Following release, serotonin undergoes reuptake by the serotonin transporter (SERT) back into the cytoplasm of serotonergic nerve terminals where it is metabolized in the cytosol by monoamine oxidases (MAOs) into 5-hydroxyindoleacetic acid (5 HIAA) or repackaged into vesicles for subsequent release (Mohammad-Zadeh, Moses, & Gwaltney-Brant, 2008). These enzymes together with the activation of autoreceptors on the terminals of serotonin neurons help to regulate serotonergic action in the central nervous system (CNS) and throughout the body.

The serotonergic system is highly plastic and continually modulating itself in order to stabilize the entire nervous system (Azmitia, 1999). Serotonergic neurons are either silent or spontaneously fire action potentials at a low regular rate of 0.8Hz. These action potentials are notably longer than other cell types and are followed by long afterhyperpolarizations (Kirby, Pernar, Valentino, & Beck, 2003). The action of serotonin

is determined by which of its 14 receptor subtypes it binds to and can result in a net excitatory or net inhibitory effect depending on the population of receptors at any given site (for review see: Barnes & Sharp, 1999). Interestingly, many of these receptors stimulate similar signaling cascades and are located in many of the same brain regions (Table 1.1), however unique pharmacological profiles have aided in teasing them apart. Particularly well studied are the 5-HT_1 receptors which, canonically, are negatively coupled to cyclic AMP. This function allows 5-HT_{1A} and 5-HT_{1B} autoreceptors located on the terminals of neurons to negatively control the release of serotonin in a feedback manner (Davidson & Stamford, 1995) but both receptors can also be found postsynaptically and can regulate a number of other functions (Mizuta & Segawa, 1988). 5-HT_3 receptors are unique among serotonin receptors in that they mediate fast synaptic transmission, acting as non-selective Na^+/K^+ ion channels (Maricq et al., 1991). The more recently characterized 5-HT_6 and 5-HT_7 receptors have been cloned and are positively coupled to adenyl cyclase. Interestingly, both 5-HT_6 and 5-HT_7 receptors have very high affinities for antidepressant and antipsychotic drugs (Ruat et al., 1993).

How can we pharmacologically study the effects of serotonin in the brain?

There are a number of compunds available for studying the the role of serotonin on neuronal function. Perhaps the most obvious substrate is 5-HT itself. While synthetic 5-HT has the advantage of being the most pyshiologicaly relevant ligand for 5-HT receptors, it has several disadvantages. First, it may result in a physiologically irrelevant response if bath applied. Bath application will result in activation of both synaptic and extrasynaptic recptors. Normally, serotonin is only released from serotonergic nerve terminals and would only activate adjacent receptors. An overflow serotonin beyond the

synaptic cleft may only occur in non-physiological or pathalogical conditions. Second, the use of 5-HT makes it difficult to identify the specific receptor responsible for any given effect. 5-HT by definition has affinity for all serotonin receptors and therefore could activate any of the receptors present on the synapse which is being studied.

One way to circumvent the problem of extrasynaptic receptor stimulation is the use of drugs that enhance synaptic serotonin specifically by only increasing endogenously released serotonin. This can be achieved through a variety of drugs including psychoto-mimetics, such as 3,4-methylenedioxy-methamphetamine (MDMA), or several antidepressants. MDMA works by increasing efflux of 5-HT from vesicles through an interaction with the vesicular transporter (Rudnick & Wall, 1992). The tricyclic antidepressant (TCA) imipramine competitively binds to the serotonin transporter to block uptake and increase serotonin in the cleft (Talvenheimo, Fishkes, Nelson & Rudnick, 1983). The selective serotonin reuptake inhibitor (SSRI) fluoxetine was developed specifically to block the uptake of radiolabeled 5-HT by transporters in rat synaptosomes. Fluoxetine is much more effective (IC_{50}= 0.6nM) at inhibiting serotonin uptake than imipramine (IC_{50}= 50mM). Fluoxetine also has the advantage of having no discernable effects on norepinephrine transport unlike imipramine (Wong et al., 1975).

These drugs are beneficial in increasing the amount of endogenous serotonin in the cleft, but lack the specificity to discern between 5-HT receptor subtypes. Over the last several decades many receptor specific ligands have been developed to better distinguish between the effects of serotonin on different receptors. At present, almost all serotonin receptors can be specifically targeted, with the exception of 5-HT_{1E}, 5-HT_{1F} and 5-HT_{5A}. 5-HT_1 receptors were among the first characterized and therefore have the most available

receptor-specific ligands. 5-HT_1 receptors can all be activated by the compound 5-CT. The effectiveness of 5-HT_1 specific drugs has been established through radiolabeled ligand binding and cAMP assays (Hoyer et al., 1992). Subtype specific agonists for 5-HT_1 receptor subtypes have also been established. 5-HT_{1A} receptors are the best characterized serotonin receptors, driven by the early discovery that these receptors can be specifically activated by the drug 8-OH-DPAT (Hjorth et al., 1982). A number of drugs have high affinity for 5-HT_{1B} receptors, but few are specific. Anpirtoline has a high affinity for these receptors (Ki= 28nM) while it does not bind strongly to 5-HT_{1A} receptors and 5-HT_2 receptors. Anpirtoline is also highly potent in rat brain tissue (EC_{50}= 55nM) as assayed by inhibition of forskolin-stimulated cAMP production (Schlicker et al., 1992). Specific agonists for the other 5-HT receptors have been developed and discovered using similar protocols, a combination of radiolabeled ligand binding and in vitro assays of cAMP production/inhibition. It is important to note that in some instances, drug affinity varies greatly depending on the species and type of tissue. For example the 5-HT_4 agonist has a 300-fold difference in affinity for receptors in the rat versus receptors in the rabbit (Kilpatrick & Tyers 1992). Nevertheless, the development of this multitude of drugs has aided greatly in the pharmacological manipulation of 5-HT receptors and has contributed significantly to the understanding of how they function in the brain.

Where is serotonin acting?

The main source of serotonin in the brain is the raphe nuclei. The raphe nuclei are located along the length of the brainstem and release serotonin broadly throughout the brain, including the frontal cortex, striatum, substantia nigra, nucleus accumbens and hippocampus. Among these pathways the projections from the raphe to the hippocampus

are of particular interest, considering that the hippocampus is a major site of adult plasticity in the brain. In addition this pathway has implications for mood disorders such as major depression (Campbell & Macqueen, 2004). The hippocampus mainly comprises glutamatergic pyramidal neurons and GABAergic interneurons; however these neurons contain receptors for multiple neurotransmitters, including serotonin. Among the most extensively studied cell groups in the hippocampus are the glutamatergic CA1 pyramidal neurons which receive both direct and indirect input from the entorhinal cortex. CA1 neurons are glutamatergic neurons that are modulated by different patterns of stimulation as well as activation of neuromodulatory receptors. These manipulations can change the strength of individual synapses as well as the output of the entire cell. While there are numerous studies investigating activity dependent plasticity in the hippocampus, relatively few have focused on the effect of neuromodulators on CA1 neurons. Increasing evidence suggests that serotonin may actually play a substantial role in the regulation of glutamate transmission in the hippocampus; a finding which has many implications in both normal and pathological behaviors.

What does serotonin do to glutamatergic transmission in the hippocampus?

One of the first studies investigating the effect of serotonin on glutamatergic CA1 pyramidal neurons was conducted by Andrade and Nicoll in 1987 They found that iontophoretically applying 5-HT onto pyramidal neurons resulted in a brief hyperpolarization, mediated by the 5-HT_{1A} receptor (Andrade & Nicoll, 1987). The magnitude of the hyperpolarization was graded along the length of the dendritic arbor, decreasing as the distance from the soma increased. On the other hand, in the presence of 5-HT_{1A} blockers, 5-HT application results in a slow excitation of the membrane and

decrease in the amplitude of the afterhyperpolarization. This effect is mediated through the 5-HT_4 receptor in the CA1 neuron (Andrade & Chaput, 1991). These findings provide evidence that serotonin can modulate glutamate neuron excitability in a bidirectional manner. Although these studies have provided important information regarding changes in intrinsic excitability of the CA1 neuron following 5-HT application, and have shaped our understanding of the roles of serotonin in the hippocampus, they may not be consistent with endogenous serotonin release in that the application method may stimulate synaptic as well as extrasynaptic receptors. Additionally, these studies do not address the effect of serotonin on synaptic glutamatergic transmission.

In addition to affecting intrinsic excitability, 5-HT has been shown to affect postsynaptic glutamatergic receptors in the hippocampus (Svenningsson et al., 2002). Glutamate receptors include ionotropic receptors, such as α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, N-Methyl-D-aspartic acid (NMDA) receptors, and metabotropic receptors. Activation and modification of these receptors are crucial for basal functioning as well as plasticity of the hippocampus. It was therefore particularly intriguing when an acute 10 minute application of 5-HT onto hippocampal slices was shown to increase phosphorylation of serine831 and serine845 on the GluA1 subunit of the AMPA receptor (Svenningsson et al., 2002). Phosphorylation of these residues is associated with an increase in function of the AMPA receptor, through increased conduction and surface expression (Man, Sekine-Aizawa, & Huganir, 2007), respectively. This finding suggests that serotonin may not only modulate transmission directly, but may also have a profound indirect impact on synaptic plasticity in the hippocampus.

To study the effect of endogenous serotonin on CA1 cells, Mlinar and Corradetti (2003) applied MDMA to hippocampal slices and evoked EPSPs by stimulating CA3 axons that synapse onto proximal CA1 dendrites. These Schaffer collateral (SC)-CA1 polysynaptic EPSPs were decreased when MDMA was applied, suggesting that serotonin can decrease excitatory synaptic transmission at this synapse. This effect was mediated by 5-HT_{1B}Rs located on axon terminals of CA1 neurons which form a local feedback loop onto neighboring CA1 pyramidal neurons (Mlinar & Corradetti, 2003). This study provided evidence that endogenous 5-HT can decrease release of glutamate in this region of the hippocampus. This regulatory mechanism may be important for preventing excitotoxicity in circumstances when glutamate is raised to harmful levels.

In addition to altering glutamate release, endogenous serotonin can affect glutamatergic transmission via postsynaptic receptors. Chronic increase of serotonin in the hippocampus via *in vivo* administration of the SSRI fluoxetine results in increased phosphorylation of S831 and S845, similar to acute bath application of serotonin in hippocampal slices (Svenningsson et al., 2002). Additionally chronic *in vivo* administration of other SSRI drugs such as paroxetine and desipramine increases the number of membrane bound AMPARs in the hippocampus (Martinez-Turrillas, Frechilla, & Del Rio, 2002). Unfortunately, both of these studies failed to identify which part of the hippocampus exhibits this form of plasticity, making it difficult to assess whether these modifications result in an actual change in synaptic AMPAR current. Nonetheless, both of these studies suggest that long-term enhancement of synaptic serotonin can cause profound changes in the population of glutamatergic receptors in the hippocampus.

Where does serotonin modify glutamate transmission in the hippocampus?

Within the hippocampus, serotonergic fibers are widely dispersed; however some regions show denser innervation than others. The stratum lacunosum moleculare (SLM) layer receives the strongest input of serotonergic fibers in the hippocampus (Vertes, Fortin, & Crane, 1999; Figure 3.1). Staining of this region reveals the presence of both fine fibers and beaded fibers, full of large synaptic boutons (Bjarkam et al., 2003). The SLM layer is the region in which layer III axons of the entorhinal cortex synapse directly onto the distal dendrites of CA1 pyramidal neurons.

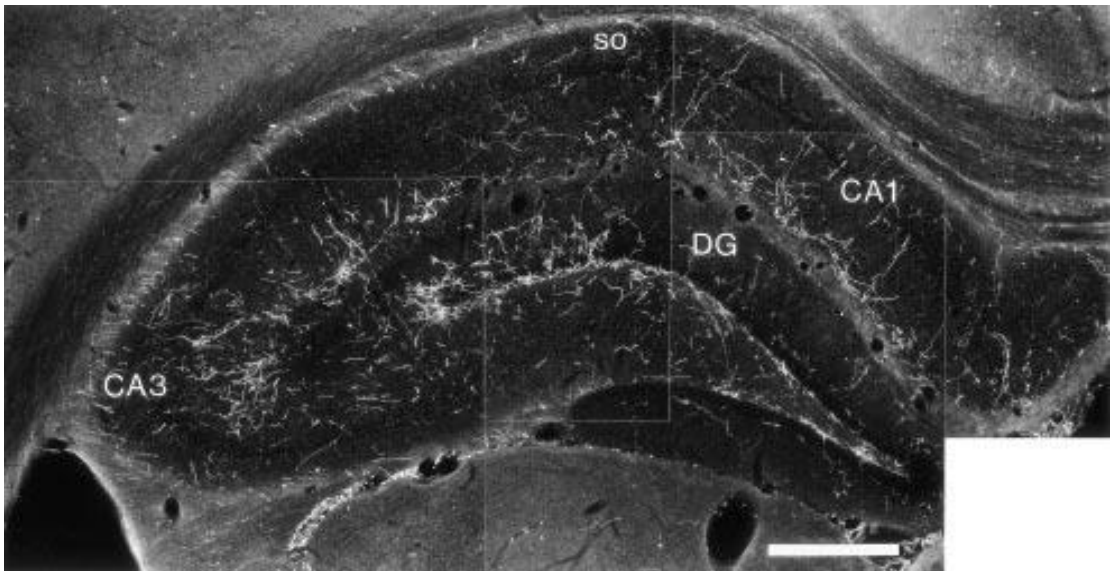


Table 3.2 Darkfield photomicrograph of 5-HT fiber staining of raphe neuron terminals in the hippocampus. Rats were injected with *Phaseolus vulgaris*-leucogglutinin (PHA-L), a retrograde tracer, into the median raphe nucleus. Dense staining is observed in the SLM layer as well as the dentate gyrus. (from Vertes, Fortin & Crane, 1999).

Despite the fact that the SLM layer is the area of the hippocampus with densest serotonin fiber innervation, studies have either examined the effect of serotonin on the hippocampus as a whole, or focused on the proximal dendrites of CA1 (SR layer) or the dentate gyrus region. Currently it is unknown whether serotonin is capable of modulating temporoammonic (TA)-CA1 synapses in the SLM and if so, through which receptor was involved. It is important, therefore, to identify serotonin receptors in CA1 pyramidal neurons that are present in this region and would be receiving the bulk of serotonergic input from the raphe in physiological circumstances.

Several 5-HT receptors have mRNA expressed in CA1 neuron cell bodies. Among them are the 5-HT_{1A-C}, 5-HT_{2C}, 5-HT_3, 5-HT_4, 5-HT_{5A-B}, 5-HT_6 and 5-HT_7 receptors (for review see Barnes & Sharp, 1999). This list can be narrowed down to receptors that exhibit binding of labeled agonists in the SLM region, to 5-HT_{1A}Rs (Chalmers & Watson, 1991), 5-HT_{1B}Rs (Aït Amara et al., 1995; Sari, 1997), 5-HT_4Rs (Compan et al., 1996), and 5-HT_7Rs (Neumaier et al., 2001). None of these studies distinguished between the postsynaptic versus presynaptic localization of the receptors, therefore receptor populations could be present on 5-HT nerve terminals, postsynaptic CA1 dendrites or interneurons in the region. A study from the Aperial lab presented at the 2010 Society for Neuroscience meeting showed the presence of 5-HT_{1B} receptors on dendrites of CA1 neurons (Liebmann et al., 2010). The presence of 5-HT_{1B}Rs at the SLM layer and the localization of these receptors at dendrites make them good candidates for further investigation. Additionally, activation 5-HT_{1B}Rs expressed in heterologous cells results in increased intracellular calcium (Giles et al., 1996) as well as phosphorylation of extracellular signal-regulated kinase (ERK) (Mendez et al., 2002), two processes known

to be important for the regulation of postsynaptic glutamate receptors (Hall & Ghosh, 2008). Unfortunately, little is known about the action of endogenous 5-HT_{1B}Rs in the hippocampus.

What actions do 5-HT_{1B} receptors exert in the hippocampus?

5-HT_{1B} receptors in the rat (which are genetically homologous to 5-$HT_{1D\beta}$ receptors in the human) are found widely throughout the brain. In the hippocampus, *in situ* hybridization experiments show the presence of 5-HT_{1B} mRNA in CA1 pyramidal cells (Bonaventure et al., 1997; Svenningsson et al., 2006). It appears that although many of the 5-HT_{1B}Rs produced in CA1 cell bodies are trafficked to the axon terminals in the dorsal subiculum, there are 5-HT_{1B}Rs localized in dendrites as well (Liebmann et al., 2010). The presynaptic 5-HT_{1B}Rs in the hippocampus can regulate serotonin (Daws et al., 2000) and glutamate (Mlinar, Falsini, & Corradetti, 2003) release as well as the release of other neurotransmitters. The function of postsynaptic 5-HT_{1B}Rs is unknown, although there is evidence for co-localization of 5-HT_{1B}Rs with AMPAR subunits in hippocampal dendritic spines (Peddie, Davies, Colyer, Stewart, & Rodríguez, 2010) (Figure 3.2). The proximity of these two receptors makes it likely that they are capable of interacting with each other. The rationale for how this could happen remains speculative. Generally, it is believed that 5-HT_{1B}Rs are negatively coupled to adenyl cyclase (Murphy & Bylund, 1989), which would lead to a decrease in PKA, the kinase that phosphorylates the S845 residue of GluA1. Additionally, there is some evidence that 5-HT_{1B}Rs are positively coupled to ERK2 (Mendez et al., 2002), a kinase known to promote exocytosis of AMPARs (Patterson, Szatmari, & Yasuda, 2010). Together, these studies suggest that 5-HT_{1B}Rs activation may lead to a cascade that can affect AMPAR function in the SLM.

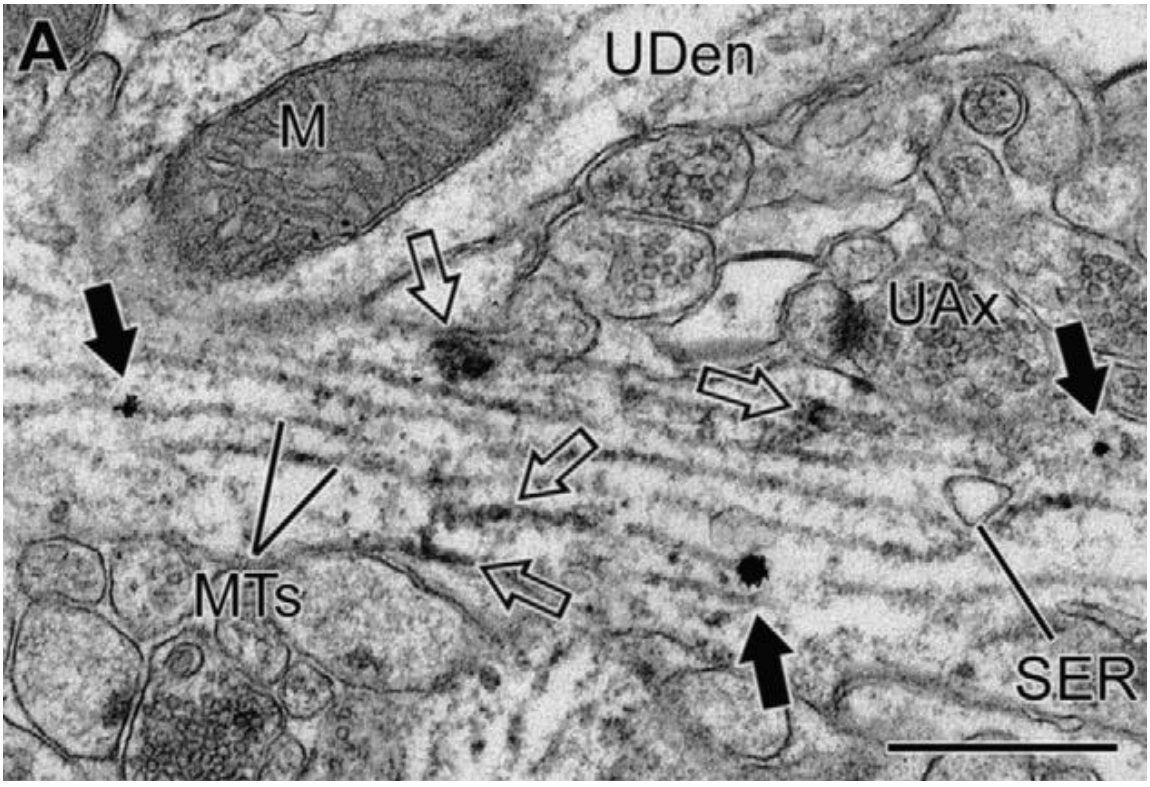


Figure 3.2 Immunolabeling of endogenous 5-HT_{1B}Rs. An electron micrograph of the dentate gyrus region reveals 5-HT_{1B}R immunoreactivity in dendrites (open arrows).Black arrows indicate GluA2 staining. M= mitochondrion, MT= microtubules, UDen= unlabeled dendrite, SER= smooth endoplasmic reticulum. Scale= 500nm. (from Peddie et al., 2010).

5-HT_{1B}Rs also play a behaviorally relevant role in the hippocampus. Performance on the Morris water maze task, a hippocampal dependent test of spatial learning and memory, is enhanced by a 5-HT_{1B}R agonist and decreased by an antagonist (Ahlander-Lüttgen, Madjid, Schött, Sandin, & Ogren, 2003). In contrast, 5-HT_{1B}KO mice exhibit *enhanced* performance on the water maze task (Malleret et al., 1999). Additionally, rats trained in the radial arm maze task, a different test of hippocampal dependent spatial memory, exhibited a deficit when treated with a 5-HT_{1B}R agonist (Buhot, Patra & Naili, 1995). One reason for the conflicting data regarding 5-HT_{1B}Rs in the hippocampus may be preferential activation of presynaptic or postsynaptic receptors depending on the task and drug used. Despite the discrepancies, it is evident that 5-HT_{1B}Rs are present in the hippocampus and play an important, albeit not yet clear, role in physiology and behavior.

Hypothesis and predictions:

I designed my experiments to test the predictions of my main hypothesis that endogenous synaptic serotonin can potentiate TA-CA1 synapses by activating 5-HT_{1B} receptors. Specifically, I predicted first, that elevation of endogenous serotonin in the extracellular space by acute application of antidepressants would potentiate TA-CA1 field excitatory postsynaptic potentials (fEPSPs) in slices from control rats. Second, I predicted that potentiation of TA-CA1 fEPSPs by acute antidepressant application would be blocked by a 5-HT_{1B}R antagonist (isomoltane) and mimicked by a 5-HT_{1B}R agonist (anpirtoline).

METHODS

Acute slice electrophysiology. Acute hippocampal slices were prepared from 4-6 week old male rats. Dissection was done in ACSF (in mM: 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1.5 $MgCl_2$, 2.5 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose). Brain slices (400µm) were cut on a vibratome and kept in a holding chamber at room temperature at the interface of physiological medium and humidified 95%O_2/5%CO_2 for >1 hr. The slices were then transferred to a submersion-type recording chamber and perfused at room temperature with ACSF (flow rate= 1ml/min). Picrotoxin (100µM) and CGP52432 (2µM) were included to block $GABA_A$ and $GABA_B$ receptors. Concentric bipolar tungsten electrodes were used for stimulation placed either in SLM to stimulate TA afferents or SR to stimulate SC afferents. Recording pipettes were filled with ACSF (3-5MΩ) and placed >500µm from the stimulating electrodes. Stimuli (100µs in duration) were delivered at 0.05 Hz. The stimulus intensity was set at 150% of threshold intensity, resulting in a fEPSP of 0.1-0.2mV. All compounds were applied by perfusion. Field EPSPs were recorded using an n.p.i. amplifier, filtered at 10kHz, and amplified 1000x prior to digitization.

Animals. Male Sprague-Dawley rats, and 5-HT_{1B}KO mice aged 4-6 weeks were used in these experiments. All animals were group housed with a 12:12 dark light schedule and food and water available *ad libitum*. Male and female homozygous 5-HT_{1B}KO mice were obtained from Rene Hen's laboratory (Columbia University) and bred to obtain male homozygous mice on a 129/sv-ter background.

RESULTS

Prediction 1: Endogenous serotonin potentiates TA-CA1 glutamatergic transmission.

In order to test this prediction, I measured TA-CA1 fEPSPs in acute hippocampal slices from 4-6 week old rats. I recorded baseline fEPSPs in control ACSF by stimulating the temporoammonic fibers coming from entorhinal cortex and recording in layer SLM about 500µm away. I recorded baseline for 30 minutes before adding an antidepressant drug (fluoxetine, citalopram, or imipramine) to the bath. Drugs were applied for approximately 60 minutes, at which point the response reached a plateau amplitude, followed by 60 minutes of wash out with control ACSF. In recording baseline responses, I adjusted the stimulation intensity to 150% of the threshold for the response and waited for a 30 minutes of continuously steady fEPSP slope before applying drug. Both fluoxetine (Figure 3.3B) citalopram (Figure 3.3A) increased the fEPSP slope. fEPSPs remained potentiated following wash out of citalopram (Figure 3.3A). Interestingly, these effects were specific to the TA-CA1 synapse, as fluoxetine did not potentiate fEPSPs from neighboring SC-CA1 synapses (Figure 3.3C) in a separate set of slices. I next designed experiments to identify the receptor responsible for the serotonin-induced potentiation I observed.

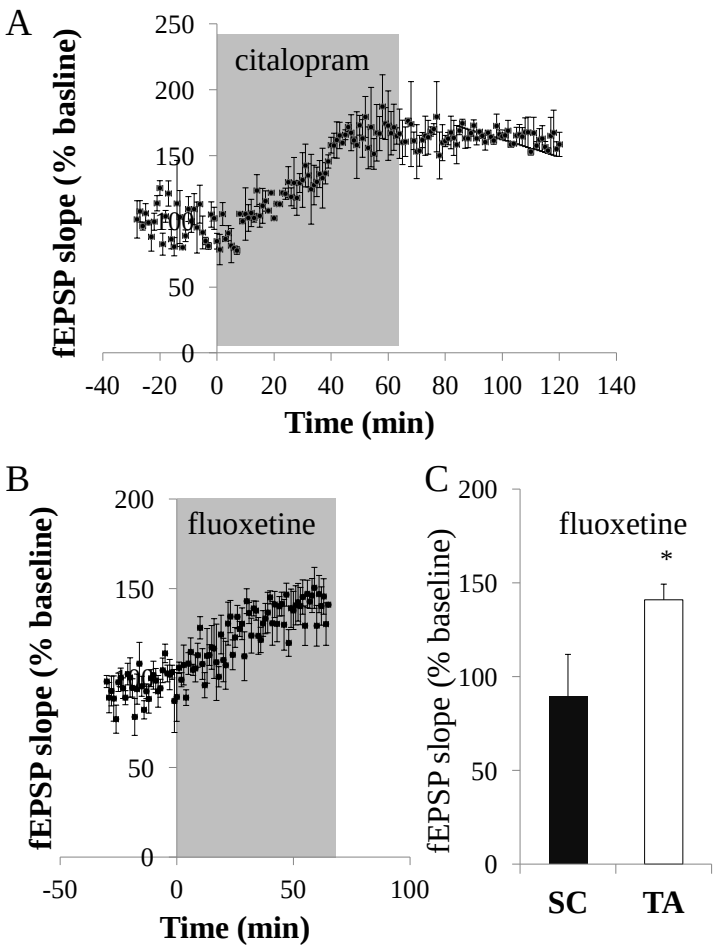


Figure 3.3 Acute application of SSRI drugs enhances the fEPSP slope at TA-CA1 synapses. A) The SSRI citalopram (10µM) increased TA-CA1 fEPSP slope by 68 % ± 18 after a 60min bath application and this effect did not wash out (64% ± 11) (n= 2 slices). B) The SSRI fluoxetine (20µM) also enhanced TA-CA1 fEPSP slope (40% ± 8) (n= 8 slices) after a 60min bath application. C) Fluoxetine (20µM) enhanced the slope of TA-CA1 fEPSPs (white bar) but not fEPSPs recorded from nearby SC-CA1 synapses (89% ± 22) (n=4 slices) (black bar). The difference in amount of potentiation between the two synapses was significant, $t(9)= -2.7$, $p= 0.02$. * indicates significantly different from each other ($p<0.05$).

Prediction 2: Serotonin induced potentiation of TA-CA1 fEPSPs is mediated by 5-HT$_{1B}$Rs

Although there are a number of receptors that may be responsible for serotonin mediated potentiation at TA-CA1 synapses, I initially focused on 5-HT$_{1B}$Rs for several reasons: mRNA for these receptors is present in CA1 neurons (Svenningsson et al., 2006) and receptor binding sites have been identified in the SLM layer (Aït Amara et al., 1995). Functionally, 5-HT$_{1B}$Rs are appealing in that they stimulate both increases in intracellular calcium (Giles et al., 1996), and phosphorylation of ERK (Mendez et al., 2002), two processes that are intimately tied to synaptic plasticity (Lisman, Schulman, & Cline, 2002; English & Sweatt, 1997).

To test the prediction that 5-HT$_{1B}$Rs are responsible for the endogenous serotonin-induced potentiation, I attempted to block the effect of antidepressant induced potentiation at TA-CA1 synapses with the 5-HT$_{1B}$R antagonist isomoltane. Following cutting, slices were incubated in normal ACSF or ACSF containing 10µM isomoltane. For control slices, I recorded fEPSPs in control saline for 30 minutes then added 2µM imipramine to the bath for 60 minutes, followed by 90 minutes of washout. Control slices exhibited an increase in fEPSP slope compared to baseline. I then used sliced pre-incubated in isomoltane and recorded baseline fEPSPs in the presence of isomoltane and after a steady fEPSP slope for 30 minutes, applied 2µM imipramine. There was no change in fEPSP slope compared to baseline (Figure 3.4A). As another test, I employed 5-HT$_{1B}$R KO mice. I found that hippocampal slices taken from 5-HT$_{1B}$R KO mice did not exhibit fluoxetine-induced potentiation (Figure 3.4B). To test whether 5-HT$_{1B}$R activation itself could mimic the effects of endogenous serotonin, I applied a 5-HT$_{1B}$R agonist to wild-type mouse hippocampal slices. I found that 50µM anpirtoline increased

the TA-CA fEPSP slope and that this effect was blocked by pre-incubation with 10μM isomoltane (Figure 3.4A).

I also tested whether 5-HT_{1B}R induced potentiation would be occluded if potentiation was first induced by endogenous serotonin. For these experiments I pre-incubated hippocampal slices in 20μM fluoxetine or 2μM imipramine before recording baseline in the presence of the respective drugs, followed by a 60min anpirtoline application. I found that none of the slices showed any anpirtoline induced potentiation in the presence of antidepressant drugs (Figure 3.4D). Although I did not test other agonists of other 5-HT receptors present in the SLM to examine whether other drugs could enhance TA-CA1 fEPSPs, previous work from our lab ruled out a role for 5-HT_{1A}Rs. Slices pre-incubated with the 5-HT_{1A}R antagonist NAN-190 showed comparable potentiation to control slices when treated with 2μM imipramine. Taken together, my results constitute strong evidence that activation of 5-HT_{1B}Rs is both necessary and sufficient to mediate serotonin-induced potentiation of TA-CA1 excitatory synapses.

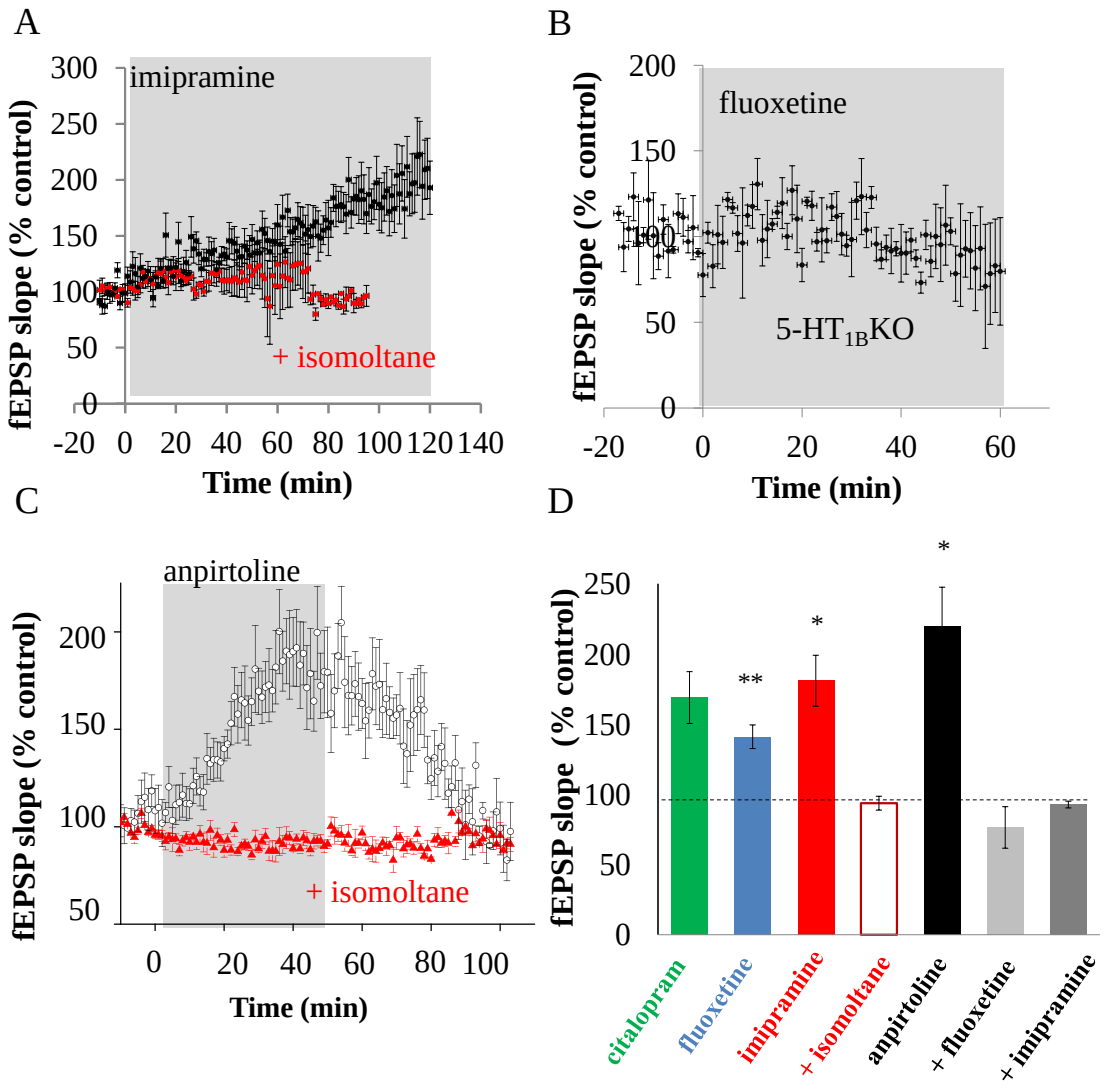


Figure 3.4 Serotonin induced potentiation at TA-CA1 synapses is mediated by 5-HT$_{1B}$Rs. A) Application of 2µM imipramine (black), resulted in an enhancement of TA-CA1 fEPSP slope (80% ± 18 of control slope) (n= 6 slices). Pre-incubation of slices with 10µM isomoltane, a 5-HT$_{1B}$R antagonist, blocked imipramine-induced potentiation (93% ± 4 of control), (red). B) Slices from 5-HT$_{1B}$KO mice did not exhibit fluoxetine induced potentiation of TA-CA1 fEPSPs (83% ± 28) (n= 3 slices). C) Bath application of 50µM anpirtoline, a 5-HT$_{1B}$R agonist, resulted in potentiation of TA-CA1 fEPSP slope (n=11

slices) (black), which was blocked when slices were pre-incubated with 10μM isomoltane (n= 5 slices) (red). D) Summary data of serotonin drug experiments. All bars are mean difference in fEPSP slope during last 10 min of drug application compared to baseline. Paired t-tests reveal that fluoxetine (blue), imipramine (solid red) and anpirtoline (solid black) all increased fEPSP slope compared to baseline. Imipramine did not increase fEPSP slope when the slice was preincubated with isomoltane (open red). Anpirtoline did not increase fEPSP slope when the slice was preincubated with 10μM fluoxetine (light gray) or 2μM imipramine (dark gray). * indicates significantly higher than baseline, $p< 0.05$, $**p<0.01$.

DISCUSSION

In these experiments I tested the prediction of my hypothesis that endogenous serotonin could potentiate glutamatergic transmission at TA-CA1 synapses. To do so, I examined the role of serotonin in glutamate mediated field EPSPs recorded at the distal dendrites of CA1 neurons in the hippocampus. This region, the SLM layer, is the region of densest innervation of serotonergic raphe neurons, yet little work has been done characterizing the effect of serotonin on these synapses. I found that acute enhancement of endogenous synaptic serotonin via bath application of antidepressant drugs can potentiate glutamatergic responses at TA-CA1 synapses and that this potentiation is mediated by 5-HT_{1B}Rs.

Serotonin enhances glutamatergic transmission at TA-CA1 synapses.

Previous work has investigated the role of serotonin in the hippocampus by iontophoretically applying serotonin or directly activating receptors using agonists. These studies provided some of the seminal accounts of the role of serotonin in the hippocampus, but are somewhat inconclusive since these methods resulted in activation of both synaptic and extrasynaptic receptors which may not be physiologically relevant. A few studies have used drugs that enhance release of serotonin, yet none have focused on the SLM layer, where serotonergic nerve terminals are predominantly located. My results show that enhancing endogenous serotonin by using antidepressant drugs that block reuptake, results in a potentiation of TA-CA1 fEPSPs. An important prediction of my model is that the potentiation should only occur at glutamatergic synapses that are proximate to sertonergic inputs. Indeed, I observed the serotonergic potentiation only at TA-CA1 synapses and not at nearby SC-CA1 synapses. This result indicates that serotonin is probably acting at a receptor that is present in the SLM but not in the SR layer, where SC-CA1 synapses are located. Also, it appears that the serotonin effect is restricted to the presynaptic or immediate postsynaptic dendritic region, as a global change in overall cell excitability would have potentiated SC-CA1 synapses as well. All three antidepressant drugs used in these experiments potentiated the glutamate response despite their varying affinities and specificity for the serotonin transporter. Citalopram is the most specific for serotonin transport inhibition with fluoxetine and imipramine having affinity for norepinephrine transporters as well (Sanchez & Hytell, 1997; Wong et al., 1975). These varying affinities do not appear to be related to the amplitude of

glutamatergic potentiation however though. The following experiments indicate that the mechanism of action is indeed through a serotonin mediated pathway.

Serotonin acts on 5-HT_{1B}Rs to enhance glutamatergic transmission at TA-CA1 synapses.

Prior work had suggested the 5-HT_{1B}Rs would be good candidates for mediating SLM-localized 5-HT effects, and accordingly I tested the role of this receptor subtype in serotonin-induced potentiation at TA-CA1 synapses. I found that pre-incubation of slices with a 5-HT_{1B}R antagonist blocked the effect of enhanced synaptic serotonin on glutamatergic transmission. Additionally, slices from 5-HT_{1B}R-KO mice did not exhibit serotonin-induced potentiation at these synapses. These data show that 5-HT_{1B}R activation is necessary to enhance glutamatergic transmission in the SLM layer. I next found that 5-HT_{1B}R activation was sufficient to enhance TA-CA1 fEPSPs, since a 5-HT_{1B}R agonist was also capable of potentiating the synapses. Serotonin induced potentiation and 5-HT_{1B}R induced potentiation also appear to occur through the same pathway in that the effect of the agonist was occluded when slices were pre-incubated with antidepressant drugs.

These results give insight into how serotonin, and specifically 5-HT_{1B}Rs, may regulate synaptic transmission in the hippocampus. It is important to understand how serotonin regulates activity at the SLM in particular because these synapses receive the bulk of raphe input into this brain region. Understanding the physiological effects of serotonin in the hippocampus can also shed light onto the normal behavioral effects of serotonin and disease states in which serotonergic transmission may be altered. Additionally, it is important to further understand the mechanism through with 5-HT_{1B}Rs

affect glutamatergic transmission in order to identify downstream signaling molecules that may also play a role in these behaviors. Finally, these results provide strong and novel support for a direct prediction of my hypothetical model that the 5-HT and glutamate systems interact in the hippocampus, and by implication, that dysfunctional regulation of this interaction may be important for understanding behavioral depression.

CHAPTER FOUR

MECHANISM OF 5-HT_{1B}R MEDIATED POTENTIATION AND INTERACTION WITH LONG-TERM POTENTIATION

INTRODUCTION

The ability of neurons to strengthen or weaken their synapses is fundamentally important for a fully functioning brain. The process by which the efficacy of synaptic transmission is altered is referred to as synaptic plasticity. Synaptic plasticity has been most extensively studied in the hippocampus, but is a highly conserved process that has been observed in everything from worm neuromuscular junctions to primate cortex. The propensity for a cell to undergo one form of plasticity versus another, or metaplasticity, can be controlled by a number of neuromodulators. The dynamic ability of neurons to alter synaptic strength is important for cell survival and the homeostatic balance of neurons and is implicated in virtually all forms of learning and memory.

The most extensively studied form of plasticity in the hippocampus is long-term potentiation (LTP). LTP results in a strengthening of the synapse and is characterized by a long lasting increase in synaptic transmission following robust stimulation. Conversely, synapses can by weakened by long-term depression (LTD). Both forms of plasticity are synapse specific and Hebbian in that they require concurrent pre- and post-synaptic activation. LTP was first observed by Bliss and Lomo, who found a 43% increase in glutamatergic transmission at the rabbit dentate gyrus following 15Hz stimulation (Bliss & Lomo, 1973). Amazingly, this potentiation lasted for six hours. A simultaneously recorded control pathway showed no potentiation. This was the first study that described that a neuron can undergo long-term, activity-dependent potentiation that only affects specific synapses.

There have been many studies since the work of Bliss and Lomo that have used a variety of stimulation paradigms in a number of different brain regions to further understand LTP. Schaffer collateral (SC) -CA1 LTP can be elicited by a number of induction protocols. SC-CA1 synapses are present in the stratum radiatum layer of the hippocampus and are the most studied synapses in the LTP field. Among the stimulation paradigms used to elicit LTP at this synapse are high frequency stimulation (HFS), which consists of 1-4 bursts of $\geq$100Hz stimuli delivered relatively close to each other ($\leq$ 5min) or theta-burst stimulation (TBS) which consists of shorter (10 pulses) but more frequent bursts (~ 10Hz) that are intended to simulate naturally occurring, physiological bursting (Larson & Lynch, 1986). Others use a pairing protocol, which pairs postsynaptic depolarization with presynaptic action potential firing at a low (~ 1 Hz) frequency (Gustafsson et al., 1987). These stimulation paradigms result in a robust release of glutamate into the synapse which then binds to postsynaptic glutamatergic receptors, including AMPA receptors and NMDA receptors. The binding of glutamate to AMPARs results in a depolarization of the cell and subsequent flow of current through the NMDAR gated ion channel, due to relief of voltage-dependent block by Mg^{2+}. This activation of NMDARs causes an influx of calcium into the cell which can stimulate second messenger pathways that result in the transcription, translation and phosphorylation of other proteins. These events can cause long lasting changes in receptor population at the synapse and allow LTP to last for months *in vivo* (Abraham et al., 2002).

How does AMPAR function contribute to LTP?

LTP has been studied at the circuit, cell, and molecule levels. The identification of molecules that are required for the induction and maintenance of LTP has contributed to

our understanding of how LTP functions within the cell, and how that cell can affect the entire circuit in which it is embedded. There have been hundreds of molecules that have been identified as being necessary for the induction and/or maintenance of LTP; however it is the modulation of AMPARs that is most important in determining synaptic strength at excitatory synapses (Derkach et al., 2007).

AMPA receptors are tetrameric, ligand-gated ion channels that conduct Na^+, K^+ and sometimes Ca^{2+}. AMPARs are composed of four subunits, GluA1-4. GluA1 and GluA2 containing receptors are trafficked in an activity-dependent manner, whereas GluA2 and GluA3 containing receptors constitutively traffic within the synapse (Hayashi et al., 2000; Shi et al., 2001). Much of the research on LTP has focused on regulation of the GluA1 subunit. There is strong evidence that LTP is dependent on increased conductance and increased trafficking of GluA1 containing AMPARs. Activation of NMDARs and subsequent insertion of AMPARs is sufficient to induce LTP in cultured hippocampal neurons (Lu et al., 2001). Additionally, LTP-inducing stimuli enhance the unitary conductance of the AMPAR (Benke et al., 1998). Conductance and trafficking of AMPARs are controlled by phosphorylation sites on the intracellular domain of the subunit. Two of the most highly implicated sites are serine 831 and serine 845. Serine 831 is phosphorylated by Ca^{2+}/calmodulin dependent kinase (CamKII) (Barria, Derkach, & Soderling, 1997) and protein kinase C (PKC) (Roche et al., 1996) whereas serine 845 is phosphorylated by protein kinase A (PKA) (Roche et al., 1996). Induction of LTP at SC-CA1 synapses results in increased phosphorylation of CamKII and S831 (Barria et al., 1997) whereas induction of LTD results in a dephosphorylation of S845 (Roche et al., 1996). It appears that both of these phosphorylation sites have a distinct and unique role

in the induction of LTP. Phosphorylation of S831 results in an increase in the unitary conductance of the AMPAR (Derkach, Barria, & Soderling, 1999) whereas phosphorylation of S845 results in increased synaptic incorporation of GluA1 containing receptors (Esteban et al., 2003).

Enhancement of AMPAR function through phosphorylation is crucial for plasticity. Double phospho-mutants of these sites have greatly reduced TBS induced SC-CA1 LTP suggesting that phosphorylation of both S831 and S845 are necessary for proper synaptic plasticity (Lee et al., 2003). Additionally, molecules known to affect AMPAR function are essential for normal LTP. Among these molecules are CamKII and extracellular regulated kinase (ERK). Blocking CamKII with KN-62 blocks LTP at SC-CA1 synapses (Barria et al., 1997), presumably because this blocks phosphorylation of S831 and subsequent increase in AMPAR conductance. Additionally, blocking ERK, a member of the mitogen activated protein kinase (MAPK) pathway, also blocks SC-CA1 LTP (English & Sweatt, 1997). Blocking this pathway with the drug U1026, blocks the exocytosis of GluA1 containing AMPA receptors following an LTP stimulus (Patterson et al., 2010), suggesting that ERK is required for synaptic insertion of AMPARs. Both of these kinases can signal a number of different events and deciphering their role in synaptic plasticity has been important to the understanding of both normal and abnormal brain function.

What is the behavioral relevance of LTP?

Depending on the circuit involved and the magnitude of the potentiation, LTP can exert a number of effects on behavior. Most behaviors regulated by LTP involve learning

and memory. Many of the studies investigating behavioral and LTP have employed the Morris water maze task. This task involves placing an animal in a large pool filled with opaque water and a slightly submerged escape platform. Through successive training trials, the animal learns its way to the escape platform using visual cues on the wall for navigation. Animals with a conditional knockout of NMDARs in CA1 cells exhibit a lack of SC-CA1 LTP and exhibit slower learning curves during the training period of the water maze task (Tsien, Huerta, & Tonegawa, 1996). Additionally, transgenic mice in which GluA1Rs cannot be phosphorylated at serines 831 and 845 exhibit both decreased LTP and a complete lack of LTD, as well as impairment in the retention of memory in the water maze task (Lee et al., 2003). LTP and LTD in other brain regions have been implicated in various other behaviors, such as monocular deprivation in the visual cortex (Sawtell et al., 2003) and fear conditioning in the amygdala (Clugnet & LeDoux, 1990) and cerebellum (Sacchetti, Scelfo, Tempia, & Strata, 2004). Understanding how LTP can be regulated is crucial to understanding the normal and abnormal expression of these behaviors.

How can other neurotransmitters modulate LTP?

In addition to affecting basal transmission, many neurotransmitters can alter the efficacy of LTP induction and/or expression. Identifying these modulators is important for understanding how LTP works in an intact physiological system and how perturbations of this system may affect behavior. In the hippocampus, depletion of norepinephrine decreases the efficacy of LTP at the perforant path-dentate gyrus synapses but not at Schaffer collateral- CA1 synapses, though neither of these pathways is affected by serotonin depletion (Stanton & Sarvey, 1985). On the other hand, a two

week *in vivo* depletion of both serotonin and acetylcholine resulted in a defect in SC-LTP and spatial learning deficits in the Morris water maze task (Matsukawa et al., 1997). Additionally, in the cortex, neuromodulators such as acetylcholine and norepinephrine can gate the strength and polarity of LTP (Seol et al., 2007). Although these studies do not provide a clear picture of how each of these neuromodulators may affect LTP, it is evident that neuromodulators such as serotonin, norepinephrine and acetylcholine are important in regulating synaptic plasticity. Identifying which neuromodulators are important for controlling plasticity at different synapses is important for both normal and abnormal behavioral function.

Do temporoammonic synapses undergo LTP?

Although it has not been nearly as extensively studied, there is evidence for LTP at temporoammonic (TA)-CA1 synapses. This synapse appears to regulate memory consolidation without affecting learning (Remondes & Schuman, 2004). Specifically, animals in which the temporoammonic pathway has been lesioned are capable of learning the location of the platform in the Morris water maze task, however they are unable to recall this information 28 days later, whereas sham-lesioned control animals are. LTP at both TA- and SC-CA1 synapses is calcium dependent and relies on protein synthesis for maintenance of the late phase (Remondes & Schuman, 2003). Interestingly, while LTP at the distal dendrites of CA1 neurons shares some common mechanisms with LTP at CA1 proximal dendrites, there appear to be some key differences. First, it appears that TA-CA1 LTP is not completely NMDAR dependent. As opposed to SC-CA1 LTP, TA-CA1 LTP is not blocked by an NMDAR antagonist (APV) (Remondes & Schuman, 2003). Rather, blockage of TA-CA1 LTP requires blocking both NMDARs and voltage-gated

calcium channels (VGCCs) (Golding, Staff, & Spruston, 2002). Additionally, TA-CA1 LTP is blocked by a $GABA_B$ antagonist but not by the combination of a $GABA_A$ and $GABA_B$ antagonist (Remondes & Schuman, 2003). This suggests that TA-CA1 synapses are tonically inhibited by GABA inputs but that a tetanizing stimulus can result in disinhibition and allow for potentiation.

While these differences appear surprising at first, considering that both SC and TA afferents synapse onto the same cells, it is conceivable that the separate sets of synapses would rely on different mechanisms for potentiation. One reason may be the large distance the TA-CA1 EPSP must travel to reach the cell body, because the synapses are at the distal dendrites and not the proximal dendrites of CA1 neurons. Additionally, there are different populations of receptors present on SC versus TA synapses, including different AMPAR/NMDAR ratios (Otmakhova, Otmakhov, & Lisman, 2002) and different densities of hyperpolarization-activated cyclic nucelotide (HCN1) channels (Nolan et al., 2004). This may differentially modulate synaptic transmission and plasticity at these sites. Additionally the neighboring inputs from other types of cells may modulate LTP differentially. For example, serotonergic innervation of the hippocampus is much more dense in the SLM layer at TA synapses than the SR layer (Bjarkam et al., 2003). The suggestion the local serotonergic innervation provides a distinctive modulatory influence on the TA synapses is supported from my data in Chapter three, which showed that serotonin is capable of potentiating glutamatergic responses at the TA- but not SC-CA1 synapse, a phenomenon mediated by $5\text{-}HT_{1B}$Rs (Figure 3.4). However, these results alone did not identify the mechanism of this potentiation nor how it interacts with electrically induced LTP.

5-HT$_{1B}$R activation results in LTP-like enhancement of AMPAR function.

Following the discovery that 5-HT$_{1B}$R activation can increase the strength of TA-CA1 synapses my colleague, Xiang Cai and I further explored the mechanism through which this occurs. Mainly, he sought to differentiate between a presynaptic mechanism (increased glutamatergic release) or postsynaptic mechanism (enhanced glutamate receptor function). His first prediction was that 5-HT$_{1B}$R activation was modulating the release of glutamate, since previous studies have shown that 5-HT$_{1B}$Rs are located mainly on presynaptic terminals (Riad et al., 2000) and are typically responsible for regulating transmitter release. Surprisingly, we found no evidence that this was the case at TA-CA1 synapse.

First, we measured paired-pulse ratio (PPR) of TA-CA1 EPSPs in slices before and after anpirtoline application. PPR is a protocol in which two stimuli are delivered in close succession to each other; the results are analyzed by comparing the second EPSP to the first EPSP. PPR is generally inversely correlated to the probability of neurotransmitter release at a given synapse. If 5-HT$_{1B}$R activation was stimulating an increase in glutamate release probability, we would predict that the potentiation would be accompanied by a decrease in PPR. We found no change in PPR after anpirtoline application, suggesting that 5-HT$_{1B}$Rs are not affecting the probability of glutamate release (Figure 4.1A). Additionally, we measured the amplitude of the fiber volley of the response before and after anpirtoline treatment. The fiber volley of the fEPSP is an extracellular measure of action current flow in axons and its magnitude increases as the number of stimulated axons increases. The fiber volley can also provide a measure of presynaptic neurotransmission efficacy by indicating whether a change in the response is

associated with a change in the number of presynaptic axons, and presumably therefore, of presynaptic terminals that are releasing glutamate. We found no change in fiber volley amplitude following anpirtoline application (Figure 4.1A), providing further support that 5-HT_{1B}R induced potentiation is not the result of a presynaptic mechanism.

The previous measures are still somewhat indirect. To get a more direct picture of glutamate release, we isolated the AMPAR and NMDAR components of the EPSC before and after anpirtoline. We predicted that if activation of 5-HT_{1B}Rs increased the release of glutamate, responses to activation of both receptor types should be increased. We found that when the cell was held at -70mV, a potential at which NMDARs are blocked by Mg^{2+}, anpirtoline enhanced the AMPAR-mediated response. However, when the cell was held at +40mV and NMDAR-mediated currents were measured, anpirtoline had no effect on the response (Figure 4.1B). This selective effect of anpirtoliine is incompatible with the predictions of a model in which 5-HT_{1B}Rs are located presynaptically. Instead, these data suggest that 5-HT_{1B}R activation selectively potentiates AMPAR responses and therefore may induce postsynaptic receptor modification.

Finally, as a further test of the hypothesis that 5-HT_{1B}R mediated potentiation occurs postsynaptically, we used microphotolysis of caged glutamate to induce small EPSP-like depolarizations (phEPSPs) at separate dendritic sites within SLM and SR. This technique allowed us to apply a constant amount of glutamate to the receptors, thus any changes observed would have to take place postsynaptically. We found that anpirtoline selectively enhanced phEPSPs at SLM but not SR (Figure 4.1C), similar to what we observed using electrical stimulation. Together, these data provide strong evidence that 5-

$HT_{1B}R$ activation results in a postsynaptic modification that specifically enhances AMPAR function at the TA-CA1 synapse, and is not the result of a presynaptic change in glutamate release. This 5-$HT_{1B}R$ induced increase in AMPAR function and lack of effect on the paired-pulse ratio is reminiscent of electrically induced LTP and may share a common expression mechanism, such as phosphorylation of GluA1 residues S831 and S845 as well as phosphorylation of molecules including CamKII and ERK.

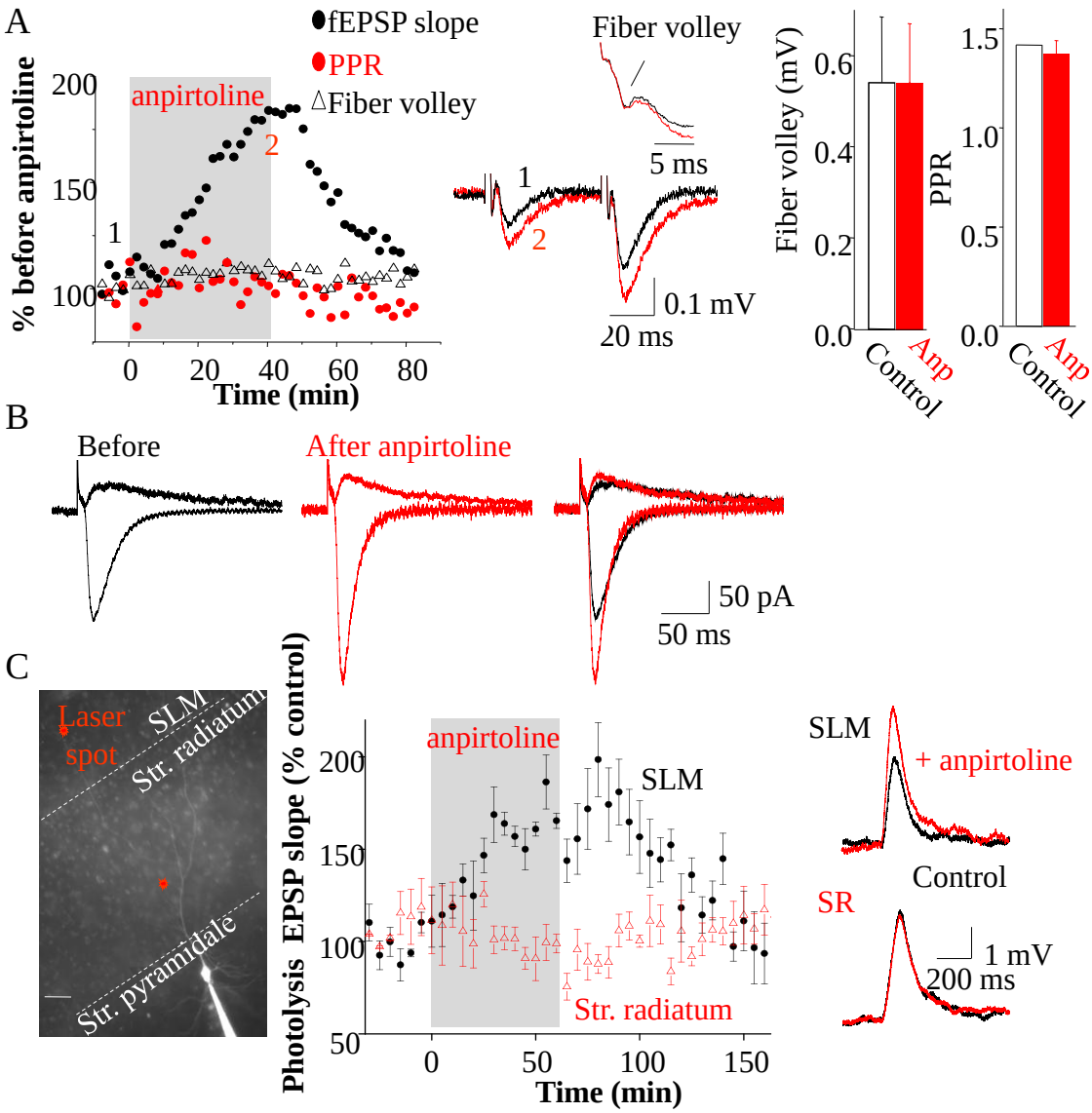


Figure 4.1 5-HT$_{1B}$R induced potentiation occurs through a postsynaptic mechanism. A) In a representative experiment, fEPSP slope (black circles) was increased in response to bath application of 50µM anpirtoline, whereas the paired-pulse ratio (red circles) and fiber volley (open triangles) remained constant (left). Pooled data of average fiber volley and average paired-pulse ratio (mean slope 2/ mean slope 1) before (white) and after anpirtoline (red), (right). B) EPSCs evoked at -70mV (bottom trace) and at +40mV (top trace) before (black) and after anpirtoline application (red). C) A CA1 pyramidal neuron

filled with Alexa 568. Red dots show location of laser spots used to induce phEPSPs (left). Photolysis induced EPSP slope increased with SLM stimulation (red) but not SR stimulation (black) following anpirtoline application (middle). Sample traces of phEPSPs evoked at SLM (top) and SR (bottom), before (black) and after anpirtoline (red), (right).

Hypotheses and predictions:

The similarities between electrically induced LTP and serotonin-induced potentiation, as described above, suggested the hypothesis that both forms of potentiation share a common expression mechanism, namely enhanced function of AMPARs through phosphorylation of the GluA1 subunit as the result of activation of CamKII and ERK. I therefore designed my experiments to test the following predictions. First, I predicted that 5-HT_{1B}R activation would result in phosphorylation of S831 and/or S845 residues on the GluA1 subunit of the AMPA receptor, as well as phosphorylation of CamKII at threonine 286 (α and β) and ERK p42/44. Additionally, I predicted that phosphorylation of these sites would be necessary for electrophysiologically observed 5-HT_{1B}R induced potentiation. Finally, I predicted that if both forms of potentiation are expressed through a common mechanism, then they would mutually occlude each other.

METHODS

Acute slice electrophysiology. Acute hippocampal slices were prepared from 4-6 week old male rats or mice. Dissection was done in ACSF (in mM: 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1.5 $MgCl_2$, 2.5 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose). Brain slices (400µm) were cut on a vibratome and kept in a holding chamber at room temperature at the interface of physiological medium and humidified 95%O_2/5%CO_2 for >1 hr. The slices were then transferred to a submersion-type recording chamber and perfused at room temperature with ACSF (flow rate= 1ml/min). Picrotoxin (100µM) and CGP52432 (2µM) were included to block $GABA_A$ and $GABA_B$ receptors. Concentric bipolar tungsten electrodes were used for stimulation and were placed either in SLM to stimulate TA afferents or in SR to stimulate SC afferents. Recording pipettes were filled with ACSF (3-5MΩ) and placed >500µm from the stimulating electrodes. Stimuli (100µs in duration) were delivered at 0.05 Hz. The stimulus intensity was set at 150% of threshold intensity, resulting in a fEPSP of 0.1-0.2mV. All compounds were applied by perfusion. Field EPSPs were recorded using an n.p.i. amplifier, filtered at 10kHz, and amplified 1000x prior to digitization.

Long-term potentiation. For LTP experiments, a 30 minute baseline was measured before delivery of high frequency stimulation (HFS). HFS consisted of 4 trains of stimuli, 100 pulses at 100 Hz, delivered 5 minutes apart.

Western blotting. Area SLM was dissected out of control and drug treated hippocampal slices using a 1mm micropuncher, pooled (2 punches from 2 slices) and homogenized in lysis buffer containing a phosphatase/protease inhibitor cocktail and sample buffer (Laemmli), boiled, and loaded into a 4-12% Bis-Tris gel. After running in 1X NuPage

MOPS SDS running buffer, the gel was transferred onto polyvinylidene diflouroride membranes in 1X NuPage transfer buffer (in 10% methanol). The membrane was blocked with 5% nonfat dry milk in buffer containing 1M Tris-buffered saline and 0.05% Tween and probed with antibodies against ser831-phosphorylated GluA1 (1:1000; Chemicon), ser845-phosphorylated GluA1 (1:1000; Chemicon), p42/44 ERK (1:1000; Cell signaling technologies) and active CamKII, pT^{286} (1:1000; Promega) at 4ºC overnight. After rinses in TBS-Tween, the membrane was incubated for 1 hr at RT in HRP-conjugated goat anti-rabbit IgG (1:1000, Sigma). The immunoblot was developed with enhanced chemiluminescence. Membranes were stripped, blocked, and reprobed with a phosphorylation-independent antibody against GluA1 (1:1000; Chemicon), MAPK (1:1000; Cell signaling technologies) or CamKII (1:1000; Cell signaling technologies). Membranes were then re-stripped, blocked and reprobed with an antibody against actin (1:1000; Cell signaling technologies) for loading control. Levels of phosphorylation (the ratio of phospho-specific optical density divided by total protein optical density) were used for statistical analysis. As a negative control for the ser831 blots, one lane was loaded with protein from an S831A mouse (see below).

Mutant animals. Two mutant animal strains were used. The S831A mouse (R. Huganir Lab, Johns Hopkins University) has an alanine knock-in mutation at serine 831 on the GluA1 subunit of the AMPA receptor, on a C57BL6J background (Lee et al. 2003). The $5\text{-}HT_{1B}$R KO mouse (R. Hen, Columbia University) is missing both copies of the gene encoding the $5\text{-}HT_{1B}$ receptor and is on a Sv129-Ter background (Saudou et al., 1994)

RESULTS

Prediction 1: Activation of 5-HT$_{1B}$Rs results in phosphorylation of ERK, CamKII and the GluA1 subunit of the AMPAR.

To test this prediction I used phospho-specific antibodies to measure phosphorylation of known signaling components involved in LTP using Western blot analysis. I chose to measure phosphorylation of S831 and S845 of the GluA1 subunit because both of these residues have been highly implicated in synaptic plasticity and are modulated by serotonin in the hippocampus (Svenningsson et al., 2002). I also measured phosphorylation of CamKII and ERK because 5-HT$_{1B}$R stimulation increases intracellular calcium (Giles et al., 1996) , a precursor to CamKII phosphorylation, as well as ERK phosphorylation (Mendez et al., 2002) in heterologous cells, and both of these signaling molecules are critical for LTP (Barria et al., 1997; English & Sweatt, 1997). I found that application of the 5-HT$_{1B}$R agonist, anpirtoline, onto acute hippocampal slices resulted in a specific phosphorylation of S831 but not S845 in the SLM region after 30 min of incubation (Figure 4.2A). I also observed a time dependent increase in phosphorylation of ERK and CamKII following incubation with anpirtoline (Figure 4.2B). The phosphorylation of S831 followed a similar time course as the anpirtoline-induced potentiation observed in the physiology experiment (Figure 4.2C). Also paralleling the physiology findings, I found no change in phosphorylation of either S831 or CamKII in the SR layer of the hippocampus following 5-HT$_{1B}$R activation (Figure 4.2C). To test whether anpirtoline specifcally acted via 5-HT$_{1B}$Rs to phosphorylate S831 and not through a non-specific effect, I examined pS831 in slices from 5-HT$_{1B}$R-KO mice and found no increase in phosphorylation with anpirtoline treatment (Figure 4.2D). I

conclude from these data that activation of 5-HT_{1B}Rs results in a phosphorylation of multiple signaling molecules involved in LTP including S831, CamKII and ERK, and that this is specific for TA-CA1 synapses and not SC-CA1 synapses. The experiments however do not answer the question of whether these signaling components are *required* for serotonin-induced potentiation.

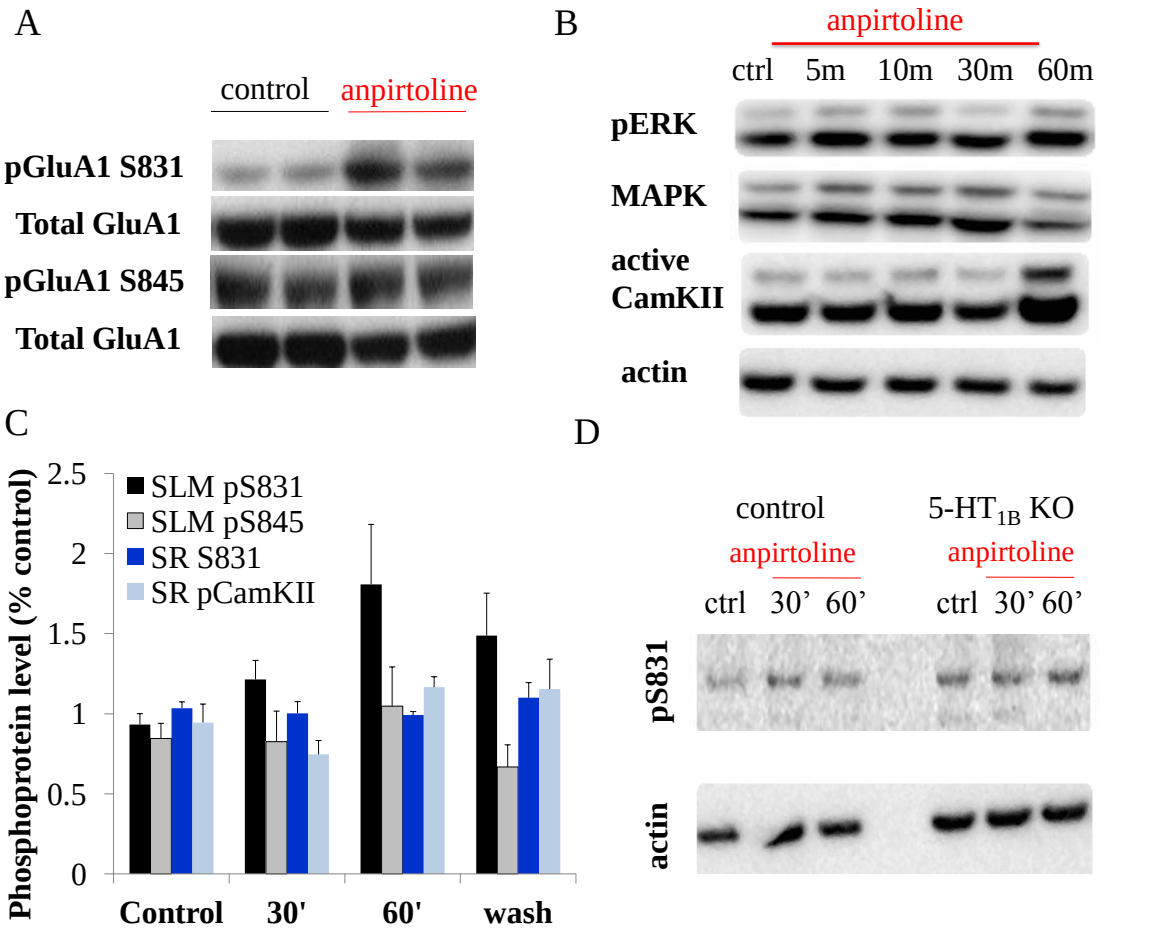


Figure 4.2 5-HT_{1B}R activation results in phosphorylation of S831 GluA1. A) Western blot using control tissue incubated in saline or 50µM anpirtoline. Phosphoprotein level of S831 GluA1 but not S845 GluA1 increased in SLM tissue following 30 min of incubation with drug. Total GluA1 protein did not change. B) Western blots exhibited a time

dependent increase in phospho-ERK and active CamKII (pT286 α and β) following application of 50μM anpirtoline. C) Quantification of western blot of SLM and SR tissue incubated in control saline or 50μM anpirtoline. S831 phosphorylation increased in a time dependent manner in the SLM and decreased after 60min of drug followed by 60min of wash (black) (n= 5 blots). There was no change in phosphorylation of S845 in the SLM (white) (n= 4 blots) and no change in either S831 (dark blue) (n= 4 blots) or CamKII (light blue) (n=4 blots) in the SR. D) Western blot of control SLM tissue and SLM tissue from 5-HT_{1B}R-KO animal in control saline and anpirtoline for 30 and 60 minutes. There was no change phosphorylation of S831 in tissue from the 5-HT_{1B}R-KO mice.

Prediction 2: Phosphorylation of serine 831 GluA1, CamKII and ERK is necessary for serotonin mediated potentiation.

To test this prediction I utilized a mutant animal with a knock-in mutation of serine to alanine at 831 on GluA1 (S831A). This mutation renders the receptor unable to be phosphorylated by CamKII or PKC at this site. In a blind experiment, I tested the effect of anpirtoline on slices from S831A mice and wild type littermates. I found that neither 5-HT_{1B}R activation with anpirtoline (Figure 4.3A) nor elevation of endogenous serotonin with fluoxetine (Figure 4.3B) could potentiate TA-CA1 fEPSPs from S831A mice, whereas both were capable of potentiating slices from wild type littermate controls. These data provide strong evidence that phosphorylation of GluA1, specifically at

serine831, is necessary for serotonin-mediated potentiation of TA-CA1 synapses through the 5-HT_{1B}R.

I next used pharmacological manipulations to test the involvement of CamKII and ERK phosphorylation in serotonin-induced potentiation. I found that application of either a CamKII inhibitor (KN-62) or an ERK inhibitor (U1206), blocked the effect of anpirtoline on TA-CA1 fEPSPs, providing evidence that their phosphorylation is necessary for 5-HT_{1B}R induced potentiation. I conclude that 5-HT_{1B}R activation triggers a signaling cascade involving increased CamKII, ERK activation and S831 phosphorylation and that blocking any of these events blocks potentiation of this synaptic response.

In summary, I have found that anpirtoline induced potentiation shares many common features with conventional LTP, however there are some key differences. The data using the S831A mouse highlight one of the differences between anpirtoline-induced potentiation and LTP. Despite the fact that anpirtoline-induced potentiation is blocked, neither the SC-CA1 LTP (Lee et al., 2010) nor the TA-CA1 LTP (Figure 4.3D) is altered in the S831A mouse. Additionally, while at most synapses an LTP-inducing stimulus results in enhanced phosphorylation of both S831 and S845, anpirtoline only increases S831 phosphorylation. Therefore it appears that although 5-HT_{1B}R activation and LTP share a common expression mechanism (enhanced AMPAR function), there are a few deviations in the pathway through which they achieve this. These differences may account for the fact that LTP persists for days to weeks, whereas serotonin-induced potentiation is reversed upon washout of the agonist in normal tissue.

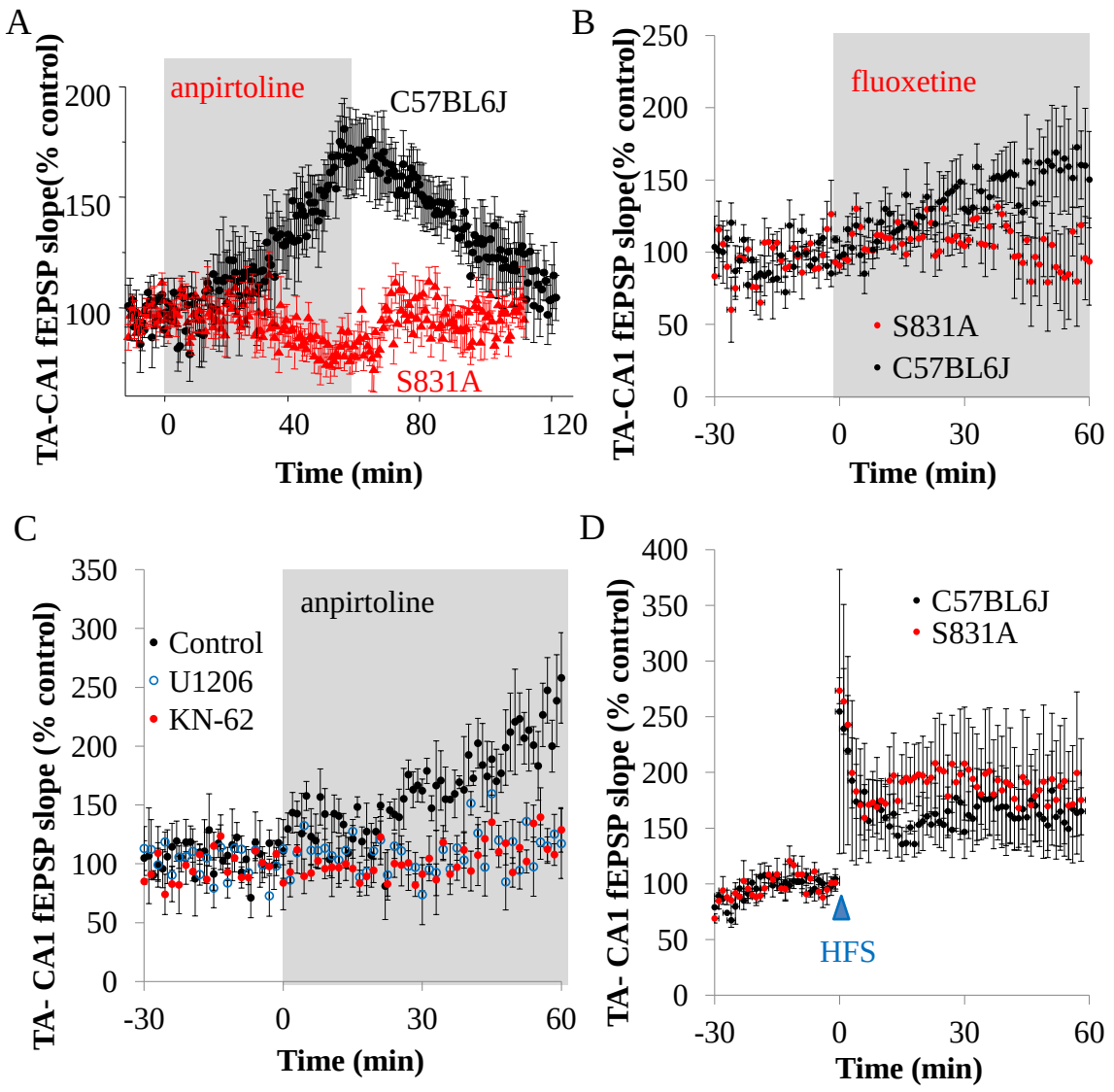


Figure 4.3 Phosphorylation of S831, CamKII and ERK is necessary for serotonin-induced potentiation at TA-CA1 synapses. A) Slices from wild-type mice (black) showed an increase in TA-CA fEPSP slope following bath application of 50µM anpirtoline which was reversed upon washout (n=7 slices). Littermate mice with a serine to alanine mutation at 831 on GluA1 (red) did not show anpirtoline induced potentiation (n= 7 slices). B) Slices from wild-type C57BL6J mice (black) showed an increase in TA-CA1 fEPSP slope with bath application of 20µM fluoxetine (n= 6 slices) but there was no

change in slices from S831A mice (red) (n= 5 slices). C) Control slices exhibited an increase in TA-CA1 fEPSP slope with bath application of 50µM anpirtoline (219 ± 27%) (n= 5 slices) (black) whereas slices pre-incubated with the MAPK blocker 20µM U1206 (115 ± 15%) (n= 6 slices) (blue) or 10µM CamKII blocker KN-62 (112 ± 18%) (n= 4 slices) (red) did not. D) Slices from WT C57BL6J (161 ± 27%) (n= 3 slices) (black) and S831A mutant (182 ± 56%) (n= 3 slices) (red) mice exhibited the same degree of potentiation at TA-CA1 synapses 60 min after 4 trains of HFS stimulation.

Prediction 3: If 5-HT_{1B}R mediated potentiation and electrically-induced LTP have the same expression mechanism, they will occlude each other.

Despite the finding that LTP and 5-HT_{1B}R mediated potentiation are not identical, they do appear to both enhance AMPAR function. Therefore I predicted that both forms of plasticity would occlude each other. To test this prediction I combined electrically induced LTP using a high frequency stimulation (HFS) paradigm and subsequent bath application of anpirtoline. I found that anpirtoline application did not further increase TA-CA1 fEPSPs in control slices that had exhibited 1hr of LTP following HFS delivery (Figure 4.4A). To determine whether this truly represented occlusion and was not a result of the fEPSP reaching saturation I lowered the stimulation intensity following LTP induction so that the fEPSP size was comparable to the pre-HFS fEPSP. I found that

anpirtoline was again unable to potentiate slices after this adjustment (Figure 4.4B). Decreasing the stimulation intensity results in the stimulation of fewer axons but does not affect the saturation of individual synapses. Therefore this method provides evidence that LTP does not act by increasing the number of presynaptic terminals that release glutamate. It is therefore possible that the AMPAR responses at individual synapses are saturated by LTP and that this explains why anpirtoline is unable to further potentiate the response.

I next performed the converse experiment of bath applying anpirtoline first, inducing potentiation of TA-CA1 synapses, then adjusting the stimulation intensity to yield a control sized fEPSP and then delivering the HFS. I found that HFS following anpirtoline induced potentiation did not result in LTP (Figure 4.4C). To ensure that anpirtoline was occluding LTP by enhancing GluA1 function at the TA-CA1 synapse and not through an indirect effect, I recorded LTP at SC-CA1 synapses which do not undergo 5-HT_{1B}R mediated potentiation. HFS at SC-CA1 synapses yielded similar potentiation in slices in control ACSF and slices treated with 50μM anpirtoline (Figure 4.4D), suggesting that anpirtoline does not occlude LTP when it is unable to potentiate the synapse. These data provide evidence that 5-HT_{1B}R induced potentiation and classically induced LTP mutually occlude each other. I conclude that despite the differences in some of the required signaling components involved in anpirtoline induced potentiation and LTP, both forms of plasticity converge on a common expression mechanism.

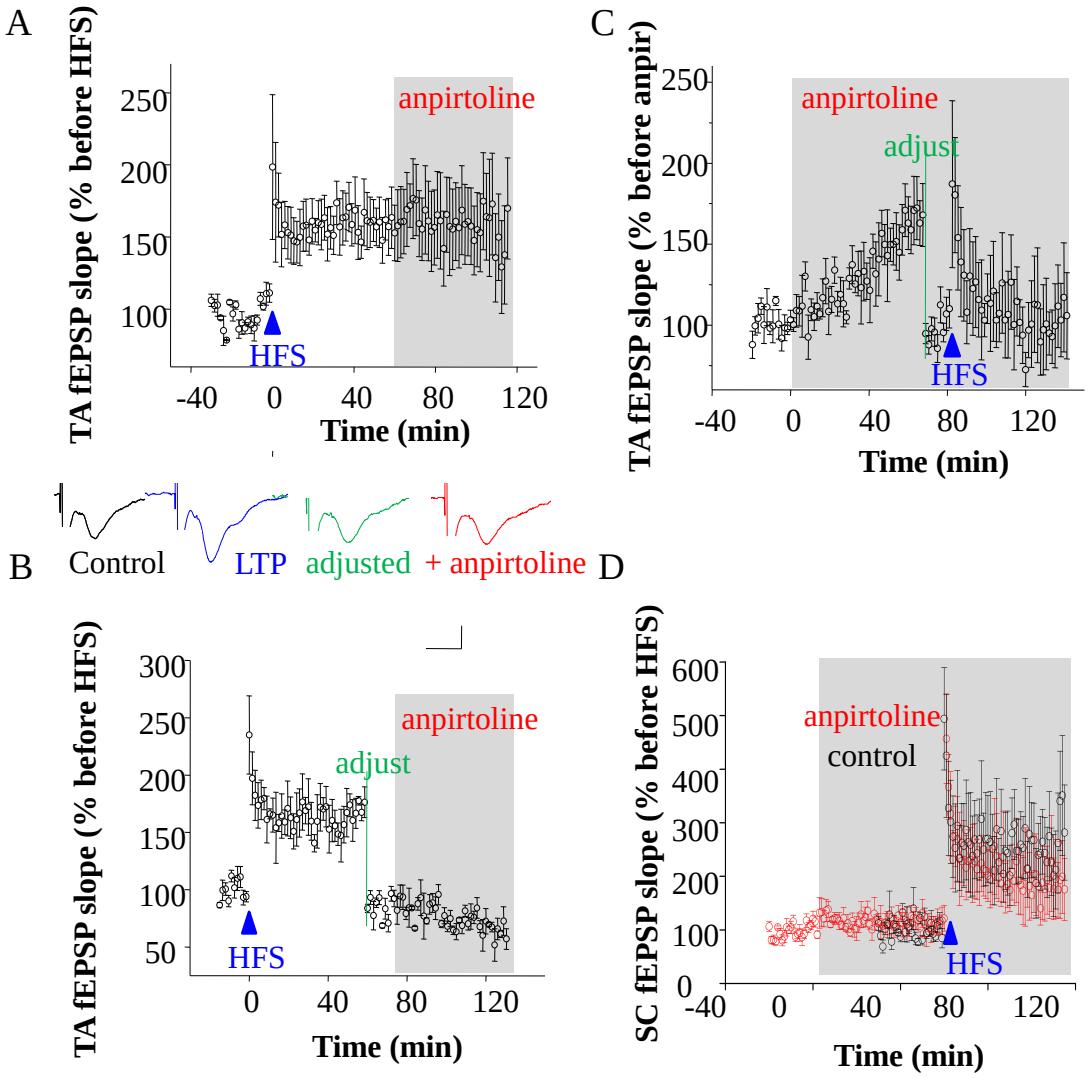


Figure 4.4 5-HT$_{1B}$R induced potentiation and electrically induced LTP occlude each other. A) Control slices exhibited a long lasting increase in TA-CA1 fEPSP slope following HFS (4 trains, 100Hz, 100 pulses, 5min apart). Bath application of 50µM anpirtoline did not further enhance fEPSP slope (n= 5 slices). B) Anpirtoline application following HFS and a stimulation reduction to obtain a response with the control fEPSP slope, did not result in potentiation of the TA-CA1 fEPSP (n= 3 slices). C) Bath application of anpirtoline increased TA-CA1 fEPSP slope, after stimulation adjustment

back to control fEPSP slope, HFS did not induce LTP (n= 4 slice). D) HFS resulted in similar potentiation of SC-CA1 fEPSP slope in control ACSF (black) (n= 5 slices) and following bath application of anpirtoline (red) (n= 7 slices).

DISCUSSION

I hypothesized that the process by which 5-HT increased the strength of TA-CA1 synapses was fundamentally the same as the process of LTP induction at these synapses. I have now tested several specific predictions of that hypothesis, and found that, in general the results are consistent with the predictions. I found that activation of 5-HT_{1B}Rs by the drug anpirtoline resulted in phosphorylation of CamKII, ERK and GluA1 at S831. These three sites are also phosphorylated by electrically induced LTP at SC-CA1 synapses and phosphorylation of the former two is necessary for both forms of potentiation. Interestingly, phosphorylation of S831 was necessary for anpirtoline induced potentiation even though it is not necessary for LTP at either SC-CA1 or TA-CA1 synapses. I also found that anpirtoline-induced potentiation and LTP mutually occlude each other. Together these findings provide support the hypothesis that 5-HT_{1B}R activation and LTP use the same expression mechanism.

After finding evidence that 5-HT_{1B}R activation results in a postsynaptic modification of AMPARs, I sought to identify the mechanism through which this occurs. Prior research has shown that chronic elevation of serotonin can elevate phosphorylation

(Svenningsson et al., 2002) and the membrane targeting (Martinez-Turrillas, Frechilla, & Del Rio, 2002) of AMPARs. AMPAR function has been widely studied in the hippocampus, particularly in reference to synaptic plasticity induced by long-term potentiation, providing a number of candidate signaling targets to explore. Additionally, activation of 5-HT_{1B}Rs is known to stimulate signaling cascades involving increases in calcium and ERK phosphorylation, both factors that are important for synaptic plasticity. These carefully studied LTP mediators combined with known serotonin receptor signaling cascades, pointed to potential players involved in the 5-HT_{1B}R mediated potentiation I had observed.

S831 but not S845 is phosphorylated following 5-HT_{1B}R activation

S831 phosphorylation by CamKII and S845 phosphorylation by PKA play an important role in synaptic plasticity [i.e., LTP]. S831 phosphorylation results in an increase in AMPAR conductance whereas S845 phosphorylation increases the delivery of AMPARs to the synapse (Derkach, Barria, & Soderling, 1999; Esteban et al., 2003). Both mechanisms contribute to LTP. My data provide evidence that the mechanisms underlying 5-HT_{1B}R mediated potentiation at least partially overlap with the mechanisms responsible for LTP. I found that bath application of anpirtoline, a 5-HT_{1B}R agonist, resulted in an enhancement of phosphorylation of S831 but not S845 or total GluA1 at the SLM of hippocampal slices. The time course of this phosphorylation paralleled the electrophysiological potentiation observed in response to anpirtoline and was accompanied by an increase in ERK and CamKII phosphorylation as well. Also, consistent with the lack of electrophysiological potentiation of SC-CA1 synaptic responses, anpirtoline did not increase S831 or CamKII phosphorylation in tissue taken

from the SR layer of the hippocampus. These data provide evidence that activation of 5-HT_{1B}Rs is capable of inducing a form of plasticity that is very reminiscent of LTP. Both result in an enhancement of AMPAR, CamKII and ERK phosphorylation which is synapse specific and does not globally affect CA1 neurons. This introduces a novel form of plasticity that has not previously been defined at this synapse or any other synapse in the brain. Moreover, these findings provide methods for identifying other regions of the brain that may be susceptible to this type of plasticity. In the future, we hope to examine phosphorylation levels of S831, CamKII and ERK in different populations of neurons in other brain regions following anpirtoline application, which would aid in the understanding of how serotonin can affect various behaviors.

S831 phosphorylation is necessary for 5-HT_{1B}R induced potentiation

Although my data showed that anpirtoline application induced phosphorylation of S831, it was plausible that activation of this pathway triggered a number of signaling cascades and that another signal could result in enhancement of the TA-CA1 synapse. For these experiments I utilized a mutant mouse model with a serine to alanine substitution at position 831, rendering it incapable of being phosphorylated by CamKII or PKC. Slices from these mutant mice did not respond to bath application of either anpirtoline or fluoxetine, whereas slices from control mice did. These data show that phosphorylation of S831 is necessary for serotonin-induced potentiation at TA-CA1 synapses. Interestingly, this animal does not appear to have a homeostatic compensation for the global mutation at this site. In general this mouse did not exhibit any gross outward abnormalities (Lee et al., 2003) though an interesting behavioral phenotype was later discovered that will be discussed in Chapter 6. In addition to S831 phosphorylation, my

data also show that phosphorylation of ERK and CamKII are necessary for anpirtoline induced potentiation. The requirement for CamKII phosphorylation is in agreement with the S831 finding, in that CamKII phosphorylates this site of the AMPAR. ERK phosphorylation on the other hand has been implicated in exocytosis of the AMPAR following an LTP inducing stimulus and has no known effect on S831 phosphorylation. Therefore it is interesting, though unclear, why blocking either ERK or S831 phosphorylation would block anpirtoline induced potentiation. It is possible that in addition to the ability of CamKII to increase ERK phosphorylation (Illario et al., 2003) ERK phosphorylation can also increase phosphorylation of CamKII, however there is no evidence for this in the literature.

5-HT_{1B}R mediated potentiation and LTP mutually occlude each other.

Although LTP and serotonin mediated potentiation do not use the exact same pathway, they are apparently expressed by the same mechanism: increased AMPAR function at the synapse. To test whether both paradigms were activating the same population of AMPARs, I induced 5-HT_{1B}R mediated potentiation and electrically stimulated LTP in a sequential manner. I found that anpirtoline treatment blocked subsequent LTP induced by HFS and that LTP induced by HFS blocked subsequent anpirtoline induced potentiation. These data suggest that 5-HT_{1B}R mediated potentiation and classic LTP have a common expression mechanism.

Additionally, these data are interesting in terms of what they may say about the effect of serotonin on LTP and memory. The TA-CA1 synapse is important for long-term memory consolidation, therefore blocking or enhancing LTP at this synapse may have

profound behavioral consequences. According to my data, drugs such as antidepressants that enhance serotonin in the synapse could have a negative effect on conventional synaptic plasticity and subsequently memory consolidation because they would activate 5-HT_{1B}Rs in this region and occlude LTP. This notion is supported by the finding that serotonin is closely tied to memory function (Buhot, Martin, & Segu, 2000). Interestingly, a different 5-HT_{1B}R agonist, CP93129, impairs memory in rats during a visuospatial task (Buhot, Patra, & Naili, 1995). Although the drug was delivered through a systemic injection, it is possible that this result was caused by an impairment of TA-CA1 LTP due to occlusion.

Serotonin mediated potentiation and LTP are not entirely the same.

Although LTP and 5-HT_{1B}R mediated potentiation appear to share common mechanisms and are capable of occluding each other, there are a few key differences between the two. First, similar to SC-CA1 LTP (Lee et al., 2010) mutating serine831 to alanine does not block TA-CA1 LTP (Figure 4.4D), however it does block anpirtoline-induced potentiation at this synapse. Second, LTP is associated with an increase in both S831 and S845 phosphorylation, whereas anpirtoline induced potentiation only increases S831 phosphorylation. Finally, the point which may tie these disparities together: LTP can last for hours and even days in some preparations, yet the effect of anpirtoline on TA-CA1 synapses can be washed out. These data suggest that 5-HT_{1B}R mediated potentiation may be the result of an enhancement in GluA1 conductance but not change in trafficking of the receptor. It is possible that the change in conductance induced by S831 phosphorylation is a somewhat transient effect since washout of anpirtoline brings the EPSP slope back to control levels. Therefore it may be that the long-term enhancement

and maintenance of synaptic transmission requires phosphorylation of both S831 and S845 on GluA1, but because 5-HT_{1B}R activation only phosphorylates S831 and not S845 there is no stabilization of the response.

In the future it will be important to explore the effect of serotonin depletion or 5-HT_{1B}R inactivation does to LTP at this synapse. Although the effects of serotonin on LTP have been studied in the SR layer of the hippocampus, our understanding of CA1 plasticity could greatly benefit from continued investigation of the SLM layer, particularly since this is the region of the hippocampus with most dense serotonin fiber innervation (Bjarkam et al., 2003). Enhancement or depletion of serotonin would also likely have interesting effects on TA-specific behaviors, such as memory consolidation in the Morris water maze. These experiments would provide insight into cognitive behaviors exhibited by patients using serotonergic drugs such as antidepressants and "ecstasy." It has previously been suggested that healthy patients treated with antidepressant medications exhibit problems with memory (Thompson, 1991). The data presented here point to a potential explanation for this problem- occlusion of LTP. Additionally, it is important to continue exploring the signaling pathways that control LTP at the TA-CA1 synapse. There may different underlying mechanisms involved with plasticity at this understudied synapse that are distinct from SC-CA1 LTP but nonetheless contribute to synaptic transmission and behavior.

CHAPTER FIVE

GLUTAMATERGIC TRANSMISSION AT THE TA-CA1 SYNAPSE IN AN ANIMAL MODEL OF DEPRESSION

INTRODUCTION

The monoaminergic theory of depression has led to the development of antidepressant drugs that work in a number of people but has left us with many gaps and unanswered questions. There remain many inconsistencies with the notion that depression is a disease caused by serotonin deficiencies. Despite what one would predict, patients depleted of serotonin do not exhibit linear decreases in mood (Delgado et al., 1994). Additionally, antidepressant medications increase serotonin immediately, yet they take weeks to become therapeutic (Katz et al., 2004). These findings suggest that serotonin is not solely responsible for depressed mood and that the therapeutic effects of antidepressants may come through a downstream target of serotonin receptor activation. Identifying the additional players involved in the etiology of depression require further in-depth analysis on depressed and antidepressant treated brains.

How can we model depression in an animal?

While imaging studies and post-mortem studies have provided a wealth of information regarding the pathophysiology of depression and the mechanism of antidepressant efficacy, they are limited. Imaging studies and post-mortem analysis are important for pointing to brain regions that may be disturbed in depression and activated with AD treatment, but are purely correlational and can only provide limited insight into which genes and molecules contribute to the etiology of the disease. Therefore, much effort has been put into the development of animal models of depression to answer these questions.

Although there have been many animal models of depression, including behavioral, lesion and genetic models, assessments of the depressed state of the animal show that many models do not meet all three forms of validity criteria (face, predictive, construct). Two behavioral models of depression that have been widely used and have passed the tests of validity are the chronic unpredictable stress (CUS) and social defeat stress (SDS) models of depression. CUS is used in both rats and mice and consists of subjecting them to mild stressors (strobe light, forced swim, social isolation, etc.) for three weeks. SDS uses a similar concept but uses the same stressor (submitting to an aggressive retired breeder rodent) for the duration of the three weeks. Both of these models of depression have face validity in that they produce phenotypes such as anhedonia and cognitive problems. They also have construct validity in that they use chronic stress to induce the phenotypes, a risk factor highly implicated in human depression. Finally, they have predictive validity, in that behavioral and biological phenotypes are rescued with chronic, but not acute antidepressant treatment (Mineur, Belzung, & Crusio, 2006; Rygula et al., 2006).

There have been many methods developed for assaying the affective state of the animal in these models, including forced swim, tail suspension, sucrose preference and novelty suppressed feeding. Among these the forced swim test and tail suspension test are among the most widely used. While these tests have been used for screening antidepressant drugs due to their ease of use, there is some concern for the lack of face validity. As stated previously, antidepressants require chronic application (3-4 weeks) to become effecting in humans, however behavioral alterations of both CUS and SDS are susceptible to changes following acute antidepressant injection in rodents (Detke,

Johnson, & Lucki, 1997; Petit-Demouliere, Chenu, & Bourin, 2005). On the other hand both the sucrose preference test (SPT) and novelty suppressed feeding task (NSF) show behavioral changes in rodents only after chronic antidepressant treatment. The SPT is a measure of anhedonia, or the inability to feel pleasure, and presents animals with a two bottle choice task in which one bottle contains normal water and one bottle contains a sucrose solution. Normal animals exhibit a high basal preference for sucrose, which is lowered in a time-dependent manner following CUS or SDS, but can be recovered with chronic antidepressant treatment (Mineur et al., 2006; Rygula et al., 2006). The NSF task requires food depriving the animal prior to testing and is conducted by placing the animal in a dark cage with food in the center that is illuminated by a bright light. Normal animals and antidepressant treated animals take less time to travel to the center to eat the food than animals subjected to these stress paradigms (Berton & Nestler, 2006).

These behavioral models allow manipulations of rodents in such a way that reproducible biological phenotypes can be produced, a feat not possible in human studies. Although CUS and SDS may not represent all forms of human depression, they may help understand at least a subset of depressed patients. Additionally, the use of behavioral assays such as the SPT and NSF tasks, allows researchers to identify signaling molecules of interest by using these tests to screen genes of interest in mutant animals. Although the monoaminergic theory of depression was developed from clinical evidence, animal models of depression have aided in filling in the many gaps. As stated previously, antidepressant drugs such as SSRIs increase serotonin immediately, yet the drugs take weeks to become effective. This raises the question, what downstream pathways are

activated following serotonin elevation? Might these downstream events make better targets for antidepressant drug development?

Are there glutamatergic disturbances associated with depression?

While there are a number of downstream factors currently being studied in regard to antidepressant induced elevations of serotonin, one newly emerging theory revolves around glutamate (Alt et al., 2006). It has become widely accepted that serotonin is capable of modulating other neurotransmitter systems, including glutamate. Serotonin and antidepressant application can increase glutamatergic release in the cortex (Aghajanian & Marek, 1997) and hippocampus (Bouron & Chatton, 1999). Serotonin can also mediate postsynaptic glutamatergic effects. Acute 5-HT application or chronic fluoxetine treatment enhances glutamate receptor phosphorylation in the hippocampus and cortex (Svenningsson et al., 2002) and can increase the membrane bound fraction of AMPA receptors in the hippocampus (Martinez-Turrillas, Frechilla, & Del Rio, 2002). Also, the tricyclic antidepressant imipramine can increase the synaptic level of AMPAR subunit GluA1 in the hippocampus (Du et al., 2004). These studies are further supported by my data presented in Chapters 3 and 4 which show that increasing serotonin concentration at the synapse is capable of potentiating glutamatergic responses in the hippocampus.

Beyond the evidence that serotonin is capable of altering glutamatergic transmission, there are a number of studies showing that glutamatergic transmission may be altered in animal models of depression and that manipulations that affect the glutamate system may result in a depressed phenotype. One study using proton magnetic resonance spectroscopy found that patients diagnosed with major depression exhibit higher levels of

cortical glutamate than do healthy controls (Sanacora et al., 2004). Conversely, glutamate/glutamine metabolism is decreased in depressed patients after treatment with electroconvulsive shock therapy (Pfleiderer et al., 2003). Although the findings regarding glutamate in depressed patients seem to conflict, probably because of difficulties of experimental design in human imaging data, they provide evidence that there is some type of alteration in glutamatergic transmission in these patients.

Evidence from animal studies has provided further support for the link between glutamate and depression. Both acute and chronic stress can increase GluA1 expression in the ventral tegmental area (Fitzgerald et al., 1996), but AMPAR mRNA is decreased in CA1 and CA3 regions of the hippocampus 24 hours after immobilization stress (Bartanusz et al., 1995). Additionally, a recent study found that animals more vulnerable to the chronic social stress paradigm of depression exhibited lower levels of GluA1 mRNA in the CA1 and dentate gyrus regions of the hippocampus compared to mice that were resilient to the stress (Schmidt et al., 2010). This group also found that a single nucleotide polymorphism in the GluA1 gene was associated with greater sensitivity to stress and decreased GluA1 expression in the hippocampus (Schmidt et al., 2010). Furthermore, mice with a knockout mutation of the GluA1 subunit of the AMPA receptor exhibit a depressed phenotype in the learned helplessness test (Chourbaji et al., 2008). Together these studies have helped to form the hypothesis that depression is associated with a decrease in AMPAR function.

This suggestion is further strengthened by the evidence that modulation of glutamate receptors directly, without alterations in serotonin, can cause an antidepressant effect in both animals and humans. An acute systemic injection of the AMPA receptor

potententiator LY392098 is capable of producing an antidepressant effect in the form of decreased immobility in both the tail suspension and the forced swim test in rodents (Li et al., 2001). Additionally, treatment with another AMPA receptor potentiator, LY451656 during the last four weeks of a five week chronic stress paradigm, blocked the depressed phenotype on the novelty suppressed feeding task, while vehicle did not (Schmidt et al., 2010). There is also emerging evidence that blockade of the NMDA glutamate receptor can result in fast-acting antidepressant effects in both human and animal studies. Subanesthetic doses of ketamine, an NMDAR antagonist, can cause antidepressant behavioral effects in the forced swim test, learned helplessness test and novelty suppressed feeding task (Li et al., 2010). Interestingly, ketamine produces an immediate effect in all of these tests even though the latter two are usually only sensitive to traditional antidepressants after chronic use. A placebo controlled, double blind study confirmed that ketamine can exert antidepressant effects in depressed patients within 72 hours of administration (Berman et al., 2000). Administration of an AMPAR antagonist blocks the antidepressant effect of ketamine, suggesting that ketamine does not function solely through NMDARs but requires activation of AMPARs (Maeng et al., 2008). Together, these studies point to a glutamatergic theory of depression in which the balance of AMPAR and NMDAR activation is necessary for maintaining a normal affective state and that manipulation of this system can result in an antidepressant effect.

Hypotheses and Predictions

I designed my experiments to test the hypothesis that basal glutamatergic synaptic strength is decreased at TA-CA1 synapses in an animal model of depression and following chronic antidepressant treatment. This hypothesis makes several predictions: 1) that the AMPA/NMDA ratio, as measured through fEPSP slope, would be decreased in CUS animals and restored to normal levels with chronic AD treatment; 2) that an indirect measure of synaptic strength, the response of TA-CA1 glutamate responses to serotonin, would be increased in animals subjected to CUS and diminished in naïve animals chronically treated with antidepressants; 3) that electrically induced long-term potentiation (LTP) would be enhanced in slices from CUS animals and comparable to controls in CUS animals chronically treated with ADs; and finally, 4) that total GluA1 levels would be diminished at the TA-CA1 synapse in CUS animals and restored following chronic AD treatment.

METHODS

Chronic unpredictable stress (CUS) procedure. Adult Sprague-Dawley rats (3-4 weeks old at start) were randomly divided into a control group and a mild CUS group. In the CUS group, animals were treated as following: Day 1, cage rotation (3 h), forced swim (5 min), food deprivation (16h). Day 2, strobe light (30 min), restraint (30 min), food and water deprivation (16 h). Day 3, strobe light (30 min), social isolation (16 h). Day 4, strobe light (30 min), restraint (30 min). Day 5, cage rotation (3 h), water deprivation (16 h). Day 6, restraint (3 h), social isolation (16 h). Day 7, cage rotation (3 h), restraint (30 min). The cycle was repeated 3 times over 3 weeks. Electrophysiological experiments

were then performed and analyzed with the experimenter blinded to the experimental condition of the animals.

AD treatment. Animals were given ADs via their drinking water in order to minimize stress associated with drug application. The concentrations of ADs were: imipramine, 100 mg/liter; fluoxetine, 80 mg/liter. Animals were housed singly and drinking water was changed every 3 days. Animals were given ADs continually for 3 to 4 weeks. Control animals received water only. Experiments were then performed and analyzed with the experimenter blinded to the experimental condition of the animals.

*Acute slice electrophysiology.*To isolate TA-CA1 responses, the dentate gyrus, and CA3 region of the hippocampal slice were removed while slices were in the holding chamber. Picrotoxin (100μM) and CGP52432 (2μM) were included to block $GABA_A$ and $GABA_B$ receptors, respectively. Concentric bipolar tungsten electrodes were used for stimulation placed either in SLM to stimulate TA afferents or SR to stimulate SC afferents. Recording pipettes were filled with ACSF (3-5MΩ) and placed >500μm from the stimulating electrodes. Stimuli (100μs in duration) were delivered at 0.05 Hz. The stimulus intensity was set at 150% of threshold intensity, resulting in a fEPSP of 0.1-0.2mV.

Long-term potentiation For LTP experiments, a 30min baseline was measured before delivery of high frequency stimulation (HFS). HFS consisted of 4 trains of stimuli, 100 pulses at 100 Hz, delivered 5min apart.

RESULTS

Prediction 1: Serotonin-induced potentiation at TA-CA1 synapses is altered in an animal model of depression and following chronic antidepressant (AD) treatment.

Multiple studies suggest that both depressed patients and animal models of depression exhibit decreased glutamatergic receptor function (Bartanusz et al., 1995; Beneyto et al., 2007; Schmidt et al., 2010) and that ADs that increase serotonin can enhance glutamatergic function (Svenningsson et al., 2002; Martinez-Turrillas, Frechilla, & Del Rio, 2002; Du et al., 2004). Additionally, the data from Chapter 4 provides a mechanism through which activation of 5-HT_{1B}Rs can enhance AMPAR function at TA-CA1 synapses. Therefore I predicted that a relative enhancement of 5-HT_{1B}R-mediated potentiation would indicate a decrease in basal AMPAR function. For these experiments I measured TA-CA1 fEPSPs in slices from chronic unpredictable stress (CUS) animals, animals administered fluoxetine during CUS, naïve animals given ADs for 3 weeks, and control littermates. I found that slices from CUS animals showed a striking change in response to anpirtoline compared to controls. TA-CA1 fEPSPs from CUS rats exhibited a doubling in the amount of potentiation induced by anpirtoline application that could not be washed out (Figure 5.1A). Interestingly, animals administered fluoxetine during CUS exhibited an anpirtoline response similar to controls (Figure 5.1C). Conversely, slices from naïve animals that were treated with fluoxetine (blue) or imipramine (red) for three weeks in their drinking water, were unresponsive to acute anpirtoline application (Figure 5.1B). These data give evidence for a profound difference between CUS, CUS+AD and AD treated animals in regards to their response to serotonin at TA-CA1 synapses and points to a change in basal synaptic strength as a possible culprit for the difference.

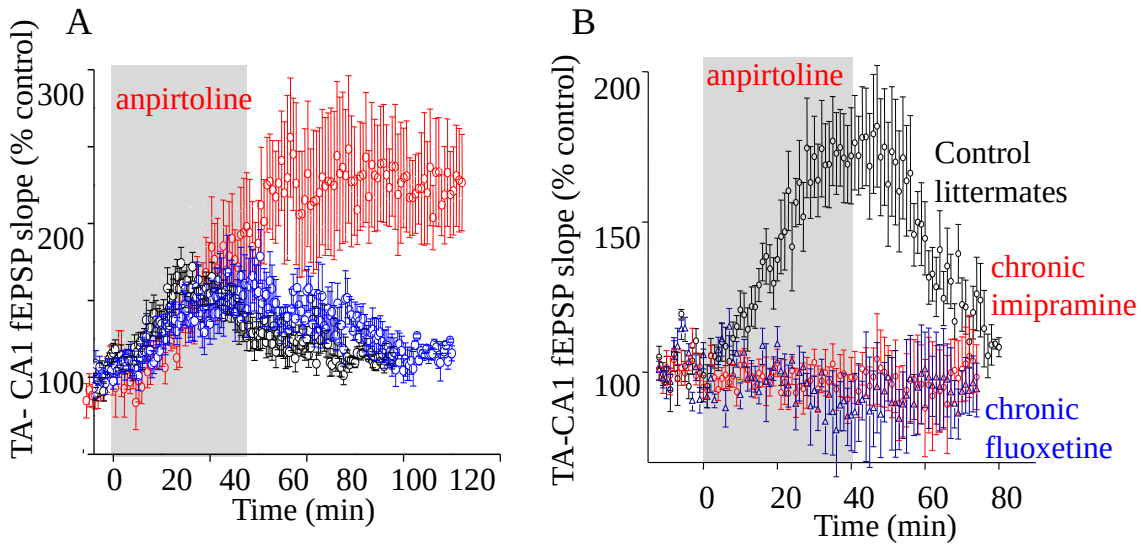


Figure 5.1 5-HT$_{1B}$R mediated potentiation is altered in an animal model of depression and following chronic antidepressant treatment. A) TA-CA1 fEPSP slope increased in slices from rats subjected to 3 weeks of CUS following 60min of anpirtoline application and 60min of wash. The magnitude of the response (red) was higher than in control slices (black) and did not return to control levels following washout of the drug (n= 5 slices). Slices from rats subjected to 3 weeks of CUS followed by 3 weeks of CUS and fluoxetine treatment exhibited an increase in TA-CA1 fEPSP slopes in response to anpirtoline, that returned to baseline after washout (blue) (n= 8 slices).B) Slices from rats treated with 80mg/L fluoxetine (blue; n= 5 slices) or 100mg/L imipramine (red; n= 9 slices) for four weeks did not display an increase TA-CA1 fEPSP slope in response to bath application of anpirtoline, whereas slices from control littermates (black; n= 7 slices) did.

Prediction 2: Basal AMPAR mediated responses are lower in TA-CA1 synapses in CUS animals compared to control and CUS + AD animals.

Vulnerability to chronic social stress is associated with a decrease in GluA1 mRNA in the CA1 and dentate gyrus regions of the hippocampus (Schmidt et al., 2010), while increasing the ratio of AMPAR to NMDAR activity in the hippocampus has been implicated as the mechanism of antidepressant action of ketamine (Autry et al., 2011). Therefore, I predicted that animals subjected chronic unpredictable stress (CUS) would display lower AMPAR to NMDAR ratios compared to control animals and this ratio would be recovered with chronic antidepressant treatment. To test the prediction at TA-CA1 synapses, I compared the AMPA receptor mediated component of fEPSPs in slices from control rats, rats subjected to CUS and rats given 3 weeks of fluoxetine treatment during the CUS procedures. I used two methods of normalizing the AMPAR response. The responses were recorded in 0 Mg^{2+} ACSF in order to produce a fEPSP with both an AMPAR and a NMDAR component. Stimulation intensity was set at threshold to evoke a response and then increased in 2mV increments until the fEPSP stopped increasing in amplitude. Next the AMPAR mediated component of the fEPSP was analyzed by measuring the slope of the first 2ms of the response following the fiber volley. Because the AMPAR response can vary from slice to slice depending on the distance between the electrodes and the number of synapses being activated, I normalized the AMPAR response to the NMDAR response, which I presumed to be unaltered by CUS. The NMDAR component was measured by washing in 50μM DNQX to block AMPARs, and then analyzing 5ms of slope from the remaining DNQX-resistant, NMDAR-mediated response. The AMPA/NMDA ratio was lower in slices from CUS animals than in control

slices (Figure 5.2A). AMPA/NMDA ratios from CUS animals chronically treated with fluoxetine did not differ significantly from the ratios in control slices (Figure 5.2A).

To examine whether the decrease in AMPA/NMDA ratio change I observed in CUS slices was the function of a decrease in AMPAR mediated current in a manner that would be independent of any change in NMDAR mediated current, I normalized the AMPAR response, as determined from the slope of the first 2ms of the fEPSP, to the amplitude of the fiber volley. The fiber volley is directly related to the number of axons that are stimulated and can be used to normalize the response to the number of synapses that are active. Slices from CUS animals trended towards a lower AMPA/fiber volley ratio when stimulated at 4mV above threshold compared to control slices (Figure 5.2B). To further analyze the relationship between AMPAR responses and the fiber volley, I fitted a straight line to the AMPAR slope versus fiber volley amplitude for all stimulation intensities for each slice. The average slope comparing these two components was significantly lower in CUS slices compared to control (Figure 5.2C, 5.2D). Slices from CUS + AD treated animals did not significantly differ from controls in either AMPA/fiber volley ratio at +4mV or AMPA/fiber volley slope. Analysis of the NMDA/fiber volley ratio indicated that the NMDAR response did not change in any of the conditions (data not shown).

Together these data suggest that synaptic strength, specifically AMPAR content, at the TA-CA1 synapses is decreased in animals subjected to chronic unpredictable stress, and chronic antidepressant treatment is able to restore this strength. These experiments are the most direct test of synaptic strength at the synapse in that they measure functional AMPAR current.

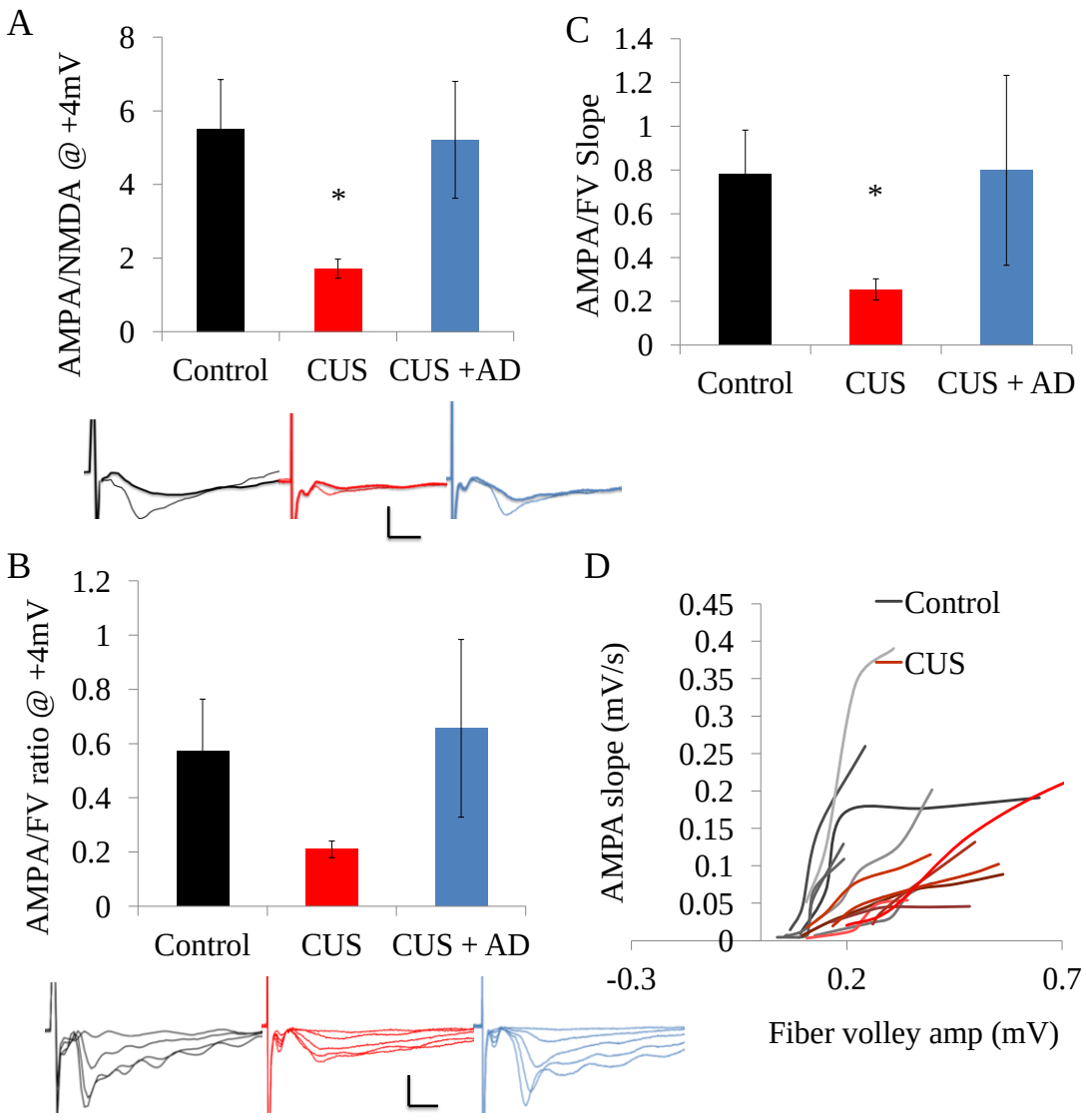


Figure 5. 2 AMPAR mediated responses are depressed in CUS animals and restored with chronic AD treatment. A) There was no significant group effect when analyzing the data using a single factor ANOVA ($F(20)= 3.55$, $p= 0.14$). However, an independent t-test showed the ratio of AMPAR slope over NMDAR slope (in 50µM DNQX) (at a stimulation intensity 4mV above threshold) is significantly lower in slices from CUS animals (red; n= 6 slices) compared to control animals (black; n= 6 slices), $t(10)= -2.8$, $p= 0.02$. There is no significant difference between CUS+AD animals (blue; n= 9 slices)

and controls. B) Ratio of AMPAR slope over fiber volley amplitude at 4mV stimulation above threshold is lower in CUS animals (red; n= 7 slices) than control (black; n= 8 slices)), and CUS+AD animals (blue; n= 9 slices). C) The slope of the line fitted to AMPAR slope versus fiber volley amplitude over all stimulation intensities is significantly lower in CUS slices (red) compared to control slices (black,) $t(9)= 2.46$, p= 0.04D). Data points for individual slices plotted as AMPAR slope compared to respective fiber volley amplitude across all stimulation intensities in control slices (black) and CUS slices (red). Scale= 1mV/10ms.

Prediction 3: Electrically induced LTP will be greater in magnitude in CUS animals than control animals and CUS animals chronically treated with ADs.

I formulated this prediction as another indirect test of basal synaptic strength at TA-CA1 synapses in CUS and CUS + AD animals. Electrically induced LTP is characterized by an increase in both conductance and surface expression AMPA receptors. Therefore, I predicted that, as with anpirtoline-induced potentiation, a lower basal level of AMPAR activity would result in a relatively larger amount of LTP in slices from CUS compared to control animals. I also predicted that CUS animals chronically treated with fluoxetine for 3 weeks would show comparable TA-CA1 LTP to control animals. Surprisingly, 60min after 4 trains of high frequency stimulation, control, CUS and CUS + AD all showed similar amounts of potentiation (Figure 5.3). One reason for the failure of this prediction may be that alterations in basal synaptic strength induced by chronic stress may respond in a complicated manner to an LTP inducing stimulus.

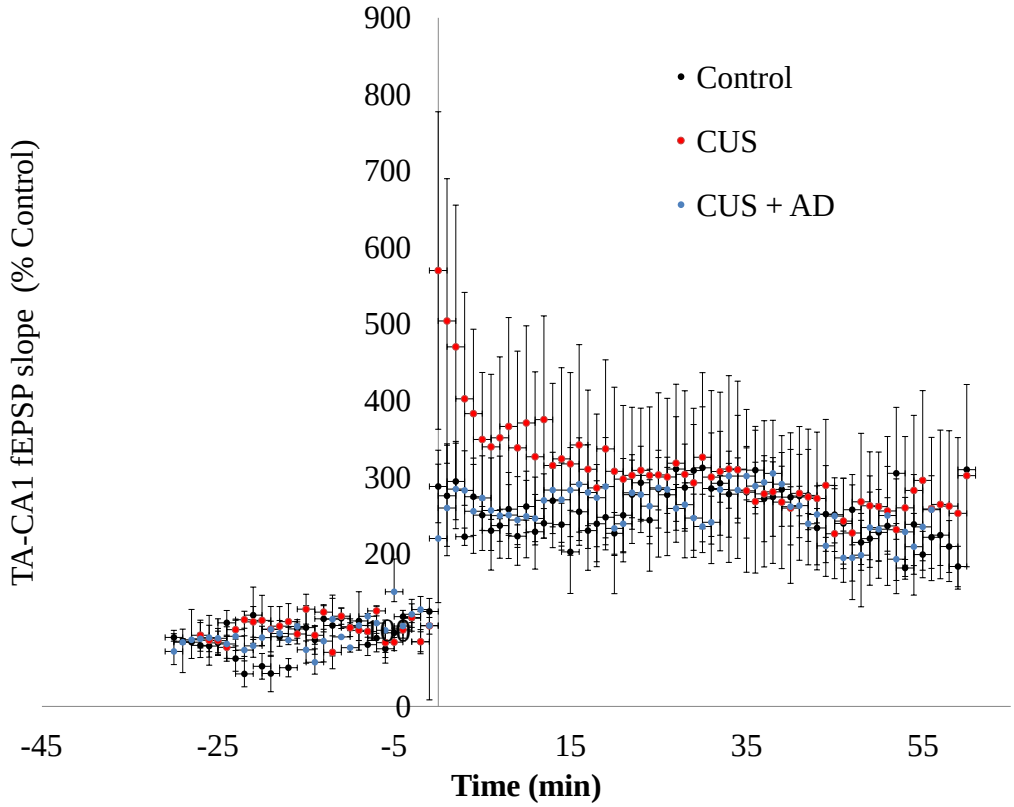


Figure 5.3 HFS induced TA-CA1 LTP is not different between control, CUS, and CUS +AD animals. TA-CA1 fEPSP slope was measured as baseline and 4 trains of HFS were given at time 0. All responses were normalized to average fEPSP slope for 10 minutes before HFS. There was no difference in the magnitude in potentiation between control (178 ± 32%), (black; n= 5 slices) CUS (227 ± 76%), (red; n= 9 slices) and CUS + AD slices (185 ± 52%), (blue n= 4 slices) 60 minutes after the tetanus.

Prediction 4: Total amount of GluA1 at TA-CA1 synapses is lower in CUS animals compared to control and CUS animals treated chronically with antidepressants.

To test this prediction I measured GluA1 protein in micropunches of tissue taken from the SLM region of the hippocampus in control, CUS and CUS + AD animals. I normalized GluA1 western blot band density to actin band density. CUS samples showed a significantly lower amount of total GluA1 protein compared to control samples and CUS + AD samples (Figure 5.4). This measure is an indicator of total amount of GluA1 present in the cell and does not differentiate between internal and external receptors. A significant decrease in total amount of protein may be due to a decreased synthesis of GluA1 protein or an increased degradation of the receptors. This total decrease in GluA1 protein is consistent with the decrease in AMPAR current seen at this synapse in electrophysiological experiments in slices from CUS animals and may result a decrease in GluA1 surface expression. These data provide additional evidence that synaptic strength is decreased following chronic stress but can be rescued with chronic antidepressant treatment.

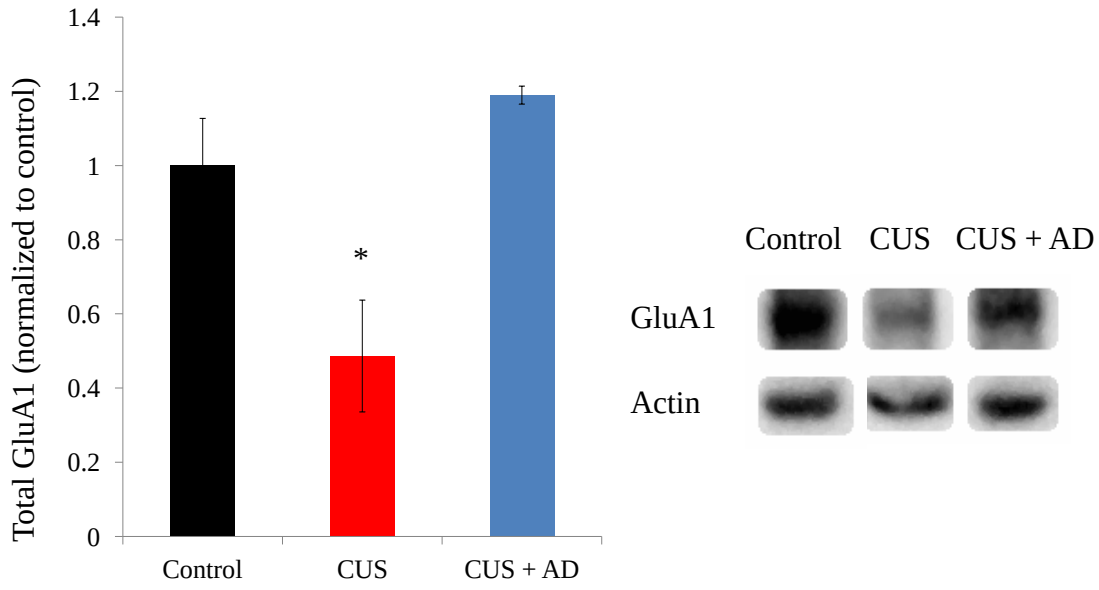


Figure 5.4 Total amount of GluA1 protein at TA-CA1 synapses is lower in animals subjected to CUS compared to control, but rescued with chronic fluoxetine treatment. GluA1 levels are normalized to actin and control tissue loaded for each western blot. A one-way ANOVA showed a significant group effect, $F(15)= 4.4$, $p= 0.03$. GluA1/Actin band density was significantly lower in CUS samples (red; n= 6 blots) compared to control samples (black; n= 6 blots), $t(10)= 2.33$, $p= 0.04$) and CUS + AD samples (blue; n= 4 blots). * indicates significantly lower than control ($p< 0.05$)

DISCUSSION

The monoaminergic theory of depression was formulated over 50 years ago, and although it has provided insight into the etiology of depression and resulted in the development of many antidepressant drugs, there are several questions left unanswered by it. Here I sought to explore the hypothesis that glutamatergic transmission is deficient in animal models of depression and that traditional AD treatments rescue this deficit through serotonergic modulation of glutamate receptors. I made several predictions based on this hypothesis and tested them in the CUS animal model of depression. I found that animals subjected to CUS exhibited decreased AMPAR-mediated field potentials at the TA-CA1 synapse compared to control animals and CUS animals treated chronically with fluoxetine. Additionally, I found that CUS animals also exhibited a decrease in total GluA1 protein in the SLM region of the hippocampus, as well as a quantitatively and qualitatively different response to serotonin-mediated potentiation at TA-CA1 synapses compared to controls. Surprisingly, control, CUS and AD treated CUS animals did not differ in TA-CA1 LTP. Using both direct and indirect measures of glutamatergic transmission at the TA-CA1 synapse, I found that synaptic strength is weakened in animals subjected to chronic stress and can be restored with chronic fluoxetine treatment.

AMPA receptor mediated current is decreased following chronic stress but recovered with chronic fluoxetine treatment.

The balance of glutamatergic receptors at a synapse determines its strength and can be altered by a number of factors. High frequency stimulation used in LTP paradigms can increase synaptic strength by increasing AMPAR function, and low frequency stimulation used in LTD paradigms can decrease synaptic strength by decreasing

AMPAR function (Lee et al., 2003). To indirectly measure synaptic AMPAR function, I recorded fEPSPs at various stimulation intensities and normalized the AMPAR mediated current to either NMDAR current or fiber volley amplitude. Both ratios were decreased in animals subjected to chronic unpredictable stress and restored in CUS animals that were chronically treated with fluoxetine. These data provide the most direct evidence that AMPARs at the TA-CA1 synapse are hypofunctional in animals that have been subjected to chronic stress; however it is hard to determine from this alone what the cause of decreased current is. It is possible that there are fewer AMPARs trafficked to the synapse, less responsive AMPARs at the synapse, fewer AMPARs being synthesized, or a degradation of AMPARs following stress. Data from Figure 5.4, showing that there is less total GluA1 protein in tissue from CUS animals provides evidence that it may be one of the latter two possibilities.

It is also difficult to determine whether the glutamatergic insult is a cause or an effect, i.e. does a genetic vulnerability of the glutamatergic system cause depression or does depression cause synaptic weakening by activating a signaling cascade? Evidence for the "causal" theory comes from genetic studies showing that knocking out the GluA1 subunit of the AMPAR results in a depressed phenotype in mice (Chourbaji et al., 2008) and that a polymorphism of GluA1 increases vulnerability to stress (Schmidt et al., 2010). Evidence for an "effected" theory focuses on the influence of stress hormones on glutamatergic neurons. Acute stress and subsequent acute activation of glucocorticoid receptors increase trafficking of AMPARs to the synapse (Conboy & Sandi, 2010). This glucocorticoid-induced increase in AMPAR function is also associated with an enhancement of working memory (Yuen, Liu, Karatsoreos, Feng, McEwen, & Yan,

2009). On the other hand, long-term stress, such as was produced by the protocol used in this study, causes atrophy of glutamatergic neurons in the hippocampus (Magarinos et al., 1996) and has detrimental effects on memory (Conrad, Galea, Kuroda, & McEwen, 1996). Most likely a combination of these factors is at play. Human studies show that while depression definitely has a genetic component, environmental factors such as stress are also strongly correlated with the disease (Sullivan et al., 2000).

These data solidify previous findings that chronic stress is connected with a perturbed glutamatergic system and that antidepressant treatments restore the strength of the synapse. The results presented in Chapter 6 will elaborate on how glutamatergic function is necessary for the behavioral effects of antidepressants. These results, together with other studies investigating the link between glutamate and depression show the promise of targeting AMPARs for newer and better antidepressant drugs.

Serotonin-mediated potentiation of glutamatergic transmission is altered following chronic stress and rescued by antidepressant treatment.

In addition to indirectly measuring glutamatergic function at TA-CA1 synapses, I indirectly measured synaptic strength by evaluating the effect of endogenous serotonin and the 5-HT_{1B}R agonist anpirtoline in CUS animals and CUS animals treated with fluoxetine. My assumption was that the weaker the synapses, the greater would be the effect of the treatments. I found that serotonin-induced potentiation was both quantitatively and qualitatively altered in animals subjected to CUS and animals given chronic AD treatment. The large increase in the amount of potentiation following anpirtoline application seen in CUS slices is probably due to a decrease in basal synaptic

strength, such that after 5-HT_{1B}R–induced phosphorylation of AMPARs, the amount of potentiation is relatively larger than in control slices because of the higher basal levels of phosphorylated AMPARs in the controls. Conversely, animals that have been chronically treated with either imipramine or fluoxetine have a chronic activation of this signaling cascade to the extent that the synapse is saturated with AMPARs and cannot be further stimulated by anpirtoline. This parsimonious interpretation of my results must be more rigorously and directly tested in future experiments.

While this hypothesis can account for the magnitude of change between the animal groups, it does not fully explain why the effect of anpirtoline cannot be washed out in the stressed animals. One possibility involves the "slot hypothesis" of synaptic plasticity, which postulates that adaptor proteins help to target receptors to specific locations in the membrane (Opazo et al., 2010). Proteins such as stargazin and PSD-95 bind to AMPARs, promote trafficking, and provide binding sites for the stabilization and clustering of the receptors in the postsynaptic density. Therefore alterations in slot proteins in depression models and antidepressant treated animals may account for the changes in stability of AMPAR responses. In fact, one study found that chronic treatment with the ADs paroxetine and desipramine increased the fraction of GluA1 subunits bound to stargazin (Martinez-Turrillas, Del Rio, &Frechilla, 2007). Additionally, my previous data suggest that anpirtoline enhances glutamatergic transmission via phosphorylation of serine 831 on the GluA1 subunit of the AMPAR (Figure 4.2,4.3) which enhances conductance of the receptor (Derkach, Barria, & Soderling, 1999). It is possible and in fact likely that other phosphorylation sites, such as S845 that regulate trafficking of AMPARs, contribute to the chronic stress and chronic antidepressant treatment effect I

have observed. Future experiments should investigate levels of adaptor proteins and S845 phosphorylation levels in CUS animals. It would also be interesting to ask whether mutations to either would change the way CUS slices respond to endogenous serotonin and anpirtoline.

It is interesting to note that I did not find a difference in the magnitude of LTP observed between control, CUS and CUS animals treated with fluoxetine. This was surprising since I predicted that a low basal level of synaptic strength in the CUS animals would translate to both an enhanced response to anpirtoline as well as increased magnitude of LTP, yet I only observed the first prediction to be true. This may be because anpirtoline induced potentiation and LTP do not completely overlap in mechanisms. 5-HT_{1B}R activation results in phosphorylation of only S831 and not S845, yet at least in Schaffer collateral LTP, both S831 and S845 phosphorylation occur. Therefore the case may be that AMPAR receptor insertion through S845 phosphorylation contributes to LTP to a greater extent than increased conductance via S831 phosphorylation, and that any changes to conductance are washed out following a tetanic stimulus that phosphorylates both. It is interesting to note that Schaffer collateral LTP is reportedly diminished in rats subjected to CUS (Alfarez, Joels, & Krugers, 2003); therefore the TA-CA1 synapse appears to behave differently than other hippocampal synapses in this respect. In future experiments it will be important to further dissect the mechanisms that underlie TA-CA1 LTP since there may key differences between TA- and SC-CA1 LTP that are important not only for understanding memory, but depression as well.

Together, these data provide interesting insight into a novel mechanism underlying the etiology of depression. Although some groups have identified glutamatergic disturbances in depression models and others have described the ability of serotonin to modulate glutamate, no group has soundly connected the monoaminergic theory of depression to the glutamatergic theory of depression. My data provide strong support for the hypothesis that an important insult of chronic stress is glutamatergic dysfunction; however chronic elevation of serotonin by AD treatment can restore this defect. The support for this hypothesis is a substantial step forward in the understanding of depression, in that it explains two previously disparate and limited theories of depression. The results presented here not only succeed in linking the two theories but also provide direction for the development of better antidepressant treatments.

CHAPTER SIX

THE ROLE OF THE 5-HT_{1B}R SIGNALING PATHWAY IN ANTIDEPRESSANT EFFICACY

INTRODUCTION

The chance discovery of iproniazid and imipramine as antidepressant drugs led to the development of a multitude of drugs targeting the monoamine system for the treatment of depression. Although studies have failed to find a consistent deficiency in the serotonergic system in depressed brains (Geracioti et al., 1997; Koslow et al., 1983), selective serotonin reuptake inhibitors (SSRIs) have become the most commonly prescribed antidepressant drugs. While SSRIs have helped a number of patients, 30% of patients remain unresponsive to these drugs (Doris et al., 1999), and those that do respond must wait weeks for therapeutic effects to begin (Katz et al., 2004). Additionally, SSRIs are associated with a number of undesirable side effects including sleep disturbances, sexual dysfunction and changes in appetite (Stahl, 1998). Surprisingly, the mechanism through which SSRIs exert their beneficial effects remains unknown. Therefore it is important to understand the signaling pathway or pathways triggered by increased synaptic serotonin and to identify which of these components underlie the therapeutic effects of antidepressant drugs.

The first step in elucidating the pathway involved in antidepressant efficacy is identifying the serotonin receptor or receptors that are involved. One promising candidate is the 5-HT_{1B}R (Murrough & Neumeister, 2011). 5-HT_{1B}Rs are seven transmembrane receptors that are coupled to their effectors by heteromeric G-proteins with an alpha subunit of the $g_{i/o}$ family. Activation of this receptor canonically results in an inhibition of cAMP production (Giles et al., 1996). Many have assumed that the most prevalent role of this receptor is as an autoreceptor, regulating release of serotonin (Boschert et al., 1993; Davidson & Stamford, 1995; Daws et al., 2000), however newer studies have pointed to a

number of other pathways that may be stimulated by 5-HT_{1B}R activation. In addition to serotonergic nerve terminals, 5-HT_{1B}Rs are also present as heteroreceptors on both glutamatergic terminals (Boschert et al., 1993) and dendrites (Peddie et al., 2010). Similarly to serotonin, 5-HT_{1B}Rs inhibit release of glutamate in various brain regions, including the dorsal raphe (Lemos et al., 2006), nucleus accumbens (Muramatsu et al., 1998) and suprachiasmatic nucleus (Pickard et al., 1999). On the other hand, the function of postsynaptic 5-HT_{1B}Rs has not yet been described. Activation of 5-HT_{1B}Rs expressed in heterologous cells results in an increase in intracellular calcium (Giles et al., 1996) and phosphorylation of extracellular regulated kinase (ERK) (Mendez et al., 2002). Therefore it is possible that postsynaptic 5-HT_{1B}Rs signal through either or both of these mechanisms. Although it is unclear exactly how both sets of 5-HT_{1B}Rs function within the nervous system, it has become increasingly apparent that one or both types are altered in depressed patients/models and involved in antidepressant efficacy.

5-HT_{1B}Rs are altered in depressed brains.

The rodent 5-HT_{1B}R is genetically homologous to the human 5-$HT_{1D\beta}$R (Adham et al., 1992) and the human receptor has been re-named the h5-HT_{1B}R. Although they share the closest sequence homology, these receptors display distinct pharmacological profiles (Hartig, Hoyer, Humphrey, & Martin, 1996). Human studies, while limited in number, have found a relationship between h5-HT_{1B}Rs and mood disorders. Huang and colleagues found a significant association of the G861C allele of the 5-HT_{1B}R in patients diagnosed with major depressive disorder, but not bipolar disorder or schizophrenia (Huang et al., 2003). Although not directly measured in that paper, an earlier paper from the group found that this polymorphism is associated with a 20% decrease in 5-HT_{1B}R

radioligand binding (Huang et al., 1999). This finding was supported by a recent study showing a significant decrease in [^{11}C]P943 binding, a selective 5-HT_{1B}R radioligand, in the ventral striatum and ventral pallidum of depressed patients (Murrough et al., 2011). Additionally, a post-mortem study found decreased levels of p11, a protein important for 5-HT_{1B}R expression, in the cingulate cortex of depressed patients (Svenningsson et al., 2006)

In agreement with the human studies, there is evidence of alterations in brain 5-HT_{1B}R levels in animal models of depression as well; however the direction of the change is unclear. The same study which found alterations in post-mortem p11 also found decreased cingulate cortex p11 in the brains of H/Rouen mice, an animal model of depression (Svenningsson et al., 2006). On the other hand, animals vulnerable to a learned helplessness paradigm displayed an *increase* in 5-HT_{1B}R mRNA in the dorsal raphe compared to non-helpless rats (Neumaier et al., 1997). These discrepancies may occur because these studies do not discriminate between pre-and postsynaptic receptor populations. Additionally, these studies are correlative and do not tease out the differences between receptor changes that cause susceptibility to depression versus those that are compensatory responses to stress. The generation of transgenic animals has aided in separating cause from effect, however the results are equally confusing. Although 5-HT_{1B}RKO animals display enhanced aggressiveness (Saudou et al., 1994) and exploratory behavior (Zhuang et al., 1999), there are few indications of disturbances in mood related behaviors. Animals with a p11 knockout exhibited decreased surface 5-HT_{1B}R levels as well as increased immobility in the tail suspension test (TST), indicating a depressed phenotype (Svenningsson et al., 2006). This study does not however

correspond with another lab's finding that male 5-HT_{1B}R knockout (KO) animals do not differ from wild type littermates in either the TST or the forced swim test (FST) (Jones & Lucki, 2005). Together, these studies implicate 5-HT_{1B}Rs in depression; however it is unclear exactly what role they play. This may, in particular, be due to the fact that the TST is an acute response to ADs and may not be reflective of the therapeutic actions of chronically administered ADs.

5-HT_{1B}Rs are modulated by ADs.

Although the data connecting 5-HT_{1B}Rs to a depressed phenotype is somewhat weak, the evidence for a role of these receptors in antidepressant efficacy is stronger. While the TST and FST have been used to evaluate basal affective state in animals, they were developed as a way to measure AD efficacy and are truly only valid in this capacity. 5-HT_{1B}RKO mice exhibit an augmented response to the AD fluoxetine in the TST compared to wild type mice (Jones & Lucki, 2005). This finding suggests that normally, 5-HT_{1B}Rs limit the effectiveness of antidepressants, presumably because the autoreceptors bind serotonin and in turn, decrease the release of serotonin from terminals. Indeed, an *in vivo* microdialysis study found that 5-HT_{1B}RKO mice exhibited increased extracellular serotonin compared to wild types mice in the ventral hippocampus and frontal cortex after treatment with the antidepressant paroxetine (Malagié et al. 2008). This finding is consistent with what has been shown with administration of 5-HT_{1B}R antagonists as well (Gobert et al., 1997). Together, these studies have led to the hypothesis that the downregulation of 5-HT_{1B}Rs is necessary for the therapeutic actions of antidepressants (Moret & Briley, 2000).

Conflicting with this hypothesis is a group of studies indicating that activation of 5-HT_{1B}Rs can induce an antidepressant effect in itself. One study found that administration of anpirtoline, a 5-HT_{1B}Rs agonist, resulted in an antidepressant phenotype in the FST. Interestingly, this same study found that a 5-HT_{1B}R antagonist blocked the antidepressant effect of imipramine on this task (O'Neill & Conway, 2001). The effect of anpirtoline on the FST was confirmed by another group that further elucidated the mechanism through which 5-HT_{1B}Rs can alter behavior (Chenu et al., 2008). They found that the antidepressant effect of anpirtoline was enhanced in the FST in animals with either a serotonergic fiber lesion, or animals depleted of serotonin with p-CPA. This suggests that heteroreceptors are responsible for the 5-HT_{1B}R-induced antidepressant effect elicited in these animals. Together these studies suggest that presynaptic 5-HT_{1B} autoreceptors limit the effectiveness of traditional antidepressants, but activation of heteroreceptors is capable of producing a therapeutic effect itself. Although this collection of research paints a clearer picture of the role of 5-HT_{1B}Rs in depression, the mechanism through which the heteroreceptors act still remains a mystery.

5-HT_{1B}Rs in the regulation of synaptic plasticity.

There is a wealth of research suggesting that the regulation of neuroplasticity is involved in the manifestation of depression and responsiveness to antidepressant treatment (Alt et al., 2006; Kempermann & Kronenberg, 2003; McEwen, 2001). Interestingly, it appears that 5-HT_{1B}Rs may play a role in regulating synaptic plasticity. The most convincing studies supporting this point have investigated the effect of 5-HT_{1B}R drugs and mutations on behavior known to be driven by synaptic plasticity. The most commonly studied behaviors include hippocampal dependent tests involving spatial

learning and memory. One study found that 5-HT_{1B}RKO mice exhibited enhanced performance on the Morris water maze test of spatial learning, as displayed by shorter latencies in the training trials of the task (Malleret et al., 1999). Conversely, treating animals with a 5-HT_{1B}Rs agonist resulted in an impairment in spatial memory in the water maze task (Ahlander-Luttgen et al., 2003) as well as the radial arm maze test (Buhot, Patra, & Naïli, 1995).

Surprisingly, there are no published studies investigating the interaction between 5-HT_{1B}Rs and long term potentiation (LTP), the form of plasticity believed to underlie these behaviors. The first assumption from these data might be that activation of 5-HT_{1B}Rs blocks synaptic plasticity while blockade of the receptor promotes synaptic plasticity. The data presented in Chapter 4 provides insight into how this may happen. I found that activation of 5-HT_{1B}Rs resulted in a form of plasticity which mimics LTP. Interestingly, this 5-HT_{1B}R-induced potentiation resulted in occlusion of conventional LTP (Figure 4.5), indicating that 5-HT_{1B}R activation can block LTP and may also subsequently block LTP-dependent behaviors. My data are further supported by the findings of others that 5-HT_{1B}Rs activation can lead to signaling cascades important for synaptic plasticity including calcium influx (Giles et al., 1996) and ERK phosphorylation (Mendez et al., 2002). Based on my own findings combined with the 5-HT_{1B}R literature, I have proposed a signaling cascade in which postsynaptic 5-HT_{1B}R activation leads to the phosphorylation of CamKII and ERK, followed by the phosphorylation of glutamatergic AMPARs at GluA1 S831. This raises the question: are the downstream mediators of 5-HT_{1B}R plasticity involved in depression and antidepressant efficacy?

ERK regulation in depression

Extracellular regulated kinase (ERK) is a member of the mitogen activated protein kinase (MAPK) pathway. ERK is activated by the Ras-Raf-Map signaling cascade and is responsible for translating extracellular events, such as receptor activation, to gene transcription and subsequently behavior (Grewal, York, & Stork, 1999). This signaling cascade plays a role in a number of biological processes including the enhancement of synaptic strength (Hall & Ghosh, 2008). Additionally, there is already substantial evidence that ERK plays an important role in depression and antidepressant efficacy. Animals chronically treated with the stress hormone corticosterone (CORT) display a depressed phenotype in the TST, which can be recovered by the antidepressant amitriptyline (Gourley et al., 2008). Interestingly, this study found that the chronic CORT treatment also decreases ERK1/2 phosphorylation in the dentate gyrus region of the hippocampus, which is recovered in animals treated with the antidepressant. Additionally, ERK phosphorylation is enhanced in animals treated with the new, unconventional antidepressant, ketamine (Li et al., 2010). Although both ketamine and amitriptyline have different points of action (NMDARs versus serotonin transporter), they both converge at the point of enhancing ERK. While the exact mechanism through which ERK regulates mood has not yet been deciphered, there are number of potential candidates including enhancement of brain derived neurotrophic factor (BDNF) and increased AMPAR activity.

AMPAR activity in the regulation of depression.

In addition to finding that 5-HT_{1B}R activation can induce ERK phosphorylation, I found that it also increased AMPAR activity. Specifically, the 5-HT_{1B}R agonist anpirtoline induces a specific phosphorylation of the GluA1 subunit of the AMPAR at residue S831 (Figure 4.2). S831 phosphorylation is enhanced following LTP inducing stimuli (Barria et al., 1997) and is responsible for enhancing the conductance of AMPARs (Derkach, Barria, & Soderling, 1999). This endpoint of the proposed 5-HT_{1B}R signaling cascade has been implicated in the etiology of depression by multiple studies. Animals with an alanine substitution at this site, rendering it incapable of being phosphorylated, display a decreased lever pressing to a reward associated cue (Crombag et al., 2008), which is indicative of abnormal motivational behavior, a trait exhibited in a number of psychiatric diseases including depression. Additionally, animals with a GluA1 knockout, exhibit increased helplessness in the learned helplessness depression paradigm (Chourbaji et al., 2008). Unfortunately, many of the other classic tests of depression have not been performed on either the S831A or the GluA1 knockout mouse.

The linkage between depression and glutamate is further strengthened by research studying the relationship between antidepressants and AMPARs. Traditional antidepressants such as SSRIs increase the phosphorylation of AMPARs at S831 as well as S845 of GluA1 (Svenningsson et al., 2002). Additionally, AMPAR potentiators are capable of producing antidepressant-like responses themselves in the TST, FST (Li et al. 2001) and novelty suppressed feeding test (Schmidt et al., 2010). Also, blocking AMPARs with a drug such as NBQX blocks the efficacy of the non-traditional antidepressant ketamine (Li et al., 2010), suggesting that these receptors are necessary

component of the therapeutic pathway. These data are supported by the findings presented in Chapter 5, showing that AMPAR function is decreased in animals subjected to chronic unpredictable stress, but can be recovered with chronic fluoxetine treatment (Figure 5.1). Together these data suggest that hypofunction of the AMPAR is associated with a depressed phenotype which can be recovered by enhancement of the receptor. It remains unclear however whether phosphorylation of S831 itself is responsible for maintaining normal functioning and whether this site is necessary for antidepressant efficacy.

Hypotheses and predictions.

I designed a series of experiments to test the hypothesis that the 5-HT_{1B}R-mediated, S831 phosphorylation-dependent plasticity process that I have described in the chapters above is necessary for the therapeutic effects of antidepressants. Specifically I tested the prediction that 5-HT_{1B}R activation is necessary for rescue of sucrose preference following a chronic stress paradigm. I also tested the prediction that phosphorylation of GluA1 S831, a downstream target of 5-HT_{1B}R activation, is necessary for antidepressant efficacy in the sucrose preference test.

METHODS

Animals. Male 5-HT_{1B}KO, S831A and C57BL6J mice aged 4-6 weeks were used in these experiments. All animals were group housed with a 12:12 dark light schedule and food and water available *ad libitum*. Male and female homozygous 5-HT_{1B}KO mice were obtained from Rene Hen's laboratory (Columbia University) and were bred to wild type sv129/ImJ mice to produce heterozygous animals. Heterozygous animals were bred to each other to produce 5-HT_{1B}KO mice and wild type littermates. Male and female homozygous S831A mice on a C57BL6/J background (backcrossed for 13 generations) were obtained from Richard Huganir's laboratory (Johns Hopkins University) and were bred to each other to produce homozygous mutant mice. Wild-type C57BL6/J mice (Jackson laboratory) were used as controls. Additionally, S831A mice were bred to C57BL6/J mice to obtain heterozygous mice. These mice then became parents for the homozygous animals used in some experiments.

Social Defeat Stress. Adult mice (> 6 weeks old) were randomly divided into a control group and SDS group. The experimental SDS mice were placed in the cage of a CD1 retired breeder mouse and allowed to be attacked for a 5min period. Next the mice were separated by a wire mesh divider but allowed to remain in visual contact for 1hr. Mice were subjected to social stress one a day for three weeks. The CD1 residents were rotated such that no intruder mouse met the same resident twice in a row.

Chronic Unpredictable Stress. Adult mice (> 6 weeks old) were randomly divided into a control group and a mild CUS group. In the CUS group, animals were subjected pseudorandomly to the following stressors: cage rotation (3 h), forced swim (5 min), food

deprivation (16h) strobe light (30 min), restraint (30 min), social isolation (16 h), 45 degree cage tilt (3h). Animals were given 2-3 stressors a day over 6-7 weeks.

Sucrose Preference Test. Mice were given an 18hr sucrose preference test. Mice were presented with two bottles made from 50mL conical tubes attached with a sipper spout. Bottles contained either 50mLs of normal tap water or 50mLs of 1% sucrose. Animals were first presented with both bottles while group housed to habituate the animals to task. For each test including the baseline, animals were individually housed overnight. Bottles were inserted 3 hours before the beginning of the dark cycle and removed 3 hours after the beginning of the light cycle. The position of the bottles was switched half way through the test to negate any side preference. Preference for sucrose was calculated as a percentage of consumed sucose-containing solution relative to the total amount of liquid intake. Any animals not showing a basal preference for sucrose (< 65%) were not used for further experiments. Sucrose preference tests were conducted once a week for the entirety of the experiment.

Novelty Suppressed Feeding Test. Novelty suppressed feeding tests were performed as previously reported (Santarelli et al., 2003)**.** The test apparatus was a brightly lit arena (60 x 60 x 35 cm) with a solid floor placed in a dimly lit room**.** The floor of the box was covered with a layer of bedding. Two laboratory chow pellets were placed on a white paper circle platform positioned in the center of the box. Mice that had been food deprived for 18 hours were gently placed in a corner of the arena. The latency to begin eating, defined as active chewing of the pellet, was recorded. A maximum time allowance was set at 400 s. Immediately after the test, animals were returned to their home cage and allowed to feed for 5 min. Food pellets were weighed before and after the 5 min, and the

amount of food consumed was calculated. Animals that ate less than 0.3 g of food within this 5-minute period were removed from all analyses, in order to ensure that only sufficiently hungry animals were included.

Tail Suspension Test. Mice were intraperitoneally injected with saline or 30mg/kg imipramine 30min prior to testing. Each mouse was taped to a wooden horizontal bar 2 inches from the base of its tail. A blind experimenter recorded the amount of time spent immobile for a 6min period.

Open Field Test. Mice were placed in a 60 x 60x 35cm plexiglass box for 5min. The box was divided into 12 squares using tape on the bottom of the box. Mice were video recorded and a blind experimenter calculated the number of wall touches, time spent in center two squares and number of line crossings for each animal.

Drugs. Animals administered antidepressants were given 80mg/L fluoxetine in their drinking water. Fluoxetine HCl was mixed with 1mL DMSO to dissolve and added to pre-weighed water pouches.

RESULTS

Prediction 1: Pharmacological block of the 5-HT_{1B}R will block the therapeutic effects of fluoxetine in animals subjected to chronic stress.

The sucrose preference test is an important measure for understanding the role of 5-HT_{1B}Rs in depression in that it is sensitive to chronic unpredictable stress (CUS) and social defeat stress (SDS) paradigms and recovers with chronic but not acute

antidepressant treatment (Rygula et al., 2006; Willner & Mitchell, 2002). Therefore, with colleagues at St. Mary's College of Maryland, I designed an experiment aiming to test the effect of 5-HT_{1B}R blockade on sucrose preference and antidepressant efficacy. These experiments were then performed by an undergraduate student, Ms. Kaitlin Galyor, under the supervision of her mentor, Aileen Bailey, as part of her senior research project.

I predicted that the 5-HT_{1B}R antagonist SB224289 would block the effects of the antidepressant fluoxetine in the sucrose preference test. To test this prediction we split animals into 5 groups: controls, SB224289 administration, SDS, SDS with fluoxetine administration, and SDS with co-administration of fluoxetine and SB224289. We found that, as previously reported (Krishnan et al., 2007), all mice subjected to SDS exhibited a decrease in sucrose preference by week 2. By week 4, SDS mice remained significantly lower in sucrose preference than control or mice treated with SB224280 alone. SDS mice treated with fluoxetine recovered their sucrose preference, however. Interestingly, SDS animals co-treated with SB224280 and fluoxetine remained significantly lower in sucrose preference than controls (Figure 6.1). These data provide evidence that 5-HT_{1B}R activation is necessary for the therapeutic effects of antidepressants in the sucrose preference task, however it is not necessary for maintaining a basal preference for sucrose.

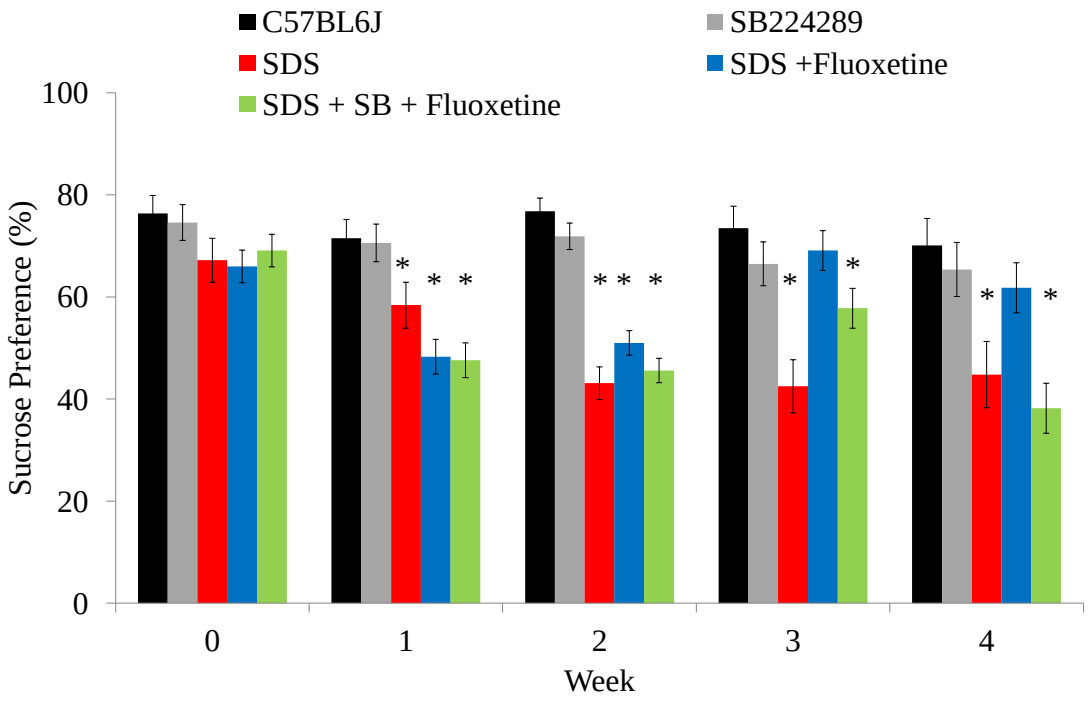


Figure 6.1 Pharmacological blockade of 5-HT$_{1B}$Rs blocks the effect of antidepressants in the sucrose preference test. A mixed ANOVA revealed an overall group effect, F (4, 25) = 47.3, p < 0.001, and a significant interaction between group and time, F (16,100) = 2.99, p <0.001. Wild type mice given normal drinking water (black) or SB224289, a 5-HT$_{1B}$R antagonist, (gray) displayed a high sucrose preference over the course of 5 weeks. At week 4 animals control mice displayed a significantly higher sucrose preference than SDS mice (red) t(8) = 9.95, p < 0.001, and SDS + SB224280 + Fluoxetine mice (green), t (11) = 4.19, p = 0.002. Animals given fluoxetine during SDS (blue) recovered their preference for sucrose and were not significantly different from controls by week 4. * indicates significantly different (p< 0.05) than control baseline.

Prediction 2: Mice lacking 5-HT$_{1B}$Rs become depressed with CUS but do not recover with chronic fluoxetine treatment.

Although much work has been done characterizing the behavior of 5-HT$_{1B}$RKO mice, no published literature has conducted a chronic stress paradigm in these animals. Additionally, it is difficult to determine what the basal affective state in these animals is. They display more exploratory and less anxiety-related behaviors in the open field task than do wild type animals (Zhuang et al., 1999) and are similar to wild types in the TST and FST measures of depression (Jones & Lucki, 2005). On the other hand, the responsiveness of 5-HT$_{1B}$R-KO mice varies following treatment with antidepressant drugs, with multiple studies indicating that they do not exhibit a normal response (Chenu et al., 2008; O'Neill & Conway, 2001). Therefore, I predicted that 5-HT$_{1B}$R-KO mice would display normal basal sucrose preference which would decrease in response to chronic unpredictable stress (CUS) but would not recover following chronic treatment with fluoxetine.

The 5-HT$_{1B}$R-KO mice were on a sv/129-ter background which was unfortunately not commercially available. For my first experiment I used KO mice bred from homozygous mutant breeders and compared them to Sv129ImJ mice. Both groups showed a high basal sucrose preference which decreased following 3 weeks of chronic unpredictable stress. Interestingly, the control mice recovered their sucrose preference following 3 weeks of chronic fluoxetine treatment during which they continually stressed, but the 5-HT$_{1B}$R-KO mice remained low in sucrose preference (Figure 6.2A). I then repeated this experiment on homozygous wild type and homozygous mutant animals that came from SV129ImJ/5-HT$_{1B}$R-KO heterozygous parents. As with the previous

experiment, both knockout mice and wild type littermates had high basal sucrose preference, indicating a normal hedonic state. Following 5 weeks of CUS both groups of animals showed a lower sucrose preference which was recovered in the wild type animals following 3 weeks of fluoxetine treatment but not in the knockout animals (Figure 6.2B). These data indicated that 5-HT_{1B}R-KO mice are not depressed basally, respond normally to chronic unpredictable stress but are unresponsive to chronic fluoxetine treatment.

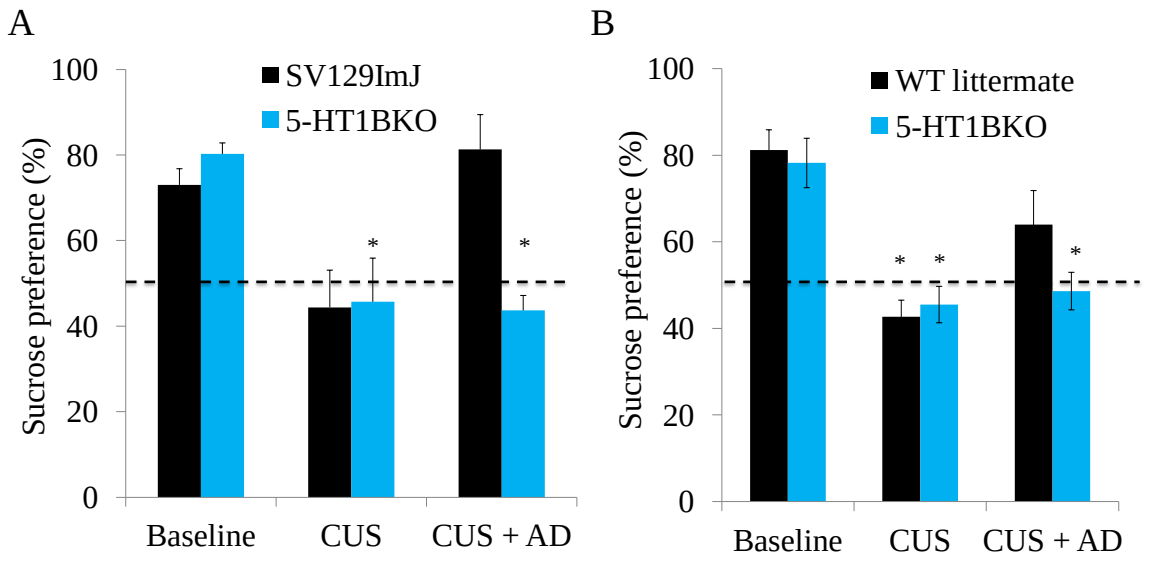


Figure 6.2 5-HT$_{1B}$R-KO do not respond to chronic antidepressant treatment in the sucrose preference test. A) A Two-factor ANVOA found a significant effect of group, F(1)= 4.11, p= 0.04, condition, F(2)= 3.25, p= 0.004, and a significant interaction, F(2)= 5.76, p= 0.007. Control mice (black) did not significantly differ from each other during any of the three conditions, but 5-HT$_{1B}$R-KO mice (blue) showed a significantly lower sucrose preference following CUS t(12)= 3.34, p= 0.006, which recovered following 3 weeks of fluoxetine treatment. B) A Two-factor ANOVA did not find a significant effect of group, but did find a significant effect of condition, F(2)= 3.40, p< 0.001, and no interaction. Wild type (black) exhibited a high sucrose preference which decrease significantly following CUS, t(8)= 6.33, p= 0< 0.001, but recovered with chronic fluoxetine treatment. 5-HT$_{1B}$R-KO mice (blue) also exhibited a high basal sucrose preference which significantly decreased after CUS, t(8)= 3.99, p= 0.004 and remained significantly lower after chronic fluoxetine treatment, t(8)= 3.55, p= 0.008.

Prediction 3: GluA1 S831A mice do not respond to chronic fluoxetine treatment.

I have proposed that 5-HT_{1B}R activation results in a downstream signaling cascade that phosphorylates serine 831 of the GluA1 subunit of the AMPA receptor. To test whether this step of the 5-HT_{1B}R signaling pathway is involved in antidepressant efficacy, I conducted a series of tests on the S831A mouse, a mouse with an alanine mutation at serine 831, rendering the site incapable of being phosphorylated. These mice do not display any gross morphological abnormalities and exhibit normal SC-CA1 LTP (Lee et al., 2010), but show decreased motivational behavior (Crombag et al., 2008). Interestingly, animals with a complete knockout of the GluA1 subunit exhibit a depressed phenotype in the learned helplessness task (Chourbaji et al., 2008), however, unlike these mice, S831A mice do not show any changes in overall GluA1 protein expression and do not exhibit changes in basal AMPAR mediated currents (Lee et al., 2010), therefore it is difficult to predict whether the basal affective state of these animals would be the same as the GluA1 knock out animals. I did however predict that if 5-HT_{1B}R mediated potentiation of AMPAR responses is necessary for the therapeutic effects of antidepressants, S831A mice that do not show this type of potentiation (Figure 4.3), would not respond to fluoxetine in the sucrose preference test.

To test this prediction I measured sucrose preference in both S831A homozygous animals bred from homozygous parents, and C57BL6/J wild type inbred mice (Jackson Laboratory) and then subjected the animals to 3 weeks of chronic unpredictable stress (CUS), followed by three weeks of CUS and fluoxetine treatment. Wild type animals exhibited a significant decrease in sucrose preference after 3 weeks of CUS compared to baseline, which recovered with 3 weeks of fluoxetine treatment. Interestingly, at baseline,

the S831A mice displayed a significantly lower sucrose preference than wild types, indicating a depressed phenotype. Therefore, these animals were not subjected to CUS but were given 3 weeks of fluoxetine treatment to assess whether the antidepressant could rescue the depressed phenotype. S831A mice treated with fluoxetine for 3 weeks remained significantly lower in sucrose preference than wild types at baseline, and trended towards being different from wild types treated with fluoxetine. Additionally, S831A mice exhibited a significantly longer latency to eat than controls, in the novelty suppressed feeding (NSF), a hippocampus-dependent task that is sensitive to chronic stress and antidepressant treatment (Dulawa & Hen, 2005). Together these data suggest that S831 phosphorylation is necessary for maintenance of basal affective state and is also necessary for the therapeutic actions of antidepressants.

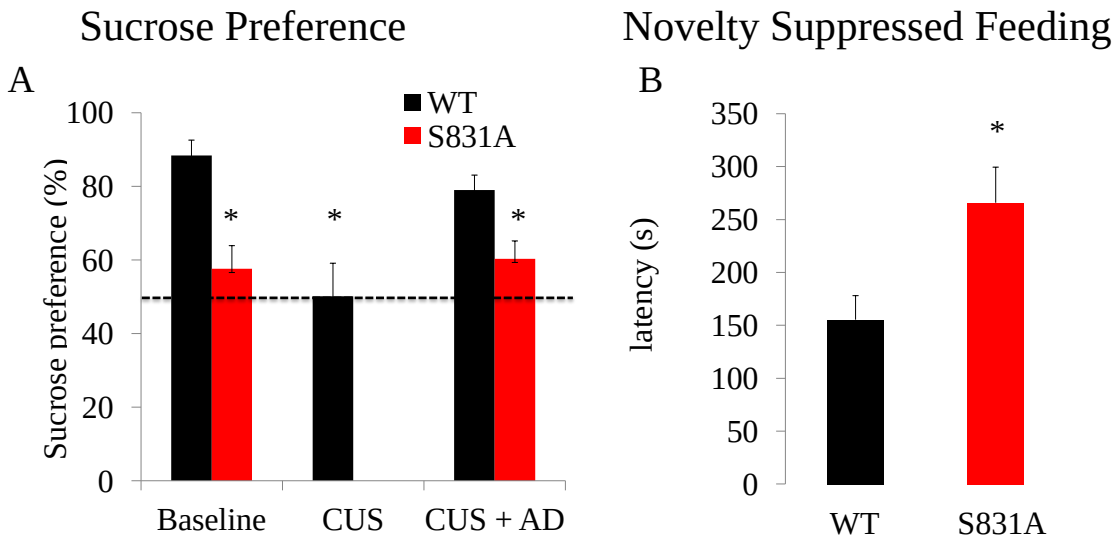


Figure 6.3 S831A mice exhibit a depressed phenotype that does not recover with chronic or acute antidepressant treatment. A) A repeated-measures ANOVA revealed a significant effect of condition on the control animals, $F(19)= 3.59$, $p < 0.001$. Control mice (black) showed a significant decrease in sucrose preference following CUS, $t(12)= 4.60$, $p < 0.001$, that recovered following 3 weeks of fluoxetine administration. Baseline sucrose preference in the wild types was significantly lower than S831A mice (red) at baseline $t(19)= 3.37$, $p= 0.003$, and after 3 weeks of fluoxetine treatment, $t(19)= 3.50$, $p= 0.002$. There was a trend for S831As treated with fluoxetine to differ from wild types in the CUS + AD condition, but the difference was not significant $t(18)= 1.95$, $p= 0.06$. B) S831A mice (red) showed as significantly higher latency to eat in the novelty suppressed feeding task compared to wild type mice (black), $t(28)= -2.10$, $p= 0.04$. *indicates significantly different than wild type ($p< 0.05$)

Following this experiment I repeated the procedure on a set of homozygous mutants and wild type littermates obtained by breeding heterozygous parents; however this experiment yielded conflicting results compared to Figure 6.3A. S831A mice were not significantly different from littermates at baseline. Therefore I again attempted to test the prediction that S831A mice are unresponsive to antidepressant treatment following CUS. Unfortunately, neither the S831A mice, nor the wild type littermates displayed a decrease in sucrose preference after 5 weeks of CUS (Figure 6.4A). This inability to decrease sucrose preference in C57BL6J mice is not unexpected, in fact others have shown that this strain does not decrease sucrose preference with up to 8 weeks of stress (Pothion et al., 2004). Additionally, the novelty suppressed feeding task performed in these mice also yielded inconclusive results. While the latencies for these mice tended to differ in the same direction as the results presented in Figure 6.3B, the differences were not significantly different (Figure 6.4B). In fact, none of the S831A mice ate for the entirety of the test (400s), and only a few wild type littermates completed the task. Therefore, I performed a series of different behavioral tests to examine the basal affective state of S831A mice compared to wild type littermates and their responsiveness to antidepressants.

S831A mice and wild type littermates did not differ from each other in the open field test in regard to time spent in the center (Figure 6.4D). This is a measure of anxiety-related behavior and therefore indicated that S831A mice are not anxious. These mice did however display fewer line crossings than littermates (Figure 6.4E), which points to a hypoactive phenotype. This may indicate a depressed phenotype, as others have shown that animal models of depression exhibit decreased line crossings and shorter distances

traveled in the open field test (Pistovcakova, Makatsori, Sulcova, & Jezova, 2005; Will, Aird, & Redei, 2003). Finally, I performed a tail suspension test in S831A mice and littermates. This test is a measure of behavioral despair, and less time spent immobile corresponds to responsiveness to antidepressant drugs (Gould, 2009). Wild type littermates administered 30mg/kg of the tricyclic antidepressant imipramine exhibited a significant decrease in time spent immobile (Figure 6.4C). S831A mice treated with imipramine showed a significant decrease in immobility compared to saline injected S831A mice as well. Together, this may signal that S831A mice are responsive to acute antidepressant treatment but not chronic treatment.

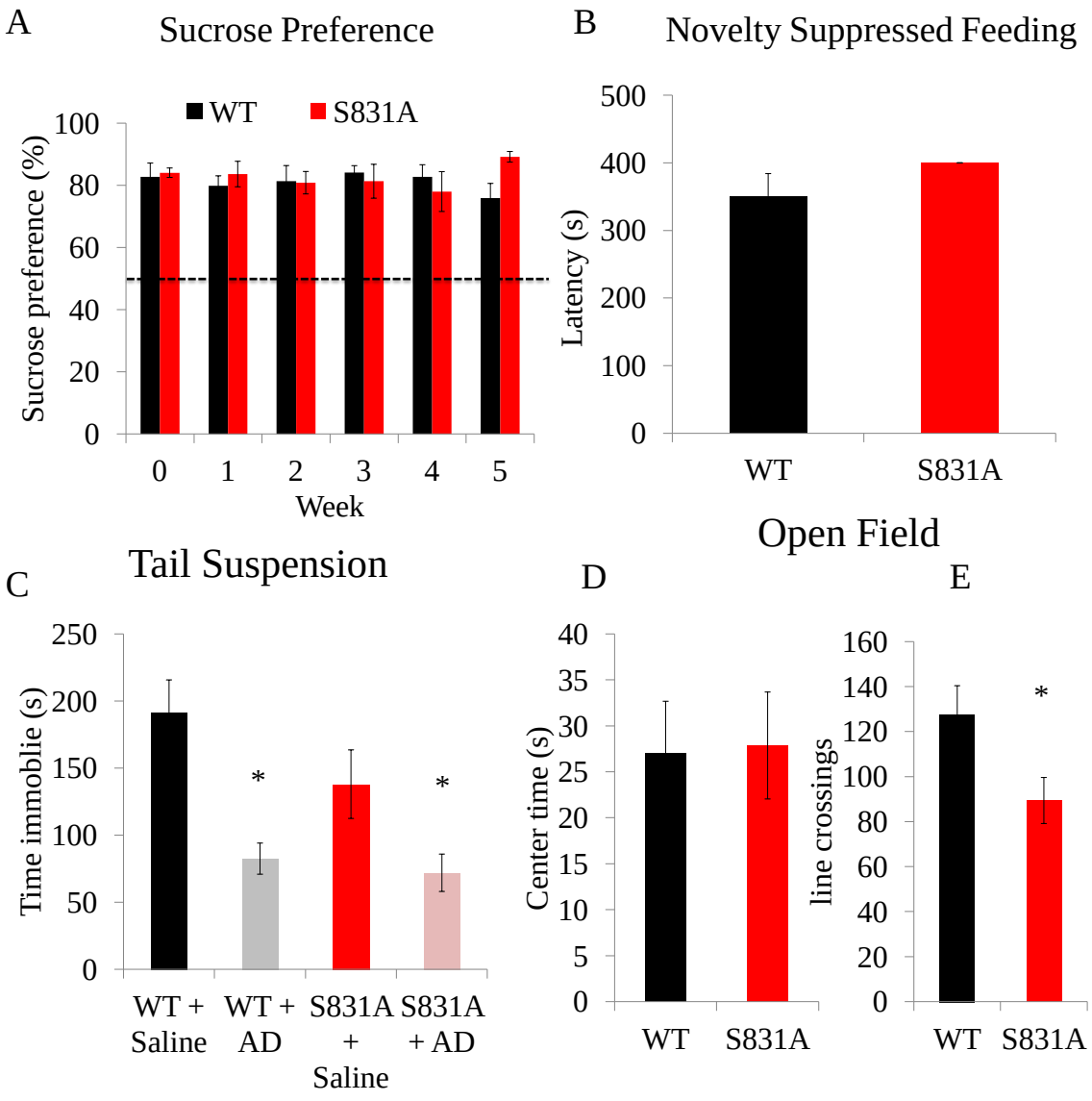


Figure 6.4 Behavioral data from S831A and wild type littermates yields conflicting results. A) There was no change in sucrose preference in S831A mice (red) or C57BL6J littermates following 5 weeks of CUS. B) S831A mice had a higher latency to eat than wild type littermates, however this was not statistically significant. C) A one-way ANOVA showed a significant group effect in the tail TST, $F(26)= 7.70$, $p= 0.001$. C57BL6J littermates treated with imipramine (gray) had a significantly lower amount of time spent immobile compared to wild types treated with saline (black), $t(10)= 4.10$, $p=$

0.002. S813A mice treated with imipramine (pink) were significantly lower in time spent immobile compared to S831A mice treated with saline (red), $t(13)= 2.35$, $p= 0.04$. D) S831A (red) did not differ from littermates (black) in regard to time spent in the center in the open field test. E) S831A mice (red) completed significantly fewer line crossings than wild type littermates (black) in the open field test, $t(13)= -2.29$, $p= 0.04$. * indicates significantly different than wildtype/saline ($p < 0.05$)

DISCUSSION

I have previously found that activation of serotonergic 5-HT_{1B}Rs results in a signaling cascade that phosphorylates the S831 residue of the AMPAR GluA1 subunit. I set to test the hypothesis that activation of the 5-HT_{1B}R and subsequent phosphorylation of S831 are necessary for the therapeutic effects of ADs. I designed experiments to test predictions of this hypothesis and found that both pharmacological and genetic inactivation of the 5-HT_{1B}R blocked the effect of fluoxetine in the sucrose preference test. Additionally, I found that S831A phospho-mutant mice exhibited a basal depressed phenotype in a number of measures and were insensitive to chronic fluoxetine treatment.

Although much work has been done on the role of 5-HT_{1B}Rs on the affective state of animals, both genetic and pharmacological manipulations have yielded inconclusive results. Most of the tests conducted on these animals have investigated their responsiveness to an acute injection of antidepressant on the forced swim test and tail

suspension test, but none have evaluated the effects of chronic stress and chronic antidepressants on these mice.

Blocking 5-HT$_{1B}$Rs does not affect basal sucrose preference, but does block response to chronic antidepressants.

The current literature using 5-HT$_{1B}$R drugs on depression tasks is incomplete and inconsistent. Thus, far researchers have only focused on tasks that are sensitive to acute and not chronic antidepressant treatments such as the forced swim test and tail suspension test. I found that 5-HT$_{1B}$R-KO mice, as previously reported (Bechtholt, Smith, Gaughan, & Lucki, 2008), and mice treated with the 5-HT$_{1B}$R antagonist SB224289, did not exhibit any difference in sucrose preference compared to wild type mice. Although 5-HT$_{1B}$R-KO mice are more aggressive than controls (Saudou et al., 1994) it appears that their basal affective hedonic state is not altered. This is somewhat in conflict with the findings of Svenningsson and colleagues who found that knocking out p11, which decreases surface 5-HT$_{1B}$R expression, results in a depressed phenotype in the tail suspension test (Svenningsson et al., 2006). This may be due to an effect of p11 that is not connected to 5-HT$_{1B}$Rs. In addition to an association with 5-HT$_{1B}$Rs, p11 also regulates a number of different molecules including annexin II (Réty et al., 1999) and acid sensing ion channels (Donier, Rugiero, Okuse, & Wood, 2005). It is also important to note that this study used the TST to indicate a depressed phenotype, even though this test is better used to measure of antidepressant response and not basal affect. This finding, in conjunction with what others have shown, suggests that 5-HT$_{1B}$R-KO mice are not depressed (Bechtholt et al., 2008; Jones & Lucki, 2005) and that alterations in 5-HT$_{1B}$Rs observed in depressed patients may be a compensatory or unrelated change, rather than the cause of depression.

Interestingly, it also appears that 5-HT_{1B}Rs are not required for the manifestation of depressive symptoms following chronic stress. Previous studies have shown that chronic stress can result in an increase in 5-HT concentration in the hippocampus (Amat, Matus-Amat, Watkins, & Maier, 1998; Keeney et al., 2006), however it does not appear that 5-HT_{1B}Rs are involved in regulating this pathway in a way that affects sucrose preference.

Although blocking 5-HT_{1B}Rs does not affect susceptibility to stress, it does appear that these receptors are necessary for AD efficacy in the sucrose preference test. Previous work has suggested that antidepressant drugs work through postsynaptic 5-HT_{1B}Rs to exert their effects on the forced swim test (Chenu et al., 2008). My data further supports this finding in that mice subjected to either social defeat stress or chronic unpredictable stress were unresponsive to fluoxetine in the sucrose preference test if their 5-HT_{1B}Rs were either pharmacologically blocked or genetically knocked out. These data provide support that 5-HT_{1B}Rs are necessary for not only acute AD action, but for chronic AD action as well. The other commonly used test that is sensitive to chronic but not acute AD treatment, the novelty suppressed feeding test, unfortunately cannot be used with these mice. 5-HT_{1B}Rs have been shown to regulate feeding and satiety in mice (Bouwknecht et al., 2001; Halford & Blundell, 1996), and which would confound a test that relies on hunger as motivation. In summary, these data present strong evidence that 5-HT_{1B}Rs are necessary for the therapeutic actions of ADs. This is in contradiction to proposals that suggest that 5-HT_{1B} autoreceptors limit the efficacy of antidepressants by titrating the concentration of serotonin in the cleft. The data presented here suggest that while this may be true, 5-HT_{1B}Rs in some capacity (e.g., acting heterosynaptically) must be present. Unfortunately, there are currently no drugs available that can distinguish

between 5-HT_{1B} autoreceptors and heteroreceptors. Based on these data and the current 5-HT_{1B}R literature, it seems that a drug with agonistic activity at heteroreceptors and antagonistic effects at autoreceptors would be the best type of antidepressant drug. Additionally, identifying signaling molecules specific to heterosynaptic 5-HT_{1B}R activation will also be beneficial in the progress of antidepressant generation.

Glutamate receptor phosphorylation is important for basal affect and antidepressant efficacy.

The GluA1 subunit of the glutamatergic AMPAR was identified as one of the downstream targets of postsynaptic 5-HT_{1B}Rs in Chapters 3 and 4. The idea that regulation of glutamate receptors are involved in antidepressant efficacy is not a novel idea; however they have not been previously linked to 5-HT_{1B}R activation except in localization (Peddie et al., 2010). Here I present evidence that phosphorylation of serine 831 of the GluA1 subunit of AMPARs is important for basal affective state and responsiveness to antidepressants. Previous studies have shown that knocking out the GluA1 subunit results in a depressed phenotype (Chourbaji et al., 2008) and enhancing AMPAR function through a potentiator can cause antidepressant effects in itself (Li et al., 2001) and cause synergistic effects when used in combination with traditional antidepressant drugs (Li et al., 2003). Interestingly, no one had measured the effects of antidepressants on GluA1 mutant mice, however. I found that animals with an alanine mutation at S831, exhibited a depressed phenotype in the sucrose preference test and novelty suppressed feeding test compared to wild type mice. Additionally, 3 weeks of fluoxetine treatment did not enhance sucrose preference in these mice, while it did in wild types that had been subjected to chronic stress. These conclusions from these tests,

which measure anhedonia and motivation, are further supported by the finding that S831A mice respond less to reward in an appetitive incentive learning task (Crombag et al., 2008). The S831A mice also exhibited a hypoactive phenotype in the open field test, a behavior that has been shown in other depression models (Pistovcakova et al., 2005). Furthermore, S831A may respond to acute antidepressant treatment in the tail suspension test even though they do not respond to chronic treatment in the sucrose preference test. It is important to note that although the tail suspension test has been widely used for the screening of antidepressants, it is somewhat controversial in that it responds to acute antidepressant injection despite the fact that humans only show responsiveness after weeks of administration. It is possible that in general effects seen in the tail suspension test are more of a side effect rather than true indication of antidepressant efficacy.

While these findings are exciting and provide added support for a novel hypothesis of depression, there are some caveats. It is important to note that the sucrose preference and novelty suppressed feeding tasks did not yield the same results when conducted in littermate controls as opposed to separate inbred controls on the same background. The sucrose preference test gave conflicting results with the finding that S831A mice and C57BL6J littermates had similar basal sucrose preferences. This experiment in the end gave inconclusive results in that neither the S831A mice nor the C57BL6J littermates lost sucrose preference after an extended chronic unpredictable stress paradigm. One reason for this may be that the C57BL6 background is somewhat resilient to stress. A paper comparing different strains of mice in the sucrose preference test found that these mice maintained a high sucrose preference even after 8 weeks of chronic stress (Pothion et al., 2004). Others have shown that these mice are capable of

losing sucrose preference, however only about half of the mice decrease preference below 65% after 4 weeks of chronic stress (Strekalova, Gorenkova, Schunk, Dolgov, & Bartsch, 2006). These discrepancies may be due to a number of factors including, temperature, lighting and time of day during which the tests are being conducted. These factors are likely to be the cause of the different results I found in the novelty suppressed feeding test as well. The second test, conducted in littermate mice, was inconclusive because of a ceiling effect. None of the S831A mice tested ate for the entirety of the test, while only a few of the control littermates completed the task. This same result was observed when the mice were given 600s to complete the test (data not shown).

The best course of action in terms of these experiments would be to conduct them in a more stable animal. Unfortunately, to date there is no transgenic rat model that could definitively answer the questions asked in this chapter. The BABL/c mouse is more sensitive to stress than other strains and therefore may yield more consistent results with chronic unpredictable stress (Cryan & Holmes, 2005). This mouse also has a serotonin transporter deficiency however (Zhang et al., 2004), which may present a confounding factor. Ultimately it does not appear that any one mouse strain is more reliable than others when factoring in consistency across different laboratories and over time (Wahlsten, Bachmanov, Finn, & Crabbe, 2006). Therefore, it seems that one of the few options is to repeat this experiment with a much larger number of animals with the goal of finding 50% of the animals to susceptible to stress. Additionally, other measurements can be made that may be more reliable than sucrose preference, such as performance in the learned helplessness paradigm, forced swim test and evaluation of coat state.

Another issue with this experiment may be a small effect size that is difficult to detect, in regard to the basal sucrose preference differences. The S831A mutation does not significantly alter AMPAR function in that both basal current and LTP is normal in these animals (Lee et al., 2010). It would be interesting to either obtain S831A, S845A double phospho-mutant animals, or GluA1 knockout animals to evaluate sucrose preference and other tests of depression that have never been measured in these animals. It seems that these animals would be better choices in testing the prediction that a deficit in glutamatergic signaling underlies the etiology of depression.

Despite the setbacks encountered in these experiments, there is some evidence that both 5-HT_{1B}R activation and GluA1 phosphorylation are necessary for the therapeutic actions of antidepressants. Together these data give credence to the hypothesis that glutamatergic dysfunction is the main culprit in the etiology of depression and antidepressants act indirectly on this system through activation of 5-HT_{1B}Rs.

CHAPTER SEVEN

DISCUSSION

Despite the millions of dollars invested in antidepressant drug development, the progress towards a better treatment has been stunted for the past several decades. Here, I sought to reconcile the oldest theory of depression, the monoaminergic theory, with new findings suggesting a role for glutamatergic dysfunction. I hypothesized that glutamatergic dysfunction underlies the etiology of depression and that traditional antidepressant medications exert their therapeutic effects by restoring this defect. First, I predicted that serotonin would be capable of modulating glutamatergic transmission. Second, I predicted that serotonin would affect excitatory synapses in a similar manner to long-term potentiation. Third, I predicted that animals subjected to a chronic stress paradigm would exhibit decreased glutamatergic function which would be restored with chronic antidepressant treatment. Finally, I predicted that the components of the serotonin-induced potentiation pathway would be necessary for the behavioral effects of antidepressants. The experiments I designed to tests these predictions support my hypothesis and provide insight into the underlying cause of depression with direction and hope for novel therapeutics.

The study of depression has centered on the monoaminergic theory of depression for over 50 years, but has made exciting new advances in the last decade. While the serotonin hypothesis of depression has aided in the generation of useful therapeutics, there are still many gaps and inconsistencies in this theory. Mainly, the lack of consistent serotonergic abnormalities in depressed brains and the long therapeutic latency of serotonergic antidepressants have caused many to search for a better theory. More recently, the neurotrophic and glutamatergic theories of depression have aided in providing insight into the sources of dysfunction in a depressed brain and targets for

novel and more effective therapies. Specifically, it appears that depressed patients and animal models of depression exhibit atrophy of the hippocampus, coinciding with changes in glutamate receptor mRNA levels. These disturbances and behavioral phenotypes are restored by both neurotrophic and glutamatergic agents. While proponents of this theory have shed a new light on depression, they have not yet reconciled their data with the mononaminergic theory of depression. How do serotonergic antidepressants work if they do not have any known affinity for glutamate receptors?

Functional consequences of serotonergic modulation of glutamate.

While previous research has shown that serotonin is capable of enhancing postsynaptic glutamatergic receptor phosphorylation (Du et al., 2004; Svenningsson et al., 2002), studies have failed to link this finding to changes in synaptic responses. Because these studies relied on western blotting of whole hippocampal homogenates, and no electrophysiology was conducted, it was difficult to deduce the functional consequences of serotonin on glutamatergic cells. Additionally, these studies did not identify the receptor population responsible for the phosphorylation of glutamatergic AMPA receptors. My results have shown that enhancing endogenous serotonin results in a specific enhancement of temporoammonic (TA)-CA1 extracellular synaptic current (detected as the fEPSP) that is the result of 5-HT_{1B}R activation. This potentiation of TA-CA1 fEPSP slope by the 5-HT_{1B}R did not occur at neighboring Schaffer collateral (SC)-CA1 synapses and only affected the AMPAR component of the response. Previous research has implicated heterosynaptic 5-HT_{1B}Rs as crucial for antidepressant efficacy (Chenu et al., 2008), although there is little understanding of the signaling pathways employed by this subgroup of receptors. Interestingly, I found that the 5-HT_{1B}R agonist

anpirtoline increases serine 831 but not serine 845 phosphorylation of GluA1 at TA-CA1 synapses, with a time course paralleling the electrophysiological potentiation. This phosphorylation of the AMPAR was accompanied by phosphorylation of CamKII and ERK as well, two signaling molecules implicated in synaptic plasticity (Hall & Ghosh, 2008) and antidepressant action (Almeida et al., 2006). These data present a novel pathway through which endogenous serotonin can activate postsynaptic 5-HT_{1B}Rs and induce potentiation of glutamatergic synapses.

Prior to the development of the glutamatergic theory of depression, the neurotrophic theory of depression had postulated that synaptic plasticity is crucial for the therapeutic actions of antidepressants. The proponents of this theory focused mainly on the role of molecules such as brain derived neurotrophic factor (BDNF) and CREB but did not identify a candidate serotonin receptor capable of increasing neuroplasticity. In my experiments I have shown that activation of the 5-HT_{1B}R can result in potentiation of glutamatergic synapses in a way that mimics long-term potentiation (LTP). It has previously been proposed that LTP, and specifically, enhanced AMPAR function, is involved in the synthesis of BDNF mRNA and cell proliferation (Bai, Bergeron, & Nelson, 2003; Kang et al., 1997; Mackowiak et al., 2002). My research provides a pathway through which traditionally antidepressant drugs may enhance plasticity and cell proliferation in the brain.

It is interesting to note that while 5-HT_{1B}R-enhanced synaptic plasticity may be beneficial for depression in human patients or animal models, it may not be so for normal patients/naïve animals. Normal animals chronically treated with antidepressants did not exhibit anpirtoline induced potentiation acutely, presumably because their synapses had

already been saturated. Additionally, activation of 5-HT_{1B}Rs resulted in occlusion of electrically induced LTP in control animals, also due to saturation. Physiologically, AD treatment may have detrimental effects on LTP-dependent behaviors. Specifically, LTP at the TA-CA1 synapse has been implicated in the consolidation of long-term memory (Remondes & Schuman, 2004). Therefore, occluding or blocking LTP by ADs may cause memory deficits. Interestingly, it has previously been shown that an intrahippocampal injection of a 5-HT_{1B}R agonist causes a deficit in learning in the Morris water maze task (Buhot et al., 1995). In fact, there is evidence that healthy volunteers administered antidepressant drugs exhibit impairments in memory (Thompson, 1991). The key difference between beneficial versus detrimental effects of antidepressants is likely to be dependent on basal synaptic strength. If synapses are weakened by chronic stress, then rescuing function with antidepressants will result in a phenotype similar to healthy patients/animals. If however synapses are already functioning normally, the stimulation of serotonin-induced potentiation results in saturation of the synapse and may prevent the synapse from responding to physiologically relevant stimuli in the future.

Depressed synapses in depressed animals?

One reason for the shift in thinking in depression research to neurotrophic factors came from the findings that hippocampal volume is decreased in depressed patients (MacQueen et al., 2003). This was further supported by animal studies finding hippocampal atrophy in animals subjected to chronic stress (Magarinos et al., 1996). NMDAR dependent excitotoxicity might be responsible for this atrophy. Glutamate levels are enhanced in both depressed patients and animal models of depression (Hashimoto, Sawa, & Iyo, 2007; Mitani et al., 2006). The higher affinity of NMDARs for

glutamate relative to AMPARs (Lester, Clements, Westbrook, & Jahr, 1990; Tang, Dichter, & Morad, 1989) means that this excess in glutamate will preferentially activate NMDARs. Overactivation of NMDARs has been a well-established cause of cell death (Mody & MacDonald, 1995) and may account for the hippocampal atrophy exhibited in depression.

Here I propose that in addition to the death of whole cells in the hippocampus, individual synapses of living cells are also weakened by chronic stress. Evidence for this comes from my finding that animals subjected to chronic unpredictable stress have lower AMPAR/NMDAR fEPSP ratios at the TA-CA1 synapse than control animals or stressed animals treated with antidepressants. It remains unknown whether the endocytosis or degradation of AMPARs is a cause or effect of chronic stress. Here I have reported a significant decrease in GluA1 in the hippocampus following chronic unpredictable stress. Additionally, both chronic and acute stress regulate the expression of AMPARs in the ventral tegmental area and prefrontal cortex (Fitzgerald et al., 1996; Yuen et al., 2009). Therefore it is possible that chronic stress induces a decrease in surface hippocampal AMPARs, increasing the ratio of NMDARs to AMPARs and making the cells more vulnerable to glutamate induced excitotoxicity. On the other hand, multiple studies have shown a decrease in GluA1 mRNA in depressed patients as well as a polymorphism in the GluA1 gene that is associated with vulnerability to stress (Beneyto et al., 2007; Schmidt et al., 2010). These findings suggest that a genetic defect in AMPAR functioning may underlie susceptibility to depression. This idea is further supported by my findings that animals with a phospho-mutation at S831 exhibit a depressed phenotype in the sucrose preference and novelty suppressed feeding tasks. Although these findings were

not replicated in littermates, this gives some indication that AMPAR hypofunctioning may underlie the behavioral deficits observed in depression.

These findings suggest that the key to treating depression is recovering AMPAR function in the brain. A recent study found that animals chronically treated with the antidepressant fluoxetine exhibited a time dependent increase in GluA1 mRNA in the hippocampus and prefrontal cortex (Barbon et al., 2011). This increase coincided with GluA1 protein increase in the hippocampus and prefrontal cortex that peaked at 2-3 weeks into administration, the same time course as the therapeutic window of antidepressants in humans (Katz et al., 2004). Therefore, it appears that decreased AMPAR function may underlie the mood changes in depression and the rate limiting factor of serotonergic antidepressants is the ability to enhance AMPAR function (Figure 7.1). This provides support for the development of new antidepressant drugs that target the glutamate system. Currently, NMDAR antagonists such as ketamine are being used in both animal models and patients, and provide an almost immediate but long lasting antidepressant effect (Maeng et al., 2008; Li et al., 2010; Li et al., 2011). Unfortunately, ketamine is a commonly abused drug and induces psychotic behavior at high doses and therefore may not be the best alternative to serotonergic drugs. Another option would be to target AMPARs; however, AMPARs are just as or more ubiquitous as serotonin receptors and using an agonist may cause non-specific effects that result in just as many side effects. AMPAR potentiators, which only enhance AMPAR function in the presence of glutamate, have been used in animal models of depression with promising results (Li et al., 2001; Schmidt et al., 2010), however it is unclear what the time course for efficacy in these experiments were and whether these drugs would work in humans. These drugs

may still be the best new avenue to explore for treating depressed patients that are resistant to serotonergic antidepressants and patients in need of immediate care. It may also be beneficial to develop a drug that specifically causes phosphorylation of GluA1 S831. Although it appears that S831 phosphorylation is necessary for antidepressant efficacy, it remains unclear if it is sufficient to induce antidepressant effects. Therefore, it would be necessary to perform further experiments before undertaking this task. It will also be important to identify downstream signaling components of serotonin induced AMPAR potentiation which may provide more specific and suitable targets for drug development.

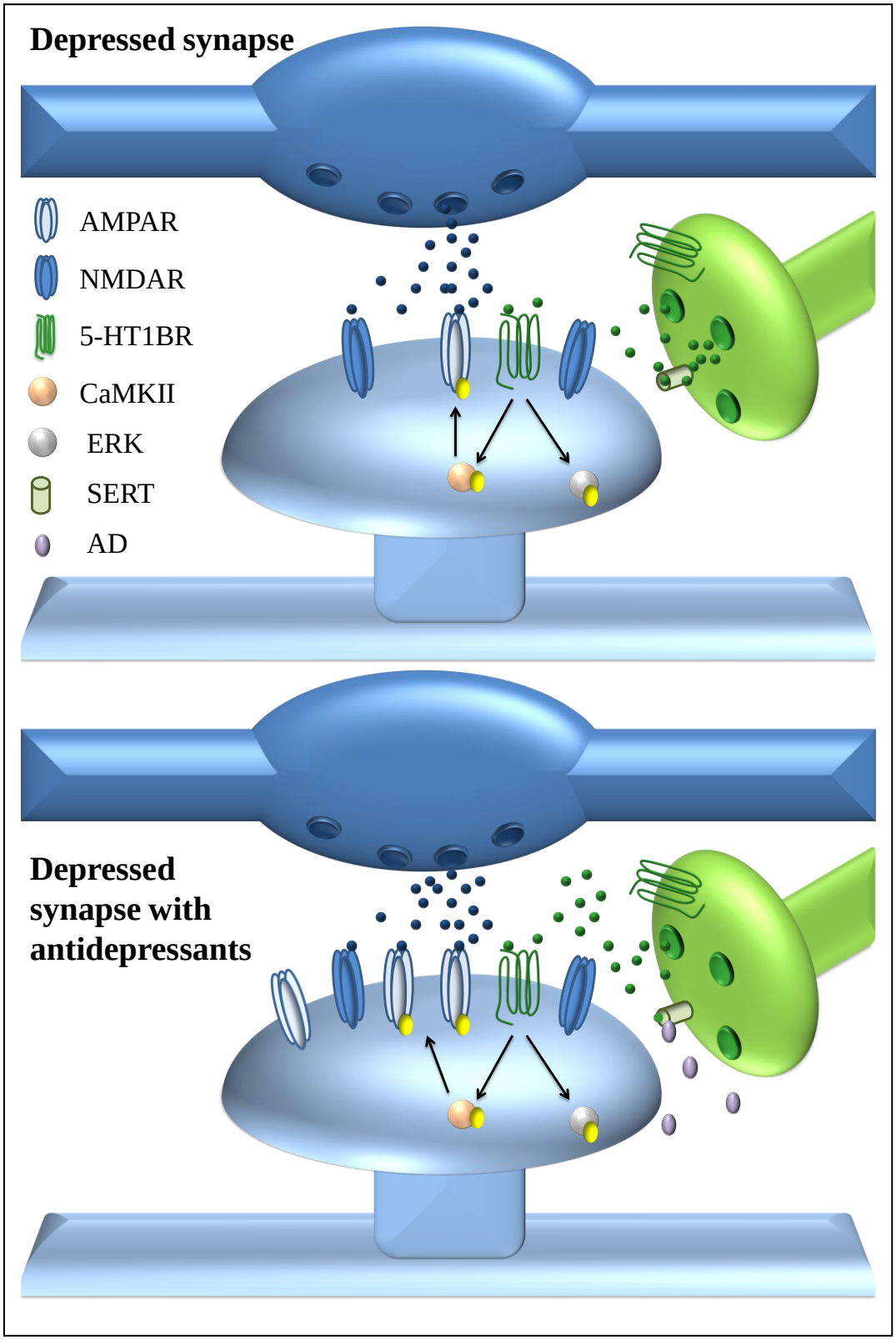


Figure 7.1 Schematic of proposed alterations in synaptic AMPARs in depressed animals/patients before and after antidepressant treatment.

What comes downstream of S831 phosphorylation?

My data have shown that in addition to GluA1 S831 phosphorylation, stimulation of 5-HT_{1B}Rs also increases phosphorylation of CamKII and ERK. It seems that both of these occur upstream and not downstream of enhanced AMPAR function, as blockers of these two molecules blocks anpirtoline induced potentiation. These molecules are also present in many cell types in many regions of the brain and therefore would likely be undesirable targets for antidepressants. One downstream consequence of enhanced AMPAR function is increased BDNF synthesis and cell proliferation (Bai, Bergeron, & Nelson, 2003; Legutko, Li, & Skolnick, 2001), it is unclear however whether phosphorylation of S831 is necessary and sufficient for this effect. One experiment currently in progress is evaluating whether S831A mice exhibit neurogenesis, as measured by BrdU staining, following chronic fluoxetine treatment. Additionally, it would be interesting to investigate whether 5-HT_{1B}R activation through anpirtoline is capable of enhancing neurogenesis itself.

It remains unclear in the neurotrophic theory of depression whether the antidepressant effects of BDNF act by increasing the birth of newborn cells, the survival of existing cells, or both. Many studies have suggested that neurogenesis is the necessary component of this pathway (Kempermann & Kronenberg, 2003; Santarelli et al., 2003), though recent findings have suggested antidepressants exert important neurogenesis-independent effects as well (Holick et al. 2008; David et al. 2009). It is possible therefore that one of the important roles of BDNF in the treatment of depression is increasing synaptic strength and consequently cell viability. In addition to AMPARs increasing BDNF synthesis, BDNF secretion can also increase surface expression of AMPARs (Li

& Keifer, 2009), therefore AMPARs and BDNF appear to form a self-perpetuating cycle. Further exploring the mechanisms and crucial components involved in this pathway may point to even more potential targets for the treatment of depression.

In conclusion, the experiments presented in this thesis support a new hypothesis that links the monoaminergic theory of depression to both the glutamatergic and neurotrophic theories of depression. Previously, these three theories provided viable explanations and antidepressant treatments for depression, yet did not provide a coherent model of depression. The results presented here provide evidence that despite popular belief, the root cause of depression may be a dysfunction in glutamatergic transmission and not serotonergic transmission. Many questions remain, including identifying the upstream processes which lead to glutamatergic dysfunction in depressed brains. However, finding a biological phenomenon which parallels affective behavior is an important piece of the puzzle. For the millions of people suffering from depression, recovery is a slow process involving many steps. The experiments presented here will hopefully not only aid in understanding this process but will enhance the efficiency of treatment for these individuals as well.

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