REGULATION OF GSK3 IN THE PATHOPHYSIOLOGY AND TREATMENT OF MOOD DISORDERS

by

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A DISSERTATION

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ABSTRACT

Mood disorders are devastating psychiatric illnesses that will affect as many as one in every five persons worldwide over the course of their lifetime. Significant gaps still remain in our understanding of these illnesses and outcomes for many patients are far from optimal. Glycogen Synthase Kinase 3 (GSK3) is a protein kinase that is increasingly recognized as playing an important role in a number of neuronal functions, as well as in pathological states such as mood disorders. GSK3 is inhibited in the brain by phosphorylation on an N-terminal serine. Several treatments for mood disorders, including lithium, antidepressants, and atypical antipsychotics increase this inhibitory phosphorylation, suggesting that it may be an important mechanism for the regulation of mood. In these studies, we investigate the role of serine phosphorylation of GSK3 in the treatment and pathophysiology of mood disorders.

To investigate the mechanisms of mood disorder treatment, we first examined activation of Akt, an upstream regulating kinase of GSK3, in response to serotonin. Both an acute increase in serotonin and chronic antidepressant treatment result in a functional activation of the Akt signaling pathway. Similarly, activation of 5-HT1A receptors were found to increase phosphorylation of GSK3 through activation of the PI3K/Akt pathway, and phosphorylation of GSK3 was found to be necessary for 5-HT1A receptor-mediated inhibition of conditioned fear memories. Antagonism of 5-HT2A receptors was found to inhibit GSK3 through activation of Akt, while activation of 5-HT2A receptors was found to increase inhibitory phosphorylation of GSK3 only in the absence of β-arrestin2.
Furthermore, loss of β-arrestin2 potentiates the antidepressant-induced increase in phosphorylated GSK3 as well as the behavioral response to antidepressants. Finally, we investigated the behavioral effects of decreased serine-phosphorylation of GSK3 using knock-in mice with GSK3 that is immune to serine phosphorylation. We found that loss of this phosphorylation resulted in increased behavioral susceptibility to induction of both manic- and depressive-like states.

The studies presented here demonstrate that serine phosphorylation of GSK3 plays a critical role in mood disorder pathology. This gives us novel insights into the regulation of mood, and may provide avenues for the development of more effective treatments for mood disorders.

Keywords: GSK3, Akt, Mood Disorders, Serotonin, Antidepressants, Behavior
DEDICATION

To my parents, David and Carol Polter. They encouraged my questions, taught me the value of hard work and of laughter, and have been with me every step of the way.
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INTRODUCTION

MOOD DISORDERS

Classification, Impact, and Symptoms

Mood disorders are a class of psychiatric illnesses that primarily consist of bipolar and major depressive disorders. Mood disorders are among the leading causes of disability worldwide (WHO 2001), with an overall lifetime prevalence of approximately 20% (Kessler et al 2005). The majority of this is due to Major Depressive Disorder (MDD), with a prevalence of approximately 17%, while the prevalence of bipolar disorder is approximately 1-3% (Kessler et al 2005; Weissman et al 1996). Major depression is the 6th leading cause of disability in the world and projected to be the 2nd leading cause by 2020 (Michaud et al 2001). It is estimated that annual costs from mood disorders in the United States, including medical expenses, lost productivity, and mortality costs from suicide are in the tens of billions of dollars (Greenberg et al 2003; Kleinman et al 2003).

Symptoms of depression consist of depressed mood, loss of interest in pleasurable activities, significant changes in weight or appetite, insomnia or hypersomnia, fatigue, decreased concentration, low self-esteem, irrational guilt, and thoughts of death or suicide (APA 1994). Persistence of most of these symptoms for at least two weeks constitutes a major depressive episode, and occurrence of one major depressive episode is sufficient for diagnosis of major depressive disorder. Multiple episodes lead to a diagnosis of recurrent
major depressive disorder. A related disorder is dysthymia, which consists of milder, long lasting depression, although there is little to distinguish the two.

Bipolar disorder (BD) is distinguished from MDD by the presence of manic episodes. A manic episode consists of symptoms of inflated self-esteem, decreased need for sleep, racing thoughts, increased goal-directed activity, and increased hedonic drive that significantly disrupt a person’s life (APA 1994). Milder forms of these symptoms that do not significantly disrupt social or occupational function are referred to as hypomaniac episodes. BD is further categorized as Type I that includes patients who have one or more manic episode, and Type II that consists of patients who have one or more hypomaniac episodes in addition to depressive episodes. Depression, although not required for diagnosis of BD, is a prominent feature of BD, especially in Type II BD. A milder form of the disease, cyclothymia, consists of cycles of hypomaniac and depressive symptoms that do not constitute a major depressive episodes.

Treatment and Outcomes

Treatment of Depression

Antidepressants have been used to treat depression for the last sixty years, since the discovery that the anti-tuberculosis medication iproniazid induced “irrational happiness” (Lopez-Munoz and Alamo 2009). It was discovered that iproniazid worked through inhibition of monoamine oxidase, preventing metabolism of monoamines such as serotonin. Since this time, antidepressant drug discovery has focused on the development of pharmacological agents capable of increasing the levels of monoamines, particularly serotonin. While monoamine oxidase inhibitors are highly effective antidepressants, they
also have a high risk of side effects and serious interaction with other drugs. The second generation of antidepressants included tricyclic antidepressants. These drugs inhibit the reuptake of serotonin and norepinephrine and are quite effective, but an unfavorable side effect profile limits their widespread use.

Modern antidepressants include selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine and serotonin-norepinephrine reuptake inhibitors (SNRIs) such as venlafaxine. These drugs are widely prescribed and have fewer side effects comparing to MAOIs and tricyclic antidepressants. They are helpful for a wide range of patients, although many patients do not proceed to a remission of all their symptoms, and some patients do not respond (Trivedi et al 2006). For patients who fail to respond to first-line antidepressants after appropriate time length of treatments, augmentation with other classes of drugs, such as atypical antipsychotics or lithium, is attempted (Marek et al 2003; Shelton et al 2010). Non-pharmacological treatments, including electroconvulsive therapy, vagal nerve stimulation, and repetitive transcranial magnetic stimulation have also proven effective for depression, although due to the higher incidence of side effects they are frequently reserved for patients who fail to respond to pharmacological treatments (Shelton et al 2010). Besides pharmacological treatments and neurostimulation, psychotherapy is an effective treatment for MDD. This was the primary mode of treatment for many years before the advent of pharmaceutical treatments. With the advance of pharmacological treatment, psychotherapy remains a role as an effective adjunctive treatment. However, even with augmentative therapies, rates of long-term recovery are disappointing. Recent large-scale studies, such as the STAR*D study, have revealed that only 67% of patients achieve remission after a year of pharmacological treatment (Rush et al 2006; Trivedi et al 2006). Therefore, despite
considerable progress in the development of antidepressant drugs and treatments, efficacy of these drugs is still less than optimal.

One major challenge in pharmacological antidepressants is that they do not provide immediate relief from depression. Indeed, antidepressants take several weeks to work. The precise reason behind this lag is unclear, but it has been hypothesized to involve adaptations in serotonin receptors (Blier 2001), neurotrophic mechanisms (Duman and Monteggia 2006), or antidepressant-induced increases in neurogenesis (Santarelli et al 2003). It is likely that it is these long-term neuroplastic mechanisms that underlie the therapeutic effect of antidepressants rather than direct changes in neurotransmitter levels. Despite the less than optimal efficacy of antidepressants, research into how they exert their actions and the links between changes in synaptic serotonin levels and long-term adaptations in neuroplasticity and behavior is vital for the identification of novel antidepressant targets, development of new drugs, and better understanding of underlying deficits in the disorder.

_Treatment of Bipolar Disorder_

BD is treated with distinct pharmacological agents from those used to treat MDD. Currently available treatments include mood stabilizers such as lithium, several anticonvulsants, and atypical antipsychotics. The precise pharmacological targets of mood stabilizers are unknown, but it is thought that they work through their effects on intracellular signaling cascades. Bipolar disorder is increasingly treated with atypical antipsychotics, which are combined antagonists at 5-HT2A and dopamine D2 receptors. Non-pharmacological methods of treating bipolar disorder have not shown great efficacy and remain to be investigated.
Outcomes for mood disorder patients

Outcomes for patients with mood disorders vary. With treatment, approximately 67% of MDD patients achieve remission within one year (Rush et al 2006; Trivedi et al 2006). A longitudinal population study showed that while half of MDD patients have no further depressive episodes, approximately 35% have recurrent episodes, and 15% of patients do not recover (Eaton et al 2008). Individual manic or depressive episodes in bipolar disorder can be successfully managed, but episodes often recur (Angst et al 2002; Kupfer 2005). This suggests that while current treatments can successfully resolve symptoms of manic or depressive episodes, the underlying disease remains present and symptoms commonly return. Both MDD and BD have much higher rates of suicide than the general population, with estimates ranging from 8-15% (Bostwick and Pankratz 2000; Guze and Robins 1970). This risk decreases considerably with treatment (Angst et al 2002; Baldessarini et al 2001; Goodwin et al 2003). In addition to increased mortality from suicide, patients with mood disorders have an increased risk of comorbid medical illnesses, such as diabetes (Knol et al 2006) and poorer outcomes in cardiovascular illness (Joynt et al 2003). Thus, mood disorders are common and serious illnesses that cause significant morbidity and mortality. A better understanding of their pathology and treatment is vital to relieving their effect.

Methods Used to Study Mood Disorders

Human Patients

As with many neurological disorders, MDD and BD can be exceptionally difficult to study in living patients. This is further complicated by the significant heterogeneity among patient populations, as well as the widespread co-morbidity of mood disorders with other
psychiatric and somatic illnesses. Despite this, important findings about the activity and connectivity of the brain have been made using functional imaging techniques such as PET and fMRI. Additional methods used to study mood disorders in human patients include the use of peripheral tissues, most commonly blood, to study expression of genes and proteins. Brain imaging and collection of peripheral tissues are minimally invasive and easily repeated, allowing changes to be followed over a lengthy period of time and correlated to changes in the patient’s behavior.

Lack of access to the human brain prevents ante-mortem study of tissues, which limits studies of protein or gene expression or high-resolution structural investigations. Postmortem brain tissue, therefore, presents a valuable resource for investigating questions of genetic and biochemical changes in the brains of depressed and bipolar patients. There are several important caveats to the use of postmortem brains. The foremost is that most patients with antemortem mood disorder diagnoses have spent considerable portions of their lives on psychotropic drugs, which induce significant changes in the brain. When differences are found between mood disorder patient brains and controls, it can be difficult to determine if they are due to disease or treatment. Additionally, the lag time between death and collection of brain tissue can make it difficult to study more labile proteins.

*Animal Models*

Another approach to the study of mood disorders is to use animal models. Although there are certainly features of mood disorders that are uniquely human, many of symptoms can be modeled with some validity in rodents. Particularly since the revolution in manipulation of the rodent genome, use of animal models has provided rapid progress. In modeling depression, behavior despair paradigms are commonly used models. In these tests,
such as the forced swim test (Kurtuncu et al 2005; Porsolt et al 1977) and the tail suspension test (Steru et al 1985), animals are placed in an inescapable, stressful situation and their efforts to escape are measured. Increased time spent actively struggling against the situation represents an antidepressant effect. Both of these measures reliably respond to antidepressants (Cryan et al 2002), although they have been criticized for the robust response animals have to acute treatment with antidepressants, as these drugs require chronic treatment in humans. A related test is the learned helplessness model, in which animals are repeatedly given an inescapable stressor, and their response when given a chance to escape is measured (Sherman et al 1979). Both acute and chronic antidepressants increase animals’ resilience in this protocol (Cryan et al 2002). Two more recent tests are novelty-induced hypophagia (Dulawa and Hen 2005) and social defeat stress (Berton et al 2006). The benefit of these tests is that they respond to chronic but not acute antidepressants; however, it is unclear whether the response is related to the antidepressant or anti-anxiety effects of these drugs.

Models of mania generally involve measuring locomotor activity or hedonic drive of animals. Many of these behaviors are sensitive to lithium and mood stabilizers (Gould and Einat 2007). Activity can be monitored at baseline levels, giving an indication of both circadian patterns and locomotor hyperactivity, or in response to psychostimulants or novelty. Additionally, susceptibility to the addictive and rewarding properties of psychostimulants can be measured using self-administration protocols and conditioned place preference paradigms. Thus, a variety of behavioral paradigms exist for modeling mood related behaviors in animals, and use of these methods, particularly in combination, can be highly useful in elucidating the pathophysiology of mood disorders. Care should be taken,
however, to avoid over-interpretation of results and continuing research into the development of novel animal models of mood will be highly valuable.

Mood Disorder Pathology

Brain Region Abnormalities

The pathophysiology of mood disorders is likely due to alteration of function in several brain regions. Changes in volume and activity have been observed in multiple regions, especially the hippocampus, prefrontal cortex, ventromedial striatum, and amygdala (Price and Drevets 2010). These regions have all been implicated in mediating the cognitive and emotional aspects of mood disorders. Decreased volume and increased activity in the orbital prefrontal cortex has been observed in mood disorder patients (Price and Drevets 2010), an effect that may lead to changes in ability to gauge reward and use that assessment to guide behavior (Padoa-Schioppa and Assad 2006; Rudebeck and Murray 2008; Schultz et al 1997). Also implicated in mood disorders is the medial prefrontal cortex. This region of the cortex receives strong input from the limbic system (Carmichael and Price 1995; Kondo et al 2005) and in turn has strong output to the hypothalamus and brainstem, which controls autonomic responses to emotional stimuli (Critchley et al 2000; Damasio et al 1990). Alterations in this circuit may contribute to chronic feelings of anxiety or unease that occur in mood disorder patients (Price and Drevets 2010). Similarly, alterations in inputs from the orbitofrontal cortex to the striatum may cause changes in goal-directed behavior in mood disorder patients (Goto and Grace 2005). One of the most well-repeated findings in patients suffering from mood disorders is a decrease in hippocampal volume in depressed patients that worsens with repeated depressive episodes and improves with treatment (Videbech and
These alterations may underlie cognitive deficits in mood disorders, as well as changes in emotional associations.

**Stress and mood disorders**

Several lines of evidence suggest that stress plays a role in mood disorders. Major depressive episodes are often preceded by stressful life events (Kendler et al 1999), while relapse of symptoms in bipolar patients is associated with stress (Hammen and Gitlin 1997). Early life stress can also render individuals more susceptible to develop depression as adults, particularly those with additional risk factors (Caspi et al 2003). Furthermore, depressed patients may have increased serum levels of the stress hormone cortisol, suggesting hyperactivity of the body’s stress response system (Wolkowitz et al 2009). Most animal models of depression rely on stressing the animal to induce a depressive-like behavioral profile. Although the mechanism of how stress may induce symptoms of mood disorders is unclear, evidence points to modulation of structural plasticity in the limbic areas of the brain. Stress has been shown to impair structural plasticity in the hippocampus of rodents and primates through cortisol signaling (McEwen and Magarinos 2001), and it has been suggested that stress-induced loss of hippocampal plasticity may be responsible for the hippocampal atrophy seen in depression (Sapolsky 2000).

**Neurotrophins**

Decreased neuroprotective factors may play a role in the pathology of depression. One crucial factor in this theory is the neurotrophin BDNF. Although it is unclear whether alterations in BDNF are a cause or effect of depression, a substantial body of evidence supports its role in the disorder.
A number of studies have reported decreases in BDNF in the rodent brain after stress (Duman and Monteggia 2006; Smith et al 1995) and in the serum of depressed patients (Karege et al 2002; Shimizu et al 2003). Chronic, but not acute antidepressant treatment increases BDNF in the rodent hippocampus (Nibuya et al 1995), and both direct infusion of BDNF into the hippocampus (Shirayama et al 2002) or peripheral injection of BDNF (Schmidt and Duman 2010) has antidepressant effects in rodent models of depression. Although loss of BDNF alone does not induce depressive-like behavior in rodents, deletion of BDNF in the forebrain (Monteggia et al 2004), and specifically in the dentate gyrus of the hippocampus (Adachi et al 2008) prevents the behavioral effects of antidepressants. These findings suggest a significant role for BDNF in the antidepressant response, but the antidepressant effects of BDNF are likely specific to brain region. Global overexpression of BDNF leads to an antidepressant-like effect but also increased anxiety (Govindarajan et al 2006). In the ventral tegmental area, BDNF has pro-depressive effects (Eisch et al 2003), and VTA-specific knockout of BDNF prevents development of a depressive state in the social defeat stress model of depression (Berton et al 2006). Additionally, evidence also implicates BDNF in bipolar disorder. Notably, a polymorphism in BDNF has been associated with increased risk of BD (Neves-Pereira et al 2002; Sklar et al 2002). Male, but not female, mice with conditional forebrain loss of BDNF show hyperactivity (Monteggia et al 2007). Therefore, BDNF may play a role in mood-related behaviors in a region-specific manner.

Because BDNF is globally expressed, future studies examining the regionally specific signal transduction pathways necessary for BDNF’s effects on behavior will be valuable in unraveling the complex role BDNF plays in regulation of mood related behavior. Abnormalities in intracellular signaling downstream of neurotrophins have also been
implicated in mood disorders. These include the Protein Kinase C (PKC) (DiazGranados and Zarate 2008; Einat and Manji 2006; Friedman et al 1993; Lenox et al 1992) and the Mitogen Activated Protein Kinase (MAPK) (Duman et al 2007; Einat et al 2003; Engel et al 2009; Mazzucchelli et al 2002; Selcher et al 2001) cascades, Bcl-2 and its substrate Bag1 (Chen et al 1999b; Einat and Manji 2006; Einat et al 2005; Kosten et al 2008; Maeng et al 2008; Rondi-Reig et al 1997; Zhou et al 2005), and Glycogen Synthase Kinase 3 (Li and Jope 2010). These pathways represent a considerable opportunity for developing novel therapeutics, especially since signaling molecules are amenable as targets of small molecule inhibitors.

Alterations in neurotransmission

Several neurotransmitter systems have been implicated in mood disorders. Dysregulation of the dopaminergic system has been suggested to underlie the anhedonia commonly seen in depression and the enhanced hedonic drive in manic states (Nestler and Carlezon 2006). In animal studies, behaviors induced by dopamine-increasing psychostimulants are used as models of mania, and psychostimulant withdrawal is used as a model of depression. Recent studies also show alterations in glutamate receptors in the postmortem brains of patients with mood disorders (Beneyto et al 2007). Thus, modification of glutamatergic neurotransmission may have promise in treating mood disorders. For example, potentiation of AMPA signaling has an antidepressant effect in the forced swim test (Knapp et al 2002; Li et al 2001; Quirk and Nisenbaum 2002), while inhibition of AMPA receptors inhibits the hyperactivity and reward learning induced by psychostimulants (Backstrom and Hyytia 2003; Harris and Aston-Jones 2003; Ossowska et al 2004; Vanover 1998). Furthermore, intravenous administration of the NMDA receptor antagonist ketamine
has a rapid and long-lasting antidepressant effect in humans (Zarate et al 2006). These data suggest that alterations in glutamate signaling may play a role in mood regulation.

While recent advances have resulted in significant strides in the understanding of mood disorders, there is a considerable amount that remains unknown. Therefore, this project has focused on the study of two crucial areas: the brain’s serotonergic system, and the signaling molecule Glycogen Synthase Kinase 3 (GSK3).

SEROTONIN SIGNALING

Serotonin and mood disorders

The early observations that antidepressants prevent the reuptake of monoamines, particularly serotonin (5-HT) led to the hypothesis that depression is caused by decreases in the levels of serotonin in the brain. Considerable studies have been done by assessing the levels of serotonin and its metabolites in the blood, CSF, and postmortem tissue of depressed patients, but no consensus has been reached. Furthermore, monoamine depletion in healthy patients has no effect on mood, although it does cause relapse of symptoms in recovered depressed patients who are taking antidepressant (Ruhe et al 2007). Although consistent alterations in serotonin levels have not been found, significant evidence from post-mortem and in vivo brain imaging suggests that brains of depressed patients have an imbalance of serotonin receptors, in general an increase in 5-HT2A receptors and a decrease in 5-HT1A receptors (Stockmeier 2003).
Metabolism, Clearance, and Circuitry

Serotonin is a monoamine neurotransmitter synthesized from the amino acid tryptophan. The rate-limiting step of this synthesis is the conversion of tryptophan to 5-hydroxytryptophan (5-HP). In the brain, this reaction is controlled by the enzyme tryptophan hydroxylase 2 (Zhang et al. 2004). Conversion of 5-HP to serotonin is achieved by the enzyme L-Amino Acid decarboxylase (Jonnakuty and Gragnoli 2008). Termination of serotonin signaling is achieved through reuptake by the 5-HT transporter (5-HTT) (Fuller and Wong 1990). Serotonin is broken down by monoamine oxidase (MAO) into 5-hydroxyindoleacetic acid (Jonnakuty and Gragnoli 2008) and excreted from the body. Thus, control of brain levels of serotonin can be altered through changes in the function of TPH2, 5-HTT, and MAO.

The majority of serotonin in the body is found outside of the brain, where it plays important roles in digestion and circulation (Berger et al. 2009; Jonnakuty and Gragnoli 2008). Only about 10% of the total serotonin is found in the brain, where it is synthesized, stored, and released from serotonergic neurons. These neurons arise in the raphe nucleus of the brainstem and project throughout the higher regions of the brain. It has been suggested that serotonin is primarily released non-synaptically in a paracrine manner. This mechanism allows a single serotonergic projection to affect a number of cells (Bockaert et al. 2006; Smiley and Goldman-Rakic 1996).

Serotonin Receptors

Serotonin exerts its wide effects on neuronal physiology through a number of receptors. At least fifteen serotonin receptors have been identified, and further diversity is
added post-genomically by RNA editing (Fitzgerald et al 1999) and alternative splicing (Bender et al 2000). Serotonin receptors are broadly grouped into 7 families based on genetic sequence homology and second messenger coupling (Hoyer et al 1994). With the exception of 5-HT3 receptors, which are ligand-gated cation channels, 5-HT receptors all belong to the G-protein coupled receptor superfamily (Bockaert et al 2006).

5-HT1 receptors (A, B, D, E, and F) couple to inhibitory G proteins (Gi), inhibiting generation of cAMP and activating inward-rectifying potassium channels that strongly inhibit neurons (Bockaert et al 2006). Of this family, the 5-HT1A and 5-HT1B isoforms play an important role as autoreceptors on serotonergic neurons, where they serve as feedback inhibitors, reducing release of serotonin. 5-HT2 receptors (A, B, and C) couple to Gq proteins, leading to phosphoinositide metabolism and activation of the phospholipase C/protein kinase C pathway. 5-HT4, 6, and 7 receptors couple to stimulatory G-protein (Gs), leading to stimulation of adenylate cyclase and accumulation of cAMP. Coupling of 5-HT5 (A and B) receptors is unclear, but they may function similarly to 5-HT1 receptors and couple to Gi protein signaling.

In addition to the canonical signaling pathways activated by these receptors, 5-HT receptors have been shown to activate multiple non-canonical pathways. Through these vast effects on cellular signaling, 5-HT plays a strong role in modulating neuronal function and behavior. Two of the serotonin receptor subtypes that are most studied for their role in regulation of mood are the 5-HT1A receptors and 5-HT2A receptors. Several studies have noted dysregulation of these receptors in depression and bipolar disorder (Stockmeier 2003). Furthermore, these receptors are vital in the treatment of depression and bipolar disorder due
to their role as mediators of antidepressant responses and targets of atypical antipsychotics. Details on 5-HT1A receptor signaling are reviewed in the next chapter.

Serotonin 2A (5-HT2A) Receptors

Expression and Localization

The gene for 5-HT2A receptors shares a high degree of homology with the other 5-HT2 receptors (Bockaert et al 2006). Immunohistochemistry and radioligand binding studies show that 5-HT2A receptors are widely expressed throughout the brain. They are highly expressed in the prefrontal cortex, particularly somatodendritically on layer V pyramidal neurons. 5-HT2A receptors are also found on dopaminergic axons in the prefrontal cortex (Miner et al 2003), which corresponds with reports that 5-HT2A receptor antagonists inhibit dopamine release (Pehek et al 2001). 5-HT2A receptors have been shown to exist on glutamatergic axons in primates (Jakab and Goldman-Rakic 1998), but this is not seen in rodents (Miner et al 2003). Additionally, 5-HT2A receptors are also highly expressed on principal cells in the hippocampus, the nucleus accumbens, the medium spiny neurons and interneurons in the striatum, and moderately expressed in the axons of the corpus callosum and internal capsule (Cornea-Hebert et al 1999). High resolution imaging shows that 5-HT2A receptors are located in the axons, soma, and dendrites of pyramidal neurons (Cornea-Hebert et al 1999; Jakab and Goldman-Rakic 1998), and only in dendrites in interneurons (Jakab and Goldman-Rakic 1998). Within pyramidal neurons, a significant intracellular pool of 5-HT2A receptors is seen (Cornea-Hebert et al 1999). The function of this pool is unknown, but it may serve as a reserve for trafficking of receptors to the membrane.
**Signaling**

5-HT2A receptors are conventionally classified by their coupling to Gq proteins. Through this coupling, 5-HT2A receptor activation leads to activation of phospholipase C and stimulation of phosphoinositide hydrolysis (Conn and Sanders-Bush 1984; Roth et al 1984; Roth et al 1986). Through this pathway, 5-HT2A receptors increase intracellular calcium and activate PKC (Takuwa et al 1989). 5-HT2A receptors have also been shown to activate non-canonical signaling, including Akt (Johnson-Farley et al 2005), Erk (Johnson-Farley et al 2005; Watts 1998; Zhong et al 2008), and Jak/Stat (Guillet-Deniau et al 1997). Studies in cultured cells show that activation of Akt and Erk by 5-HT2A receptors is calcium dependent, suggesting that this lies downstream of activation of the canonical PLC pathway (Johnson-Farley et al 2005). *In vivo* studies show that although 5-HT2A receptor agonists do not effect GSK3, antagonizing these receptors results in a strong increase in inhibitory phosphorylation of GSK3 (Li et al 2007b; Li et al 2004).

One recent area of study in 5-HT2A receptor signaling has been of its interaction with the scaffolding proteins, such as β-arrestin2 and PSD-95, and the selectivity of signaling cascades. Signaling of 5-HT2A receptors to increase phospho-ERK by non-hallucinogenic ligands is dependent on β-arrestin-mediated signaling (Schmid et al 2008; Yuen et al 2008). Interestingly, the hallucinogenic 5-HT2A agonist DOI does not depend on β-arrestin2 for signaling to ERK (Schmid et al 2008), although it is dependent on PSD-95 (Abbas et al 2009). This line of study has thus far focused primarily on signaling to Erk, the role of scaffolding proteins in 5-HT2A receptor coupling to other signaling pathways remains to be identified.

*Physiology and Behavior*
5-HT2A receptors have profound effects on physiology and behavior. In the prefrontal cortex, activation of 5-HT2A receptors increase spontaneous excitatory (Aghajanian and Marek 1997; Zhou and Hablitz 1999) and inhibitory (Zhou and Hablitz 1999) post-synaptic potentials of Layer V pyramidal neurons. The effect on EPSPs has been shown to involve asynchronous release of glutamate (Aghajanian and Marek 1999) and is independent of postsynaptic 5-HT2A receptor activation (Beique et al 2007). Furthermore, 5-HT2A receptors can affect the excitability of neurons through modulation of ion channels coupled to Gq-mediated activation of PKC (Carr et al 2002). Through this modulation of excitability, 5-HT2A receptors are poised to have a profound role in regulation of behavior.

Indeed, 5-HT2A receptors have been implicated in several behavioral domains. Most notably, cortical 5-HT2A receptors mediate the effects of hallucinogenic drugs (Gonzalez-Maeso et al 2007), an effect that is modeled in mice by the head-twitch response. This response is dependent on 5-HT2A receptor signaling through the PSD-95 scaffolding protein. 5-HT2A receptors are also involved in control of locomotion (Brookshire and Jones 2009; Halberstadt et al 2009), as 5-HT2A receptor activation induces hyperactivity in rodents, presumably through enhancement of dopamine release (Pehek et al 2001).

5-HT2A receptors have also been implicated in anxiety and regulation of mood. 5-HT2A receptor knock-out mice exhibit drastic reductions in anxiety (Weisstaub et al 2006) and selective 5-HT2A receptor antagonists have been shown to have an antidepressant effect in rats (Jiang et al 2009; Patel et al 2004; Zaniewska et al 2010) and mice (Pandey et al 2010a). Furthermore, the antagonist bip-1 can potentiate the effect of a tricyclic antidepressant in the rats (Pandey et al 2010a). Atypical antipsychotics are increasingly used to treat bipolar disorder and as augmentative treatments in treatment resistant depressed
patients (Ostroff and Nelson 1999; Reeves et al 2008), with a hypothesized mechanism of its
dual antagonistic actions on both 5-HT2A and D2 receptors (Meltzer et al 1989). Addition of
an atypical antipsychotic to an SSRI antidepressant can rapidly induce an antidepressant
response in patients who do not respond to SSRIs alone. Further investigation of signaling
pathways that mediate the behavioral effects of 5-HT2A receptors may lead to improved
therapeutic treatments for depression and bipolar disorder.

GLYCOGEN SYNTHASE KINASE 3

Identification of Glycogen Synthase Kinase 3

Glycogen Synthase Kinase 3 (GSK3) is a serine/threonine protein kinase that has
been shown to play an important role in neuronal signaling and has been highly implicated in
the regulation of mood (Li and Jope 2010). GSK3 is a signaling intermediate of both
neurotrophin signaling (Mai et al 2002) and a number of different neurotransmitter systems
(Beaulieu et al 2007; De Sarno et al 2006; Li et al 2004) and regulates a wide array of
cellular functions. As such, it is uniquely poised to play a critical role in mood-related
pathology.

GSK3 was originally identified and isolated as a kinase negatively regulating the
metabolic enzyme glycogen synthase (GS) (Embi et al 1980). Two isoforms of the enzyme,
GSK3α and GSK3β, have been identified. The two isoforms are virtually identical in their
catalytic region with differences in structure outside of this (Woodgett 1991), most notably a
glycine rich N-terminal in GSK3α but not GSK3β. A splice variant of GSK3β known as
GSK3β2 containing a 13 amino acid insertion in the C-terminal region of the protein has
been identified in the brain (Mukai et al 2002). Structural studies have suggested that this
insertion may alter conformation of the substrate binding pocket, but the distinct function of this variant remains unclear (Mukai et al 2002). Early studies demonstrated that GSK3α and β are enriched in the brain in comparison to other tissues, providing tantalizing evidence that GSK3 may have effects beyond control of metabolism (Woodgett 1990). Further research has shown that GSK3 isoforms are widely distributed throughout the rodent brain, primarily in neurons (Leroy and Brion 1999; Perez-Costas et al 2010). Expression of GSK3 peaks early in development, around postnatal day 10 (Leroy and Brion 1999).

Further insights into GSK3’s function and structure revealed that GSK3 has a unique mechanism of action on its substrates. Early studies of its action on GS revealed that priming phosphorylation of GS by casein kinase was required for GSK3 to phosphorylate GS (Woodgett and Cohen 1984). The consensus sequence for substrate phosphorylation by GSK3 is S/TXXXS/T-p, with a primed serine or threonine (Fiol et al 1987) and three non-specific amino acids preceding the phosphorylation site. Over the years since the discovery of GSK3α and β, a number of substrates have been identified, and these protein kinases have been shown to play a crucial role in a wide array of cellular processes beyond metabolism.

Regulation of GSK3

Regulation of GSK3 by phosphorylation

In the mammalian brain, GSK3 is primarily regulated by post-translational modifications that affect the activity of the enzyme or access to substrates. One of the most common mechanisms of GSK3 regulation is by phosphorylation. There are several phosphorylation sites on GSK3, allowing several mechanisms of regulation by upstream signaling. Phosphorylation on Tyr279 of GSK3α and Tyr216 of GSK3β (Hughes et al 1993)
is generally required for activity of the enzyme, while phosphorylation on Ser21 of GSK3α and Ser9 of GSK3β (Sutherland et al 1993) strongly inhibits activity of the enzyme. A more recently identified phosphorylation site on Thr390 of GSK3β appears to be inhibitory (Thornton et al 2008).

Tyrosine phosphorylation of GSK3 was first reported in vitro (Hughes et al 1993; Wang et al 1994) and has since been shown to occur in cells and tissues. In vitro, tyrosine phosphorylation is required for activation of the enzyme (Hughes et al 1993), although it is not clear if this is the case in intact tissue. The mechanism of tyrosine phosphorylation is not clear and may vary between cells and tissues, either mediated by growth-factor activated tyrosine kinases Fyn (Lesort et al 1999) and Pyk2 (Hartigan et al 2001), or as a result of autophosphorylation (Cole et al 2004; Wang et al 1994). The majority of GSK3 appears to be tyrosine phosphorylated in resting cells, as incubation with phosphatase inhibitors does not result in an increase in phosphorylation (Cole et al 2004). Tyrosine phosphorylation of GSK3 represents an important mechanism for activation and stabilization of the enzyme.

In contrast to tyrosine phosphorylation of GSK3, phosphorylation of Ser21 on GSK3α or Ser9 on GSK3β is inhibitory. Early studies indicated that isolated GSK3 was inactivated by incubation with Ribosomal S6 Kinase (RSK2) and reactivated by incubation with phosphatases (Sutherland et al 1993). Later, phosphorylation of Ser-9 and inhibition of GSK3β was reported in intact cells (Stambolic and Woodgett 1994). Serine phosphorylation of GSK3 turns the N-terminal of the enzyme into a primed pseudo-substrate, which prevents access of substrates to the active site of the enzyme (Frame et al 2001). Serine phosphorylation of GSK3 can be induced by a number of upstream kinases. The most well-established of these is Akt, a downstream target of the PI3-kinase cascade (Cross et al 1995),
but serine phosphorylation of GSK3 has also been shown to be mediated by RSK2 (Sutherland et al 1993), PKC (Goode et al 1992), and Protein Kinase A (Fang et al 2000). This phosphorylation can be reversed by activation of protein phosphatases such as PP2A (Shaw et al 1997) and PP1 (Peineau et al 2007). Serine phosphorylation of GSK3 occurs in response to growth factors such as insulin (Cross et al 1995; Welsh and Proud 1993), epidermal growth factor (EGF) (Saito et al 1994), insulin-like growth factor 1 (IGF-1) (Cross et al 1994), and BDNF (Mai et al 2002), as well as in response to activation of G-Protein Coupled Receptors (GPCR) (Beaulieu et al 2009; Li et al 2004) and NMDA receptor activity (De Sarno et al 2006; Peineau et al 2008). The regulation of GSK3 in response to such a wide variety of stimuli indicates the importance of serine phosphorylation of GSK3.

**Regulation by Protein-Protein Interactions**

Another mechanism of regulation of GSK3 is through the formation of protein complexes. The most well known of these is the so-called “destruction complex” involved in the regulation of β-catenin levels. The scaffolding protein Axin facilitates the formation of a complex consisting of GSK3, APC, and β-catenin (Ikeda et al 1998; Rubinfeld et al 1996; Yost et al 1996). GSK3 phosphorylates β-catenin, targeting it for ubiquitination and degradation (Aberle et al 1997). Activation of Wnt signaling inhibits this complex, allowing β-catenin to accumulate, translocate into the nucleus, and activate transcription. Inhibition of GSK3 by Wnt signaling does not occur through serine phosphorylation of GSK3 (Ruel et al 1999), but by binding to a separate protein known as GBP or FRAT (Li et al 1999; Yost et al 1998) which physically prevents GSK3 from binding to Axin (Ferkey and Kimelman 2002; Fraser et al 2002). Signaling to GSK3 via protein-protein interaction in the Wnt pathway appears to be isolated from signaling to GSK3 via inhibitory serine phosphorylation, as
manipulations of one pathway do not affect the other. Thus, regulation of GSK3 by distinct mechanisms allows the enzyme to serve as a nexus for a variety of signaling pathways with minimal crosstalk between them.

*Regulation by Intracellular Location*

GSK3 can also be regulated by changes in intracellular location. Although originally thought to be a soluble cytosolic protein, GSK3 is also found in the nucleus and mitochondria of cells and the levels of GSK3 therein can be dynamically regulated. Levels of nuclear GSK3 protein are increased in cells during S-phase of the cell cycle (Diehl et al 1998); after treatment with endothelin-1 (Haq et al 2000); or in response to apoptosis-inducing stimuli such as staurosporine or heat shock (Bijur and Jope 2001). Nuclear localization of GSK3 does not appear to correlate with serine or tyrosine phosphorylation (Bijur and Jope 2001). Nuclear GSK3 exhibits much lower levels of Ser21/9 phosphorylation than cytosolic GSK3, suggesting a high level of activity (Bijur and Jope 2003a). Nuclear GSK3 targets a number of transcription factors to a variety of effects including increasing (Watcharasit et al 2002) or decreasing (Chu et al 1996; Grimes and Jope 2001) transcription factor activity, or directing export from the nucleus (Beals et al 1997). GSK3 is also present in the mitochondria (Hoshi et al 1996; Perez-Costas et al 2010). As in the nucleus, mitochondrial GSK3 exhibits reduced serine phosphorylation and thus increased activity compared to cytosolic GSK3 (Bijur and Jope 2003a). Total levels of GSK3 in the mitochondria are relatively stable (Bijur and Jope 2003b); however, mitochondrial GSK3 can be regulated by phosphorylation in response to growth factors (Bijur and Jope 2003b) or by binding to other proteins (Watcharasit et al 2003).
Functions of GSK3

Metabolism and insulin signaling

GSK3 was originally identified as one of several kinases responsible for phosphorylating and inactivating glycogen synthase, thus decreasing glycogen synthesis. Further investigations showed that insulin increases glycogen synthesis through phosphorylation and inhibition of GSK3 (Cross et al 1995; Parker et al 1983; Welsh and Proud 1993). GSK3 also phosphorylates other metabolic enzymes including ATP citrate lyase (Hemmings et al 1982) and mitochondrial pyruvate dehydrogenase (Hoshi et al 1996). Phosphorylation of the latter enzyme inhibits its activity, providing a mechanism by which GSK3 impairs cellular energy production.

In addition to regulation of glycogen synthase, GSK3 plays a role in regulation of other substrates of insulin signaling. GSK3 phosphorylates (Welsh and Proud 1993) and inhibits (Singh et al 1996) eukaryotic Initiation Factor 2B (eIF2B), a factor required for initiation of protein translation. Inhibition of GSK3 is a crucial step in the promotion of cell growth and protein translation by insulin. GSK3 also phosphorylates insulin receptor substrate-1 (IRS-1), which turns IRS-1 into an inhibitor of insulin receptor signaling (Eldar-Finkelman and Krebs 1997). This has been suggested to be a potential mechanism of the development of insulin resistance. Thus, GSK3 plays an important role in metabolic signaling, and may be a therapeutic target in pathological conditions such as diabetes.

Apoptosis and Cellular Resilience

GSK3 plays a unique and paradoxical role in apoptosis (Beurel and Jope 2006). In the intrinsic pathway, GSK3 amplifies apoptosis by promoting the actions of pro-apoptotic factors such as Bim (Hongisto et al 2003), Bax (Linseman et al 2004), and p53 (Watcharasit
et al 2003; Watcharasit et al 2002) while inhibiting the actions of protective factors such as HSF-1 (Chu et al 1996). In contrast, GSK3 plays an anti-apoptotic role in the extrinsic apoptosis pathway, through upregulation of NFκB (Schwabe and Brenner 2002) and inhibition of death receptor signaling (Sun et al 2008). Inhibition of GSK3 has been shown to be neuroprotective in cellular models of Alzheimer’s disease (Takashima et al 1993), Parkinson’s disease (Chen et al 2004; King et al 2001) and excitotoxicity (Liang and Chuang 2007) as well as in animal models of ischemia (Nonaka et al 1998; Roh et al 2005) and Huntington’s disease (Wei et al 2001).

Gene Expression

Another cellular function that GSK3 plays an important role in is the regulation of gene expression. GSK3 phosphorylates a number of transcription factors (Jope and Johnson 2004; Liang and Chuang 2006), including AP-1 (Boyle et al 1991; de Groot et al 1993; Nikolakaki et al 1993; Ozaki and Chuang 1997), HSF-1 (Chu et al 1996), NFκB (Hoeﬂich et al 2000; Schwabe and Brenner 2002), allowing it to have broad effects on cellular function through induction and repression of gene expression. The prototypical transcription factor regulated by GSK3 is β-catenin. As previously discussed, GSK3 activity leads to downregulation of β-catenin (Yost et al 1996) in the nucleus, preventing its activation of TCF/LEF genes. Downregulation of β-catenin leads to changes in activity of other transcription factors such as Cyclin D1 (Tetsu and McCormick 1999), WISP-1 (Xu et al 2000), and c-myc (He et al 1998). Thus, through regulation of the transcriptional activities of β-catenin, GSK3 can affect a range of developmental processes.

Another transcription factor regulated by GSK3 is cyclic AMP Response Element Binding Protein, or CREB. CREB is a major transcription factor thought to be a crucial
player in synaptic plasticity, neuronal excitability, and the formation of long-term memories (Benito and Barco 2010), as well as in pathological processes such as depression (Blendy 2006). GSK3 negatively regulates CREB through phosphorylation on Ser129, and this regulation has been repeatedly shown to have a negative effect on CREB activity (Bullock and Habener 1998; Grimes and Jope 2001; Liang and Chuang 2006). Due to GSK3’s requirement of substrates to be primed, CREB must be phosphorylated on Ser133 prior to phosphorylation on Ser129 by GSK3 (Grimes and Jope 2001). Ser133 phosphorylation results in activation of CREB (Chrivia et al 1993; Gonzalez and Montminy 1989; Sheng et al 1991) and occurs downstream of neurotrophic factors (Ginty et al 1994). Thus, GSK3 does not serve to constitutively inhibit CREB activity, but rather to halt the effects of CREB that has been activated by growth factors.

Neuronal Development

GSK3 has also been strongly implicated in regulation of neurodevelopmental processes. Through regulation of the transcription factors Cyclin D1 (Diehl et al 1998), GSK3 has a strong influence on progression through the cell cycle. Many of the other transcription factors regulated by GSK3, such as β-catenin and CREB, have also been shown to regulate developmental processes (Lonze and Ginty 2002{Hur, 2010 #49}). Several factors involved in early development of the nervous system are inhibited by GSK3, including Notch (Espinosa et al 2003) and Sonic Hedgehog (Jia et al 2002). Similarly, signaling molecules that induce neuronal differentiation such as FGF (Shimizu et al 2008) and BDNF (Mai et al 2002) are strongly inhibitory towards GSK3. The importance of GSK3 in developmental is highlighted by a recent study showing a drastic increase in neuronal progenitors and decrease in embryonic neurogenesis in GSK3α/β conditional knock-out
mice. Neuronal progenitors isolated from these animals had increased proliferation, which could be rescued by reducing signaling through the β-catenin, Shh, and Notch pathways (Kim et al 2009). The importance of GSK3 in neurodevelopmental processes may extend into adulthood, as knock-in mice bearing S21/9A mutations in GSK3α and β exhibit deficits in adult neurogenesis (Eom and Jope 2009).

**Regulation of Circadian Rhythms**

Circadian rhythms are another area of function that is strongly regulated by GSK3. Overexpression of the GSK3 ortholog shaggy in drosophila shortens the circadian period, while reduction of Shaggy activity lengthens the period. This effect is due to a direct interaction between Shaggy and the circadian proteins TIMELESS (Martinek et al 2001) and dPER (Ko et al 2010). Similar effects of GSK3 on circadian period are seen in mammalian cells, with GSK3 directly regulating the timekeeper protein Per2 (Iitaka et al 2005; Kaladchibachi et al 2007). Another component of the mammalian circadian clock regulated by GSK3 is the transcription factor Rev-Erbα, which is phosphorylated and stabilized by GSK3β, leading to expression of circadian genes such as bMAL (Yin et al 2006). Furthermore, levels of phosphorylated GSK3β are regulated throughout the circadian cycle in the mammalian superchiasmatic nucleus, the location of the brain’s light-sensing pacemaker (Iitaka et al 2005). Given that circadian genes are expressed and regulated over the circadian period in every tissue in the body, this represents a novel area through which GSK3 can affect cellular function.

**Regulation of Synapses and receptors**

One emerging area that GSK3 is implicated in regulating is synaptic plasticity and neurotransmitter receptor function. NMDA-receptor dependent long-term depression (LTD)
at hippocampal CA3-CA1 synapses induces Ser9 dephosphorylation of GSK3β, which in turn maintains LTD by promoting internalization of AMPA receptors (Peineau et al 2007). Furthermore, mice expressing transgenic GSK3β bearing an S9A mutation rendering it immune to inactivating phosphorylation have enhanced LTD (Dewachter et al 2009). GSK3 is also an important player in long-term potentiation (LTP). Several studies have shown that induction of LTP at dentate gyrus and CA1 synapses increases serine phosphorylation of GSK3 (Hooper et al 2007; Peineau et al 2007). Overexpression of GSK3β inhibits LTP at CA1 and dentate gyrus synapses, an effect that is reversed with lithium (Hooper et al 2007). In transgenic mice expressing S9A-GSK3β, late LTP is impaired (Dewachter et al 2009). Thus, GSK3 plays an important role in synaptic plasticity.

In addition to regulating synaptic plasticity a few recent studies have suggested that GSK3 may also play a role in pre-synaptic function, as increasing GSK3 activity inhibits exocytosis of glutamate at perforant path-CA3 synapses, an effect that occurs through modulation of calcium channels (Zhu et al 2010; Zhu et al 2007). Another way in which GSK3 affects synaptic plasticity is through regulation of glutamate receptors. GSK3 promotes surface expression of NMDA receptors in dissociated hippocampal neurons, and inhibition of GSK3 increases internalization of these receptors (Chen et al 2007a). In contrast, active GSK3 promotes internalization of AMPA receptors (Du et al 2010; Peineau et al 2007) through interaction with the kinesin motor system (Du et al 2010). The GSK3-AMPA interaction is crucial for LTD, as a synthetic peptide that blocks the interaction also blocks LTD (Du et al 2010). Furthermore, GSK3β has recently been shown to directly interact with 5-HT1B receptors and is necessary for the activation of the receptor (Chen et al 2009). As this receptor is found at presynaptic terminals of serotonergic and other neurons
where it inhibits neurotransmitter release, regulation of 5-HT1B receptors by GSK3 presents a novel mechanism by which GSK3 can affect neurotransmitter dynamics in the brain.

GSK3 and mood disorders

*Regulation of GSK3 by Mood Disorder Treatments*

Interest in GSK3 as a potential target in the treatment of mood disorders arose when it was discovered that lithium, a gold-standard mood stabilizer used in the treatment of bipolar patients, potently inhibits GSK3 (Klein and Melton 1996; Stambolic et al 1996) through competition for a magnesium binding site (Ryves and Harwood 2001). Additionally, lithium inhibits GSK3 through increasing serine 21/9 phosphorylation (Chalecka-Franaszek and Chuang 1999), an effect that has been shown to occur in rodents chronically treated with therapeutic levels of lithium (De Sarno et al 2002) as well in the peripheral blood cells of bipolar patients (Li et al 2007a). Several potential mechanisms have been proposed for the increase in phosphorylation of GSK3 induced by lithium, including inhibition of protein phosphatase 1 (Zhang et al 2003), and stimulation of Akt through disruption of interactions between β-arrestin2, protein phosphatase 2A, and Akt (Beaulieu et al 2008a). GSK3 can also be inhibited by the mood stabilizer and anticonvulsant valproic acid (VPA). Treatment of cultured cells (Aubry et al 2009; Chen et al 1999a; De Sarno et al 2002) or rats (Leng et al 2008) with VPA increases phosphorylation of Akt and subsequent phosphorylation of GSK3, although this is not seen in all cell types (Jin et al 2005; Jonathan Ryves et al 2005; Phiel et al 2001). The activation of Akt and inhibition of GSK3 in response to VPA and lithium appear to occur through separate pathways, as co-treatment of rats with both drugs results in a potentiated signaling response compared to treatment with either drug alone (Leng et al
Therefore, regulation of GSK3 appears to be a common step in the effects of two major mood stabilizers.

In addition to regulation by lithium and valproate, GSK3 is also regulated by pharmaceutical mood disorder treatments that affect serotonergic systems. Acute treatment of mice with the serotonin releasing agent d-Fenfluramine, the tricyclic antidepressant imipramine (Li et al 2004), or the SSRI fluoxetine increases Ser9 phosphorylation of GSK3β in the brain (Beaulieu et al 2008b; Li et al 2004). Additionally, chronic treatment of rats with fluoxetine or the SNRI venlafaxine has recently been shown to increase Ser9 phosphorylation of GSK3β (Okamoto et al 2010). The increase in serine phosphorylation of GSK3β induced by serotonin enhancers is likely due to activation of 5-HT1A receptors (Li et al 2004). Although the precise mechanism of GSK3 phosphorylation in response to serotonin and antidepressants is still unknown, it may be through activation of Akt, as 5-HT and 5-HT1A receptors have been shown to activate Akt in cell culture (Cowen et al 2005).

Antipsychotics, which are used to treat Bipolar Disorder, as well as to augment antidepressant treatment, can also potently inhibit GSK3 through increased phosphorylation. Traditional antipsychotics, such as haloperidol, have shown conflicting evidence on their ability to regulate GSK3 through phosphorylation, with several studies showing increased serine phosphorylation of GSK3 in animals treated with haloperidol (Alimohamad et al 2005; Roh et al 2007), and others showing no effect (Li et al 2007b). More conclusive evidence exists in favor of regulation of GSK3 by atypical antipsychotics. Several distinct atypical antipsychotics have been shown to increase phosphorylated GSK3 in cells (Aubry et al 2009) as well as in the rodent brain (Alimohamad et al 2005; Li et al 2007b; Roh et al 2007). This is likely due to antagonism of 5-HT2A receptors, as it occurs at low doses of drug that are
sufficient for binding to 5-HT2 receptors, but less efficient to D2 receptors (Li et al 2007b). Furthermore, treating animals with a combination of fluoxetine and risperdone augments the increased phospho-Ser-GSK3 to a higher level than that induced by fluoxetine alone (Li et al 2007b), suggesting that inhibition of GSK3 may be a mechanism of antidepressant augmentation by 5-HT2A receptor antagonists.

*Regulation of GSK3 in Animal Models of Mood Disorders*

Inhibition of GSK3 as a strategy for treating bipolar disorder and depression has shown considerable promise in preclinical models of behavioral features of these diseases. Acute treatment of mice with peptide and small molecule inhibitors of GSK3 have antidepressant effects in the forced swim test (Gould et al 2004; Kaidanovich-Beilin et al 2004; Rosa et al 2008) and tail suspension test (Beaulieu et al 2008b) and reverses depression-like behaviors caused by serotonin deficiency (Beaulieu et al 2008b). Similarly, mice with only one copy of GSK3β (O'Brien et al 2004) or with genetic deletion of GSK3α (Kaidanovich-Beilin et al 2009) exhibit reduced immobility in the forced swim test. Inhibition of GSK3 also has anti-manic effects in behavioral models. Small-molecule inhibitors (Gould et al 2004) or haploinsufficiency of GSK3β (Beaulieu et al 2004) reduce amphetamine-induced hyperactivity. The mechanisms by which inhibition of GSK3 influences mood are still unknown and remain to be studied. However, in view of the multiple functional aspects of GSK3, it is likely that GSK3 plays a role in multiple processes that affect mood.

While significant evidence has been shown for anti-depressant and anti-manic effects of GSK3 inhibition, fewer studies have addressed whether increased GSK3 activity adversely affects behavior. Mice overexpressing constitutively active GSK3β with an S9A mutation
have reduced brain size and minor impairments in spatial memory (Spittaels et al 2002) as well impaired inhibitory avoidance learning (Dewachter et al 2009). Mice transgenically overexpressing GSK3β have increased reactivity as demonstrated by startle response and increased hyperactivity in an open field (Prickaerts et al 2006). Although evidence thus far is still sparse, these studies suggest that increased activity of GSK3 may be an underlying factor of behavioral disorder.

**GSK3 in Mood Disorder Patients**

Alterations in GSK3 have also been shown in studies of human mood disorder patients. A study of post-mortem brains from depressed and non-depressed suicides showed increased activity of GSK3β and decreased activity of its upstream inhibitory kinase Akt in the postmortem pre-frontal cortex of the depressed but not the nondepressed subjects (Karege et al 2007). In isolated platelets, bipolar patients, but not those with major depression, had decreased GSK3β phosphorylation, levels of which were restored by treatment (Pandey et al 2010b). A single nucleotide polymorphism of the human GSK3β gene (-50T/C) appeared to have effects on age of onset, but not overall susceptibility to bipolar disorder (Benedetti et al 2004a; Benedetti et al 2004b). This locus has also been associated with treatment response in bipolar (Benedetti et al 2004b; Benedetti et al 2005) and depressed (Adli et al 2007) patients, although other studies have found no effect of the SNP (Szczepankiewicz et al 2006). Together, the emerging data suggests that GSK3 activity may be increased in patients suffering from bipolar disorder.

Based on the above discussed background information, this project was designed to better understand the role GSK3 plays in both the pathophysiology and treatment of mood disorders. We first examined the consequences of loss of GSK3 serine phosphorylation on
mood-related behaviors. We then used a combination of biochemical and behavioral
techniques to examine the signaling mechanisms in regulation of GSK3 by serotonin, as well
as the necessity of this regulation for the behavioral effects of antidepressants.
Figure 1. Regulation of GSK3 activity by inhibitory phosphorylation. (A) Substrates must be primed in order to be phosphorylated by GSK3. (B) Phosphorylation of GSK3α/β on Serine 21/9 turns the N-terminal into a primed pseudo substrate, preventing access of primed substrates to the active site
5-HT1A Receptor-Regulated Signal Transduction Pathways in the Brain

by

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Abstract

Serotonin is an influential monoamine neurotransmitter that signals through a number of receptors to modulate brain function. Among different serotonin receptors, the serotonin 1A (5-HT1A) receptors has been tied to a variety of physiological and pathological processes, notably in anxiety, mood, and cognition. 5-HT1A receptors couple not only to the classical inhibitory G-protein-regulated signaling pathway, but also to signaling pathways traditionally regulated by growth factors. Despite the importance of 5-HT1A receptors in brain function, little is known about how these signaling mechanisms link 5-HT1A receptors to regulation of brain physiology and behavior. Following a brief summary of the known physiological and behavioral effects of 5-HT1A receptors, this article will review the signaling pathways regulated by 5-HT1A receptors, and discuss the potential implication of these signaling pathways in 5-HT1A receptor-regulated physiological processes and behaviors.
Introduction

Serotonin (5-Hydroxytryptamine, 5-HT) is a monoamine neurotransmitter that plays important roles in physiological functions such as sleep, feeding, sexual behavior, temperature regulation, pain, and cognition, as well as in pathological states including mood disorders, anxiety disorders, psychosis, and pain disorders. Serotonergic neurons originate in the dorsal and median raphe nucleus of the brainstem. Ascending projections from these neurons reach throughout the brain to release serotonin in a paracrine manner, allowing serotonin released from one terminal to activate receptors on a number of post-synaptic cells and modulate a variety of neuronal activities [4].

There are at least 16 different types of serotonin receptors, and these are broadly grouped into sub-families based on their primary signaling mechanisms [5]. 5-HT1 receptors (A, B, D, E, and F) classically couple to the inhibitory G proteins (Gi/o) that inhibit adenylyl cyclase/protein kinase A (PKA) signaling cascade. 5-HT2 (A, B, C) receptors couple to Gq protein that stimulates the phospholipase C (PLC)/protein kinase C (PKC) signaling cascade and enhance intracellular calcium signaling. 5-HT3 receptors (A, B, C) are unique among serotonin receptors as they are ligand-gated ion channels. 5-HT4, 6, and 7 receptors couple to the stimulatory G protein (Gs) and activate adenylyl cyclase/PKA. Signaling of 5-HT5 (A and B) receptors is less clear, but evidence suggests that it may be linked to Gi/o proteins and inhibition of adenylyl cyclase/PKA [4, 6]. Further diversity of serotonin receptors is derived from transcriptional modifications, such as mRNA editing [7] and alternative splicing [8]. Among these subtypes of serotonin receptors, the 5-HT1A receptors were the first to be cloned and are widely studied for their roles in regulation of mood, anxiety, and cognition [1-3]. Below, we will summarize the known functions of 5-HT1A receptors, placing an
emphasis on the regulation of signal transduction pathways by 5-HT1A receptors in neurons and brain.

Characterization and Localization of 5-HT1A Receptors

5-HT1A receptors are among the best characterized serotonin receptors due to the early discovery of its encoding gene and the availability of selective ligands. The gene for the human 5-HT1A receptors was cloned in 1987 as a single intronless gene, and was identified as a G protein-coupled receptor (GPCR) based on sequence homology to the β2-adrenergic receptor gene [9]. Subsequent binding studies identified the gene as encoding for the 5-HT1A receptors [10].

In the mammalian brain, 5-HT1A receptors are divided into two distinct classes based on their location. The 5-HT1A autoreceptors are located on the soma and dendrites of serotonergic neurons in the raphe nucleus [11-16]. Activation of 5-HT1A autoreceptors suppresses firing of serotonergic neurons and thus reduces activity-dependent serotonin release [17]. The 5-HT1A heteroreceptors are located on non-serotonergic neurons, primarily of the limbic areas, such as on the dendrites and soma of glutamatergic pyramidal neurons [16]; axon terminals of GABAergic [18, 19], and cholinergic [20] neurons. Activation of 5-HT1A heteroreceptors on these distinct neurons usually reduces neuronal excitability and firing [21, 22]. The heteroreceptors are particularly enriched in the hippocampus, where immunohistochemistry and radioligand binding have demonstrated high receptor levels in the stratum radiatum of CA1 and the granule cell layer of the dentate gyrus, and at moderate levels in CA3 [11-15]. 5-HT1A heteroreceptors are also strongly expressed in the entorhinal cortex, frontal cortex, and lateral septum, and moderately expressed in the amygdala,
superior colliculus, piriform cortex, and interpeduncular nucleus, as well as in several hypothalamic and thalamic nuclei [11-15]. The broad distribution of 5-HT1A receptors suggests that they have a variety of functions in brain.

Functions of 5-HT1A receptors in neuronal activity

*Synaptic physiology and plasticity*

In neurons, activation of 5-HT1A receptors activates the G protein-coupled inwardly-rectifying potassium (GIRK) channels, an action that profoundly hyperpolarizes neurons [23, 24] and decreases firing [25-28]. 5-HT1A receptors also reduce calcium currents and evoked calcium influx [29-31]. The combination of these two effects allows serotonin to rapidly and effectively silence neuronal transmission.

5-HT1A receptors also play a role in regulation of post-synaptic activity. Activation of 5-HT1A receptors reduces N-Methyl-D-Aspartate (NMDA) receptor mediated currents in the prefrontal and visual cortex [25, 32-34]. In cortical pyramidal neurons, this action of 5-HT1A receptors involves down-regulation of surface NR2B subunit of NMDA receptors [33]. Activation of 5-HT1A receptors also reduces α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor currents [35] as well as surface expression of GluR2/3 subunits of AMPA receptors [36]. Through these actions, 5-HT1A receptor activation reduces excitatory postsynaptic potentials (EPSPs) in several brain regions [28, 31]. 5-HT1A receptors also have effects on prolonged changes in the strength of synapses, known as synaptic plasticity. In the visual cortex and the dentate gyrus of the acute brain slices, 5-HT1A receptor agonist 8-Hydroxy-N,N-dipropyl-2-aminotetralin (8-OH-DPAT) blocks long term potentiation (LTP) [37] [26], one of the best studied forms of synaptic
plasticity induced by robust activation of a synapse, whereas the 5-HT1A receptor antagonist NAN-190 reverses the \textit{in vivo} inhibitory effect of antidepressants fluvoxamine and milnacipran on LTP in area CA1 of the hippocampus [38, 39].

However, the effect of 5-HT1A receptors in synaptic plasticity may depend on the type of activation in specific brain regions, as direct activation of 5-HT1A receptors in the dentate gyrus of the hippocampus results in increased glutamatergic output of granule cells [40]. Conversely, recordings in the intact dentate gyrus reveal decreased LTP when 5-HT1A autoreceptors are activated, and thus reducing release of serotonin in the dentate gyrus, or 5-HT1A heteroreceptors in the dentate gyrus are blocked [41]. The direct effect of 5-HT1A receptors in the dentate gyrus is thought to be a result of silencing inhibitory interneurons [41]. Thus, the effects of 5-HT1A receptors on synaptic plasticity may also be tied to state-dependent alterations in GABAergic tone [42, 43].

While it seems clear that 5-HT1A receptors can profoundly affect synaptic physiology and plasticity through changes in membrane potential and alteration of excitatory and inhibitory tones, the signaling mechanisms mediating the effect of 5-HT1A receptors to the induction or long-term maintenance of synaptic plasticity are not completely understood, and remain to be elucidated.

\textit{Neurogenesis and neuroprotection}

Adult neurogenesis is increasingly recognized as an important process in the maintenance of normal neuronal function [44], and 5-HT1A receptors have been shown to regulate neurogenesis in the subgranular zone of the dentate gyrus. Activation of 5-HT1A receptors increases proliferation of neuronal progenitors [45] and promotes development of neural precursors into adult neurons [46], whereas 5-HT1A receptor antagonists decrease
neurogenesis in the dentate gyrus [47]. This effect of 5-HT1A receptors is not prevented by serotonin depletion, suggesting that this is a direct function of 5-HT1A heteroreceptors [48]. The effect of 5-HT1A receptors on neurogenesis may have important roles in maintaining normal contextual memory formation that requires ongoing neurogenesis [49], as well as mediating antidepressant action as it may be mediated by neurogenesis [50].

5-HT1A receptors also have important function in neuroprotection in both neuronal cell cultures [51-59] and in the mammalian brain [60, 61]. In animal models of ischemia [60-63] and Parkinson’s disease [64], 5-HT1A receptor agonists have shown promise as potential neuroprotective therapies. The neuroprotective effect of 5-HT1A receptors is dependent on the activities of the growth factor-associated signaling molecules mitogen-activated protein kinase (MAPK) and Akt [65-67], and involves inhibition of NMDA receptor-mediated excitotoxicity by reducing calcium influx and glutamate release [57, 58, 63].

Functions of 5-HT1A receptors in Behaviors

Anxiety

5-HT1A receptors are particularly influential in anxiety-related behaviors [68]. Systemic administration of 5-HT1A receptor agonist 8-OH-DPAT and partial agonists buspirone and gepirone generally decreases anxiety in rodents, as observed in the elevated plus maze and social interaction tests [69]. The effects of 5-HT1A receptor agonists on anxiety in rodents appear to be ligand-specific. The structurally similar ligands buspirone and gepirone are consistently anxiolytic [69-71], although gepirone may only be effective after chronic treatment [72], while mixed results have been found with 8-OH-DPAT [69, 71, 73]. The anxiolytic effect of buspirone after local injection to the hippocampus is task-specific
since it reduces anxiety-like behaviors in the elevated plus maze and the open field [70], but not in the social interaction test [74]. Buspirone has demonstrated clinical efficacy for generalized anxiety disorder [75, 76], but it remains to be determined how the ligand-, temporal-, spatial-, and task-specific regulation of anxiety by 5-HT1A receptor agonists determines their therapeutic implication in anxiety disorders.

Some of these questions have been addressed using genetically modified animals. 5-HT1A receptor knockout mice exhibit increased anxiety-like behaviors in the elevated plus maze, elevated zero maze, open field test, and novel object exploration [77-79]. The impaired performance of these mice in anxiety-related tasks is likely due to an enhanced fear response in aversive environments [80], but not due to changes in exploration or behavioral inhibition [81]. Furthermore, restoring 5-HT1A receptor function to the forebrain of 5-HT1A knockout mice rescues anxiety-like behaviors, suggesting a crucial role for heteroreceptors in regulation of anxiety and fear [82]. This rescue does not occur if forebrain 5-HT1A receptors are restored after postnatal day 20, whereas elimination of forebrain 5-HT1A receptors after postnatal day 80 has no effect on anxiety [82], further suggesting that 5-HT1A receptor signaling early in life plays a crucial role in the development of the brain’s fear and anxiety systems [83].

Depression

5-HT1A receptors also regulate mood-related behaviors, particularly those related to depression. Sub-chronic administration of the 5-HT1A receptor agonists 8-OH-DPAT and azapirones reduces depressive behaviors in the forced swim test [71, 84] and tail suspension test [85]; and chronic administration of 8-OH-DPAT reduces depressive behavior in the novelty-suppressed feeding test [50]. In the learned helplessness model of depression,
systemic injection of 5-HT1A agonists reversed escape deficits induced by inescapable stress [86], and this effect can be observed after direct injection of an agonist into the septum or after ascending serotonergic fibers are destroyed [87, 88], suggesting that the antidepressant-like effect is due to action of 5-HT1A heteroreceptors. However, one study reports reduced escape deficits in the learned helplessness model after intra-dorsal raphe infusion of 5-HT1A receptor agonist [89], suggesting that 5-HT1A autoreceptors may also contribute to an antidepressant effect.

5-HT1A receptors are likely important mediators of antidepressant responses. In animal studies, 5-HT1A receptor antagonists alone do not alter depressive behaviors, but they prevent the antidepressant effect of desipramine in the forced swim test [90] and fluoxetine in the tail suspension test [85], and 5-HT1A receptor knockout mice fail to respond to fluoxetine in the novelty-suppressed feeding test [50]. It is also thought that 5-HT1A autoreceptor desensitization is a necessary step for the chronic effect of antidepressants in animals and in humans [91]. In accordance with these finding, 5-HT1A receptor knockout mice have intrinsic antidepressant-like behavioral phenotypes [77, 78]. However, a recent study suggests that autoreceptor desensitization alone is not sufficient for the response of antidepressant to occur, but a sufficient basal serotonergic tone at a low autoreceptor level is necessary for a better response to antidepressant [92].

Evidence from human studies also supports a role of 5-HT1A receptors in the pathology and treatment of depression. Well-replicated postmortem and brain imaging studies have shown that 5-HT1A receptors are reduced in crucial areas of brain in depressed subjects, including the dorsal raphe nucleus, hippocampus, amygdala, and prefrontal cortex [91, 93]. Additionally, postmortem brains of suicide subjects have decreased 5-HT1A
coupling to G-proteins and reduced activity of signaling molecules associated with 5-HT1A receptors [94]. A single nucleotide polymorphism of the 5-HT1A receptor gene that alters expression levels of the receptors has been associated with both susceptibility to depression and responsiveness to antidepressants [91]. Although it has not determined whether the diminished function of 5-HT1A receptors in depressed patients is a cause or an effect of the disease, the results from these studies, combined with the strong evidence from animal studies, suggest that 5-HT1A receptors play an important role in mood regulation. A better understanding of this role, and of the signaling molecules mediating it, may yield more effective and faster acting antidepressant treatments.

Learning and Memory

Many studies have investigated the role of 5-HT1A receptors in learning and memory. The most consistent effect has been shown in passive avoidance learning, a paradigm in which animals are trained to avoid natural behavioral responses to stressful stimuli by associating those responses with an aversive stimulus, such as a shock. Systemic administration of several 5-HT1A receptor agonists suppresses performance [95-99], whereas 5-HT1A receptor antagonists facilitates learning [100], suggesting that 5-HT1A receptors play a role in controlling inhibitory learning. Regulation of passive avoidance learning is primarily a function of heteroreceptors, as the 5-HT1A receptor agonist 8-OH-DPAT is effective both when injected into the entorhinal cortex [101], and after endogenous serotonin is depleted [97, 98].

In fear conditioning, a hippocampus- and amygdala- dependent paradigm in which animals learn to associate a cue or a context with an aversive stimulus, both systemic and local 8-OH-DPAT injections into the hippocampus or the medium raphe nucleus inhibit
contextual fear conditioning [102, 103]. In agreement, 5-HT1A receptor knockout mice have enhanced contextual fear conditioning [81, 104], an effect that can be reversed by selectively expressing and activating 5-HT1A receptors in the dentate gyrus [104].

While there is a consensus that 5-HT1A receptor activation inhibits learning in the passive avoidance and fear conditioning paradigms, both paradigms depend on learning being induced by activation of fear circuits in the brain [105]. Given the fact that 5-HT1A receptors alter these fear circuits and regulate anxiety [80], it is difficult to determine if the effects of 5-HT1A receptors in these learning paradigms are a reduction of anxiety or suppression of learning. However, the effect of 5-HT1A receptors on cognition does not appear to be solely due to the receptor’s strong effect on anxiety. In the Morris Water Maze, a spatial memory task that is not intertwined as strongly with anxiety and fear, 5-HT1A receptor agonists also show inhibitory effects on learning and memory [106-109].

5-HT1A receptor-regulated Signal transduction pathways

Canonical 5-HT1A receptor-regulated signaling pathway

Early studies identified that 5-HT1A receptors couple to the inhibitory G-proteins (Gi/o) [112]. Agonist binding to the receptor exchanges GDP for GTP on the alpha subunit of Gi/o (Gia/Goα) [110], active of which primarily functions to inhibit adenylyl cyclase, resulting in decreased cyclic adenosine monophosphate (cAMP) production and PKA activity [111] (Figure 1). Experiments in mammalian hippocampal membranes demonstrated that serotonin inhibited forskolin-stimulated cAMP accumulation through 5-HT1A receptors [112-113]. This effect has been duplicated in hippocampal and cortical neuron cultures [114], and in cells expressing 5-HT1A receptor gene [21, 115-118]. In the brain, only 5-HT1A
heteroreceptors have been shown to couple to Giα-induced inhibition of adenylyl cyclase, as 5-HT1A autoreceptors located in the dorsal raphe nucleus do not inhibit adenylyl cyclase [119]. The spatial preference may account for the expression of signaling components in specific brain areas. For example, 5-HT1A receptors primarily couple to Giα3 in the dorsal raphe nucleus and to Goα in the hippocampus [120]. In addition, the desensitization properties of 5-HT1A autoreceptors in the dorsal raphe nucleus are far more sensitive than are heteroreceptors in the limbic areas [121-123], which may also affect its coupling to the signaling pathway.

In the passive avoidance paradigm, the 8-OH-DPAT-induced decrease in PKA activity in the hippocampus causes increased protein phosphatase-1 activity and a reduction of training-induced phosphorylation of Ca2+/calmodulin-dependent protein kinase II (CaMKII), and this signaling effect is accompanied by an impairment of performance [124]. Therefore, inhibition of adenylyl cyclase and PKA activity may mediate 5-HT1A receptor-regulated behaviors.

Activation of 5-HT1A receptors also activates G protein-coupled inward rectifying postassium channels (GIRKs) [125] in the hippocampus [23, 126, 127] and in the dorsal raphe nucleus [119, 128]. Given that 5-HT1A receptors do not couple to inhibition of adenylyl cyclase in the raphe nucleus [119], the robust activation of inward potassium currents in the dorsal raphe nucleus by 5-HT1A receptor agonists is unlikely to be an adenylyl cyclase-dependent response. Instead, activation of GIRKs is primarily mediated by G protein βγ subunits upon receptor activation [129]. The ability of 5-HT1A receptors to activate GIRK-induced hyperpolarizing currents allows them to have a strong effect on
neuronal firing and excitability [128], a physiological process that may be linked to 5-HT1A receptor-regulated behaviors [104].

Despite of the well-established coupling of 5-HT1A receptors to the Giα/oα-mediated adenylyl cyclase-cAMP-PKA and the Gβγ-mediated GIRK pathways, the functions of these canonical signaling mechanisms in 5-HT1A receptor-regulated neuronal activity and behaviors have not been studied in detail. The complex signal transduction mechanisms in brain require combined pharmacological, biochemical, and molecular techniques to elucidate the role of each component within the signaling pathway, which may have limited the in vivo approaches to define their roles in 5-HT1A receptor-regulated functions in neurons and in brain as a whole. Additionally, signaling mechanisms other than the canonical pathways may have substantial effects in mediating functions of 5-HT1A receptors, among which the signaling pathways traditionally associated with growth factor receptors have been increasingly recognized for their association with 5-HT1A receptors.

5-HT1A receptors and Mitogen Activated Protein Kinases (MAPK) signaling pathway

MAPKs are known for their roles in growth and survival [130], and they are critical regulators of development and plasticity in the central nervous system [131, 132]. The MAPK family includes extracellular signal-regulated kinases 1 and 2 (ERK1/2, also known as p42 and p44 MAPK), p38-MAPK, and c-Jun N-terminal kinase (JNK) [133, 134]. Of these MAPKs, ERK is particularly affected by 5-HT1A receptors. ERK is traditionally activated by growth factor tyrosine kinase receptors. These receptors activate the small molecule GTPase Ras, which activates Raf1, which in turn phosphorylates and activates MAPK/ERK kinase 1 and 2 (MEK1/2) [135] (Figure 1). MEK is a direct upstream protein kinase regulator of ERK, activation of which phosphorylates and activates ERK [131].
Activation of this pathway leads to changes in downstream protein kinases, such as the Ribosomal S6 kinase (RSK) [136], and transcription factors such as Myc [137] and the oncogene Elk1 [137]. Phosphorylation of proteins by ERK in neurons results in receptor and ion channel activation, gene expression, and neuroplasticity [132], all of which may alter behaviors. One interesting example is activation of the transcription factor CREB by the ERK substrate serine/threonine protein kinase RSK [136, 138, 139]. CREB is a widely studied transcription factor for its gene expression function and the underlying roles in stress, anxiety, and depression [140], its regulation by the ERK signaling pathway suggests that ERK may have important impact in mood-related behaviors. The behavioral effects of the MEK/ERK signaling pathway have been reported in several studies, with MEK inhibitors causes diverse behavioral changes in animals, ranging from hyperactivity, reduced or increased anxiety, and depressive-like behavior [141-144], and MEK inhibitors also block the behavioral effect of antidepressants [145]. The diverse effect of MEK inhibitors may be due to the multiple regulators and substrates linked to MEK/ERK, and to further dissect the behavioral effects of this signaling pathway, it is important to understand the specific regulators to each component of this signaling pathway.

5-HT1A receptors were first reported to activate ERK by phosphorylation in non-neuronal cells expressing 5-HT1A receptors [146, 147]. This effect of 5-HT1A receptors is sensitive to pertussis toxin-induced inhibition of G-proteins [66, 146-149], suggesting that G-protein-coupled signaling mechanism is involved in the initiation of ERK activation by 5-HT1A receptors. As in growth factor-regulated ERK activation, 5-HT1AR-induced ERK activation is mediated by the small GTPases Ras and Raf [66, 146, 149, 150] and active MEK [66], a signal cascade that requires the calmodulin-dependent endocytosis of receptors.
as an intermediate step [150]. Additionally, activation of ERK by 5-HT1A receptors in non-neuronal cells can be mediated by the phosphatidylinositol 3-kinase (PI3K) and phosphatidylcholine-specific phospholipase C (PLC) in a G-protein-dependent manner [66, 146, 147], but the details of signal transduction from G-protein-dependent PI3K to ERK during 5-HT1A receptor activation are not completely known.

Despite consistent findings in cell systems with heterologous expression of 5-HT1A receptors, effects of 5-HT1A receptors on ERK activity vary in cells of neuronal origin. In hippocampal-derived differentiated HN2-5 cells, 5-HT1A agonists increase ERK phosphorylation and activity, an effect that is dependent on the small GTPases Ras and Raf, MEK and calcium mobilization [54, 56]. However, this effect of 5-HT1A receptors was not found in primary culture of hippocampal neurons [151] or fetal rhombencephalic neurons [65], and in differentiated raphe neurons, 5-HT1A receptors are coupled to a Gβγ subunit-dependent decrease in MEK activity and ERK phosphorylation [152]. Many factors may affect the response of ERK to 5-HT1A receptor activation in cells. For example, high receptor density in cell culture seems to be required for 5-HT1A receptor-induced activation of ERK [153], but the preferred coupling of 5-HT1A receptors to a specific G protein subtype and its availability in the tested cells may also affect the response [154]. Although determinants that associate 5-HT1A receptors to ERK remain to be identified, results from these studies suggest that 5-HT1A receptor-mediated regulation of ERK in neurons could be highly selective. This can be particularly important in the brain because of the diversity of brain regions and neuron types that are home to 5-HT1A receptors.

Indeed, several studies have suggested that activation of ERK by 5-HT1A receptor activation is not a universal response in brain. Consistent findings have shown that 5-HT1A
receptor agonists rapidly but transiently increase phosphorylation of ERK in the hypothalamus [155-158], and this effect of 5-HT1A receptors is likely an intermediate step for 5-HT1A receptor-induced elevation of oxytocin, adrenocorticotropin (ACTH), and prolactin [155]. In contrast, 5-HT1A receptor activation decreases ERK phosphorylation in the hippocampus [155-157, 159]. Although the underlying significance of this negative coupling of ERK to 5-HT1A receptors in the hippocampus is unclear, ERK is known as a crucial regulator in cognition and an important mediator of synaptic plasticity [131, 132]. Inhibition of hippocampal ERK activity could potentially play a role in 5-HT1A receptor-mediated alterations in synaptic plasticity or in 5-HT1A receptor-induced disruption of cognition. Findings for regulation of ERK phosphorylation by 5-HT1A receptor activation in other brain areas, such as the cerebral cortex, amygdala, and dorsal raphe nucleus, are less consistent. In the frontal cortex, 5-HT1A receptor agonists are reported to increase ERK phosphorylation in some studies [157, 158], but have no effect in other studies [159]. In acute prefrontal cortical slices, activation of neither 5-HT1A nor NMDA receptors alone affects ERK, but simultaneous activation of both receptors results in a decrease in ERK phosphorylation [25], suggesting that crosstalk between different neurotransmitters, receptors, and signaling mechanisms coordinates the regulation of ERK in the cortex. Detailed studies in defined cortical areas and neuron types are warranted to further understanding of a relation between 5-HT1A receptors and ERK signal transduction in the cortex. In contrast to studies in differentiated raphe neurons showing a decrease in ERK activity by 5-HT1A receptor activation [152], systemic treatment with 5-HT1A receptor agonist results in a transient increase in ERK activity in the dorsal raphe nucleus [155, 156], whereas selective activation of 5-HT1A autoreceptors may also indirectly affect ERK
activity through regulation of serotonin release in other brain areas [157]. Therefore, regulation of ERK activity by 5-HT1A receptors in brain is divergent and complicated. Experiments focusing on localized 5-HT1A receptor activation or using transgenic mice with spatial- and temporal- 5-HT1A receptor modification will be useful in further delineating specific regulation of ERK by 5-HT1A receptors in brain.

With the prominent effect of 5-HT1A receptors in regulating anxiety, mood, and cognition, and the evidence showing its brain region-selective effect on ERK, further investigation of the role of ERK in mediating 5-HT1A receptor-regulated neuronal activity and behaviors may help define the specific function of MAPK signaling pathway in brain, as well as the therapeutic potentials of modulating this 5-HT1A receptor-regulated signaling pathway.

5-HT1A receptors and the Akt signaling pathway

Another growth factor-regulated signaling pathway, the PI3K and Akt pathway, can also be regulated by 5-HT1A receptors. When tyrosine kinase receptors are activated by growth factors, they recruit PI3K to activate phosphoinositide-dependent kinase (PDK), which phosphorylates and activates Akt [160] (Figure 1). Akt is a well-known regulator of cell survival as activation of Akt by growth factors mediates insulin-stimulated growth responses and promotes survival against apoptotic stimuli [160]. In brain, Akt has been increasingly recognized as a crucial mediator in neurotrophin and neurotransmitter actions [161, 162]. As with ERK, Akt is a protein kinase that phosphorylates a variety of substrates, such as downstream protein kinases and transcription factors.

Glycogen synthase kinase 3 (GSK3) is a protein kinase that is primarily phosphorylated and inactivated by Akt [163] and several other protein kinases, such as PKC
GSK3 is a potential molecular target in several psychiatric disorders, particularly mood disorders, as the mood stabilizer lithium is a selective inhibitor of GSK3 [166, 167]. Inhibition of GSK3 by pharmacological or genetic means mimics the effects of antidepressants [168, 169] and anti-manic drugs [169, 170], whereas impaired regulation of GSK3 results in behavioral abnormalities reminiscent of states of mania and depression [171, 172]. Another relevant group of Akt substrates is the Forkhead box O transcription factors (FoxOs). In response to growth factors, active Akt phosphorylates and inactivates FoxOs by exporting them out of the nucleus [173]. In both invertebrate and animal brain, FoxOs can be phosphorylated and inactivated by serotonin via the PI3K/Akt-dependent mechanism [174, 175], and the brain FoxO3a subtype can be inactivated by the antidepressant imipramine [175] and down-regulated by lithium [176]. In addition, mice with FoxO deficiency exhibit antidepressive and anxiolytic behavioral phenotypes [175]. Therefore, regulation of protein substrates by Akt in brain plays a critical role not only in neuronal growth and survival, but also in the maintenance of neuronal activity and behavior.

In non-neuronal cells, activation of heterologously expressed 5-HT1A receptors increases Akt phosphorylation that represents the active state of Akt [66, 67, 177]. Similar to regulation of ERK, regulation of Akt by 5-HT1A receptors is sensitive to Gi/o and is mediated by PI3K and Ras [66]. Additionally, activation of Akt by 5-HT1A receptors can be inhibited by cAMP and restored after inactivation of PKA [67], suggesting that 5-HT1A receptor-induced inhibition of adenylyl cyclase-cAMP-PKA signaling pathway is also involved in activation of Akt. This finding are in line with other studies showing that cAMP can induce dephosphorylation and inactivation of Akt via PKA-dependent activation of protein phosphatases [178].
5-HT1A receptor agonists have consistently shown to increase Akt phosphorylation in neuronal cells, including hippocampal derived HN2-5 cells [54], primary hippocampal neurons [151, 179], and primary fetal rhombencephalic neurons [65]. As in non-neuronal cells, 5-HT1A receptor-induced Akt activation in neurons is sensitive to pertussis toxin and is dependent on PI3K [151]. Therefore, regulation of Akt and its down-stream targets is potentially a signal transduction mechanism that mediates the physiological and behavioral functions of 5-HT1A receptors.

Regulation of Akt by 5-HT1A receptors in the mammalian brain has not yet been reported; however, some indirect evidence does suggest an effect of 5-HT1A receptors in regulating Akt. For example, systemic treatment of mice with the 5-HT1A receptor agonist 8-OH-DPAT robustly increased the N-terminal serine phosphorylation of GSK3, a major Akt-regulated mechanism [163], in several brain regions [180], and the serotonin-induced increase in GSK3 phosphorylation can be blocked by a 5-HT1A receptor antagonist [180]. Additionally, in the mouse brain, enhancing synaptic serotonin resulted in increased phosphorylation of Akt, an effect that was blocked by intracerebroventricular injection of a PI3K inhibitor [175]. However, none of these studies directly examined if 5-HT1A receptors regulate Akt in the mammalian brain, and it is also not known if an effect of 5-HT1A receptors on Akt is brain region-specific. Additional studies are also needed to determine if regulation of Akt signaling pathway by 5-HT1A receptors has an impact in 5-HT1A receptor-regulated neuronal activity and behavior.

Taken together, increasing evidence suggests that 5-HT1A receptors are linked to not only the conventional Gi/o-mediated signaling pathway, but also MAPK and Akt signaling pathways that are associated with neuronal development and survival. Additional studies are
needed to elucidate brain region- and cell type-specific signaling mechanisms regulated by 5-HT1A receptors as they may diversely mediate the physiological and behavioral functions of this major serotonin receptor. A better understanding of the signal transduction mechanisms associated with 5-HT1A receptors may lead to discovery of novel drug targets for the treatment of pathological conditions associated with abnormal activity of 5-HT1A receptors.

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Figure 1. 5-HT1A Receptor-regulated Signal Transduction Pathways. 
5-HT, serotonin; 5-HT1A, serotonin 1A receptors; Gi, inhibitory Guanine nucleotide binding protein; AC, adenyl cyclase; cAMP, cyclic adenosine monophosphate; GIRK, G-protein coupled inward rectifying potassium channel; GFR, growth factor receptor; PKA, protein kinase A; CREB, cAMP response element binding protein; MEK1/2, MAP and ERK kinase 1/2; ERK 1/2, extracellular signal-regulated kinase 1/2; RSK, ribosomal S6 kinase; PI3K, phosphotidylinositol-3 kinase; PDK, phosphoinositide dependent kinase; GSK3, glycogen synthase kinase-3; FoxO, forkhead box O transcription factor
Forkhead Box, Class O Transcription Factors in Brain: Regulation and Behavioral Manifestation

by


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Format adapted for dissertation
Abstract

Background: The mammalian FoxO transcription factors function to regulate diverse physiological processes. Emerging evidence that both BDNF and lithium suppress FoxO activity suggests a potential role of FoxOs in regulating mood-relevant behavior. Here, we investigated whether brain FoxO1 and FoxO3a can be regulated by serotonin and antidepressant, and whether their genetic deletion affects behaviors.

Methods: C57BL/6 mice were treated with d-fenfluramine to increase brain serotonergic activity, or with the antidepressant imipramine. The functional status of brain FoxO1 and FoxO3a was audited by immunoblot analysis for phosphorylation and subcellular localization. The behavioral manifestations in FoxO1 and FoxO3a deficient mice were assessed via the Elevated Plus Maze Test, Forced Swim Test, Tail Suspension Test, and Open Field Test.

Results: Increasing serotonergic activity by d-fenfluramine strongly increased phosphorylation of FoxO1 and FoxO3a in several brain regions, and reduced nuclear FoxO1 and FoxO3a. The effect of d-fenfluramine was mediated by the PI3K/Akt signaling pathway. Chronic, but not acute, treatment with the antidepressant imipramine also increased the phosphorylation of brain FoxO1 and FoxO3a. When FoxO1 was selectively deleted from brain, mice displayed reduced anxiety. In contrast, FoxO3a deficient mice presented with a significant antidepressant-like behavior.

Conclusion: FoxOs may be a transcriptional target for anxiety and mood disorder treatment. Despite their physical and functional relatedness, FoxO1 and FoxO3a influence distinct behavioral processes linked to anxiety and depression. Findings in this study reveal important new roles of FoxOs in brain and provide a molecular framework for further investigation of how FoxOs may govern mood and anxiety disorders.
Introduction

FoxO (forkhead box, class O) belongs to the large family of forkhead transcription factors that are characterized by a conserved “forkhead box” DNA-binding domain (1-3). Four mammalian FoxO transcription factors, FoxO1, FoxO3a, FoxO4, and FoxO6 (4-8), have been identified as orthologs of DAF16, an insulin-responsive transcription factor involved in regulating longevity of worms (9) and flies (10).

The activity of FoxO transcription factors is largely controlled by posttranslational regulation. Activation of growth factor receptors activates phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway which results in phosphorylation of FoxO proteins at three serine/threonine residues – threonine 24, serine 256 and serine 316 for FoxO1, and threonine 32, serine 253 and serine 315 for FoxO3a (11-16). Phosphorylation of FoxOs results in redistribution from the nucleus to the cytosol and therefore prevents their binding to DNA (11, 15-18). Besides inhibition by Akt-induced phosphorylation, phosphorylation by other protein kinases, nuclear acetylation, and proteasomal degradation also contribute to the multiple levels of posttranslational regulation of FoxO transcriptional activity (19-23).

Studies in mammalian cells have shown that activation of FoxO transcription factors can induce the expression of genes involved in apoptosis (11, 16, 24-26), cell survival and differentiation (27-29), cell cycle arrest (30-33), and resistance to oxidative stress (34-37). Consistent with their capacity to regulate diverse networks of genes, the FoxOs have been linked to a number of human diseases, such as cancer (4-7, 21, 38-41) and diabetes (42-44). FoxOs also have diverse roles in developmental and reproductive biology, in which FoxO1-null mice dying on embryonic day 10 from impaired vasculogenesis (45, 46), whereas FoxO3a-null mice exhibiting premature ovarian failure (46, 47).
Fewer studies have explored the function of FoxOs in the brain and its link to neuropsychiatric diseases. In neuronal tissues, FoxO3a induces the expression of the proapoptotic Bcl-2 interactive mediator (Bim) (26, 48-52). Ischemia in the brain has been shown to elevate the level of active FoxO3a (49, 53, 54). Neurotrophins, such as BDNF, activate signaling pathways leading to phosphorylation and inactivation of FoxOs (48, 55, 56). More recently, a study in *C. elegans* (57) revealed that activation of serotonin receptors led to inhibition of FoxO transcriptional activity via activation of Akt, which appeared to be an important mechanism in stress modulation in worms. Additionally, we have also reported that FoxO3a transcriptional activity in mouse brain is inhibited by the mood stabilizer lithium (58). We therefore hypothesized that FoxO is an important mediator of behaviors normally regulated by neuromodulators. In this study, we sought to test this hypothesis by examining the regulation of mouse brain FoxO1 and FoxO3a by serotonin and antidepressant and by testing anxiety- and mood-relevant behaviors in mice lacking FoxO1 or FoxO3a.

Materials and Methods

*Animals and treatments*

The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham approved the experimental protocol using mice. Adult male C57BL/6 mice (8-10 weeks of age, Frederick Cancer Research, MD) were used for pharmacological treatments. After a one-week accommodation in the university animal facility, mice were treated with d-fenfluramine or imipramine dissolved in saline and injected intraperitoneally (i.p.). For intra-cerebroventricular (i.c.v.) injection, mice were anesthetized with ketamine and xylazine (100 mg/kg:10 mg/kg) to placed a guide cannula (2.2 mm) stereotaxically.
(posterior 0.8 mm and left 1.6 mm to Bregma). Five days later, the PI3K inhibitor LY294002 (5 nM) or vehicle (10% DMSO) was infused into the left ventricle via an internal cannula. Animals were given d-fenfluramine (i.p.) 90 min after LY294002. At the end of treatment, mice were rapidly decapitated and brain regions (cerebral cortex, hippocampus, and striatum) were immediately dissected in ice-cold saline and used for immunoassays.

_FoxO1 and FoxO3a mouse strains_

See details in Supplemental Materials.

_RT-PCR_

Brain RNA was extracted with Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA) and chloroform, precipitated with isopropanol, washed with 75% ethanol, and stored at -80°C in DEPC-treated water. RT-PCR was performed using the SuperScript III One-step RT-PCR system (Invitrogen Life Technologies, Carlsbad, CA) with the forward and reverse primers of FoxO1 (5’-CCTGTCGTACGCCGACCTCATCAC-3’ and 5’-GTCCATGGACGCAGCTCTTCTCCG-3’). PCR products (15 ml) were separated on a 2% agarose gel, visualized by ethidium bromide staining, and photographed with Fluor-S MultImager (Bio-Rad, Hercules, CA).

_Protein preparation and immunoblotting_

Proteins from brain homogenate or cortical nuclear extracts were prepared as previously described (48, 59). Proteins were resolved in 7.5-10% SDS-polyacrylamide gels, and immunoblotted with antibodies to phospho-Ser256-FoxO1, FoxO1, phospho-Ser253-FoxO3a, phospho- Thr308-Akt, phospho-Ser473-Akt, Akt (Cell Signaling Technologies, Danvers, MA), phospho-Thr32-FoxO3a, FoxO3a, CREB (Upstate Biotech, Lake Placid, NY), and b-tubulin (Sigma-Aldrich, St. Louis, MS). Following a reaction with horseradish
peroxidase-conjugated goat anti-mouse or antirabbit IgG, the immunoreactions were detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ), and protein bands were quantified with a densitometer.

**Immunohistochemistry**

The immunohistochemistry method was as previous described (59). Briefly, brains were immersion-fixed in Bouin’s fixative overnight at 4°C, processed in paraffin, and 4 mm brain sections were prepared on a microtome. Deparaffinized sections were incubated with anti-FoxO1 or anti-FoxO3a, labeled with horseradish peroxidase-conjugated anti-rabbit IgG, and counter-stained with Hoechst 33,258. Immuno-fluorescence in brain sections was viewed with a digital confocal microscope and photographed using a 100x objective. Negative controls of FoxO1 and FoxO3a immunostains were obtained from Brain FoxO1 knockout mouse and FoxO3a-deficient mouse, respectively.

**Behavior tests**

Male and female mice, 10-14 weeks old, were subjected to the Forced Swim Test (FST), Tail Suspension Test (TST), Elevated Plus Maze Test (EPMT), and Open Field Test (OFT) (see details in Supplemental Materials). No more than two behavior tests were conducted within one week and mice were kept in their home cage with food and water between tests.

**Statistical analysis**

All data are presented as mean ± SEM. Statistical analyses were conducted using unpaired Student’s t-test or one-way analysis of variance (ANOVA) for significant differences (p<0.05). Any unexplained outlier value greater than ±2 standard deviations from
the mean of the group was excluded. Any significant difference by ANOVA was followed by a post hoc test.

Results

To assess whether FoxOs can be regulated by serotonin in the mammalian brain, we first measured phosphorylation of brain FoxO1 and FoxO3a after serotonergic activity was increased by administration of d-fenfluramine, a drug that selectively stimulates serotonin release and blocks serotonin reuptake. C57BL/6 mice received an acute treatment with d-fenfluramine (50 mg/kg, i.p.) for 0.5, 1, and 2 hr. Following the dfenfluramine injection, the Akt sensitive phosphorylation and the total levels of FoxO1 and FoxO3a in homogenates from cerebral cortex, hippocampus, and striatum were detected by immunoblots (Figure 1). Acute d-fenfluramine treatment greatly increased the level of phospho-Ser256-FoxO1 in the cerebral cortex, hippocampus, and striatum (Figure 1a). The effect of dfenfluramine was statistically significant in all tested brain regions when data from the 2-hr d-fenfluramine treatment were compared to saline controls (Figure 1b). Dfenfluramine did not significantly change the level of total FoxO1, although a trend towards a decrease was observed in the hippocampus and striatum. Thus, the initial data suggest that a surge of synaptic serotonin can rapidly increase brain FoxO1 phosphorylation.

Similar to the effect on FoxO1, d-fenfluramine treatment greatly increased the levels of phospho-Ser253-FoxO3a and phospho-Thr32-FoxO3a in the cerebral cortex, hippocampus, and striatum (Figure 1c). The level of phosphorylated FoxO3a after 2-hr d-fenfluramine treatment was significantly higher than saline controls, whereas the level of total FoxO3a was not changed by d-fenfluramine in the tested brain regions (Figure 1d). The above
Experiments were conducted using a high dose of d-fenfluramine (50 mg/kg) that is comparable with our previously published results (60). Mice tolerated this dose of d-fenfluramine well with 100% survival rate. To examine if the response of FoxOs to d-fenfluramine is dose-dependent, mice were also treated with different doses of d-fenfluramine (0, 1, 5, 10, 30 mg/kg) for 2 hr (Figure 1e). Except FoxO1 in the cerebral cortex, which was more sensitive to a lower dose of d-fenfluramine (1 mg/kg), a dose range of 10-30 mg/kg was sufficient to induce a 50% increase in FoxO1 phosphorylation in the hippocampus and FoxO3a phosphorylation in both cerebral cortex and hippocampus. To assure maximal effect in all experiments, we then used 50 mg/kg d-fenfluramine for all experiments and data analysis in this study.

Phosphorylation of FoxOs at the Akt-sensitive serine/threonine residues is known to cause translocation of FoxOs from the nucleus to the cytosol, resulting in transcriptional inactivation (11, 14-17). We therefore measured the nuclear and cytosolic FoxO1 and FoxO3a in the cerebral cortex after d-fenfluramine treatment. Both immunoblots and immunostained images indicated that d-fenfluramine treatment reduced nuclear FoxO1 and FoxO3a (Figure 1f, 1g), and the effect of d-fenfluramine was statistically significant when compared to saline treatment (Figure 1f). These results suggest that dfenfluramine can inactivate FoxO1 and FoxO3a in the brain, likely through an increase in the inhibitory phosphorylation.

Since phosphorylation of FoxOs is primarily regulated by the growth factor-activated PI3K/Akt signaling pathway, we measured Akt phosphorylation, an indicator of Akt activation, after d-fenfluramine treatment. Similar to the effects on FoxOs, dfenfluramine caused a rapid increase in phospho-Thr308-Akt and phospho-Ser473-Akt in the cerebral cortex,
hippocampus, and striatum (Figure 2a). The level of Akt phosphorylation after 2-hr d-fenfluramine treatment was significantly higher than saline controls, but d-fenfluramine had no effect on the level of total Akt in the tested brain regions (Figure 2b).

To determine if the above changes resulted from PI3K activation, mice were treated with the PI3K inhibitor LY294002 (5 nM in 1 mL i.c.v.) 90 min prior to d-fenfluramine treatment. LY294002 moderately reduced the d-fenfluramine-induced increases in phospho-Thr\textsuperscript{308}-Akt and phospho-Ser\textsuperscript{473}-Akt in both cerebral cortex and hippocampus. It also reduced d-fenfluramine-induced increases in phospho-Ser\textsuperscript{256}-FoxO1 and phospho-Ser\textsuperscript{253}-FoxO3a, with the effect significant in the cerebral cortex and moderate in the hippocampus (Figure 2c and Table 1). The above results suggest that increasing brain serotonergic activity by d-fenfluramine activates Akt at least partially via PI3K to phosphorylate and inactivate FoxO transcription factors in the mouse brain.

The above findings, taken in conjunction with reports that FoxOs are regulated by the mood regulating BDNF (48, 55) and the mood stabilizer lithium (58), prompted us to hypothesize that FoxOs may be a transcriptional target of mood disorder treatment. We therefore examined if the monoamine-regulating antidepressant imipramine regulates brain FoxOs. An acute imipramine treatment (30 mg/kg, i.p. for 1 hr) did not cause a significant change in either phosphorylated or total FoxO1 and FoxO3a in the tested brain regions, except a trend toward an increase in phospho-Ser\textsuperscript{256}-FoxO1 was noted in the hippocampus (Table 2).

Since the therapeutic effects of antidepressants usually occur after prolonged treatment, we then tested if chronic imipramine treatment (20 mg/kg, i.p.) may regulate brain FoxOs. Before testing brain FoxOs, mice were subjected to the FST after receiving chronic
imipramine treatment (23 days) to confirm an antidepressant-like effect of this treatment. The result demonstrated that the chronic imipramine treatment used in our experiment reduced the immobility time when compared to saline treatment (Figure 3a). Interestingly, chronic imipramine treatment (28 days) caused significant increases in phospho-Ser256-FoxO1 and phospho-Ser253-FoxO3a in the cerebral cortex, hippocampus, and striatum, whereas the levels of total FoxO1 and FoxO3a were not affected by chronic imipramine treatment (Figure 3b and 3c). This effect of imipramine was accompanied by a moderate increase in phospho-Thr308-Akt but no change was observed in the level of total Akt. Thus, a therapeutically relevant imipramine treatment can inhibit FoxO1 and FoxO3a in mouse brain.

Serotonin and antidepressants are key regulators of anxiety and mood. Since both serotonin and imipramine increased the inhibitory phosphorylation of FoxO1 and FoxO3a in mouse brain, we hypothesized that mice lacking brain FoxOs may exhibit an anxiolytic or antidepressive behavioral phenotype. To test this hypothesis, we examined behavioral phenotypes of brain FoxO1-knockout and systemic FoxO3a-deficient mice. Although systemic FoxO1 knockout mice die on embryo day-10 from impaired vasculogenesis (45), the survival of brain-targeted FoxO1 knockout mice has not yet been reported. In order to measure FoxO1-mediated behavioral phenotypes, we generated brain-targeted FoxO1 knockout mice (Figure S1). These mice survived and remained viable through adulthood and bred normally.

To measure anxiety-like behavior, the FoxO1 knockout mice were subjected to the EPMT. In male mice, the time spent in open arms was significantly different among wildtype controls, heterozygous, and homozygous FoxO1 knockout mice. Both heterozygous and homozygous FoxO1 knockout mice stayed a significantly longer time in open arms of the
maze during the test period when compared to wild-type controls, whereas there was no
difference between the heterozygous and homozygous FoxO1 knockout mice (Figure 4a).
Although time in closed arms was only mildly shorter in FoxO1 knockout mice, it was noted
that the frequency to entering closed arms was significantly lower in FoxO1 knockout mice
than in wild-type controls. Results shown in Figure 4a were a summary of three separate
experiments and similar results were observed in all three experiments. These results
demonstrate that FoxO1 plays a role in mediating anxiety-like behavior in mice.

To further test if the anxiolytic phenotype in FoxO1 knockout mice is gender-
specific, three groups of female mice were subjected to the EPMT. Although time spent in
open arms and in closed arms were not different among female wild-type controls,
heterozygous, and homozygous FoxO1 knockout mice, a statistically significant difference
was observed in the frequency of entering closed arms, in which female heterozygous and
homozygous FoxO1 knockout mice had significantly lower frequency than wild-type
controls (Figure 4b). Thus, it appears that both male and female FoxO1 knockout mice carry
an anxiolytic behavioral phenotype, but the phenotype is more pronounced in males than in
females.

When FoxO1 knockout mice were subjected to the FST, somewhat unexpectedly,
both heterozygous and homozygous FoxO1 knockout male mice displayed prolonged
immobility in the FST when compared to wild-type controls (Figure 4c), suggesting an
increase in depressive behavior in mice lacking brain FoxO1. Similarly, a prolonged
immobility was also observed in female homozygous FoxO1 knockout mice. To confirm the
depressive behavior in FoxO1 knockout mice, another group of male wild-type and
homozygous FoxO1 knockout mice were tested in the TST. Homozygous FoxO1 knockout
mice again displayed prolonged immobility (Figure 4d), confirming the depressive phenotype of these mice. The increased immobility time in brain FoxO1 knockout mice was unlikely a result of impaired locomotor activity since FoxO1 knockout mice and wild-type controls had similar travel distance and velocity in the baseline Open Field Test (OFT) (Figure 4e). When the locomotor response of mice to d-amphetamine treatment (4 mg/kg, i.p., 30 min) was tested, the travel distance and velocity in wild-type and FoxO1 knockout mice were increased by 348.23±47.21% and 275.04±43.19%, respectively. Although FoxO1 knockout mice showed a trend towards slightly lower response to d-amphetamine, the difference was not significant.

To test if FoxO1 and FoxO3a have similar effects on anxiety- and mood-relevant behaviors, these behavior tests were applied to a line of mice systemically lacking FoxO3a (FoxO3a-deficient) (61). During the EPMT, both male and female FoxO3a-deficient mice tended to stay longer times in the open arms, but no significant difference was detected among wild-type, heterozygous, and homozygous FoxO3a-deficient mice (Figure 5a). This result suggests that FoxO3a has less impact on anxiety-like behavior than FoxO1. When tested in the OFT, the total travel distance and velocity were also similar among wild-type, heterozygous, and homozygous FoxO3a-deficient mice (Figure 5b), indicating normal locomotor activity in FoxO3a-deficient mice.

Interestingly, male homozygous FoxO3a-deficient mice, in strong contrast to the brain FoxO1 knockout mice, had a significantly lower immobility in the FST when compared to both heterozygous FoxO3a-deficient and wild-type mice (Figure 5c). This antidepressant-like behavioral phenotype required complete deletion of FoxO3a since heterozygous FoxO3a-deficient mice did not display a significant decrease in immobility. Similarly to male
mice, a significantly lower immobility was also observed in female homozygous FoxO3a-deficient mice when compared to wild-type controls.

To test if the low expression of FoxO3a in FoxO3a-deficient mouse brain contributes to the antidepressant phenotype, we measured the levels of cytosolic and nuclear FoxO3a after wild type mice were treated with the antidepressant imipramine (20 mg/kg/day, i.p.) for 28 days. Comparing to saline-treated wild-type mice, an increase in the level of cytosolic FoxO3a and a corresponding decrease in the level of nuclear FoxO3a was noticeable in the cerebral cortex of mice treated with imipramine (Figure 5d), therefore suggesting that the lowered level of FoxO3a may contribute to the antidepressant behavior.

Furthermore, to compare the response of FoxO3a-deficient mice versus comparable wild type mice to antidepressant, a group of homozygous FoxO3a-deficient and littermate wild-type mice received daily injection of saline or imipramine (20 mg/kg/day, i.p.) for 23 days. When tested in the FST, there was a significant difference in immobility among the four groups of mice with different genotypes and treatments (Figure 5e). When multiple comparison with Bonferroni correction was conducted using the wild type saline-treated mice as control, imipramine significantly reduced immobility in wild type mice (p<0.0018), and FoxO3a-deficient mice, either without or with imipramine treatment, also exhibited reduced immobility (p<0.0019 and p<0.0001, respectively). On the other hand, when using the FoxO3a-deficient mice with saline treatment as control, the immobility in imipramine-treated mice, either wild type or FoxO3a-deficient, was not different from control.
Discussion

In this study, we tested the regulation of brain FoxO1 and FoxO3a by serotonin and imipramine, and the behavioral manifestations of genetic insufficiency of each. We focused on FoxO1 and FoxO3a because they are abundant in the adult brain (62-64).

The findings that enhancing brain serotonergic activity by d-fenfluramine treatment increases the inhibitory phosphorylation of FoxO1 and FoxO3a in several regions of mouse brain conform with findings in C. elegans by Liang et al. (57), and we confirm that serotonin can regulate FoxO transcription factors in mammalian brain. Although serotonin often is not considered as a primary activator of the PI3K/Akt signaling pathway, several studies in cultured cells and in invertebrates have shown that stimulation of serotonin receptors can result in phosphorylation and activation of Akt (57, 65, 66). The results shown in this study support the conclusion that enhancing serotonin activity exhibits a regulatory effect in Akt-regulated FoxO1 and FoxO3a in brain. Although the specific serotonin receptors mediating this effect remain to be identified, our observations imply that brain FoxOs are important transcription factors on which signals from serotonergic neurotransmission and neurotrophic activity converge. In our experiments, the PI3K inhibitor LY294002 partially reduced the effect of d-fenfluramine. It is possible that the low dose LY294002 is not sufficient to penetrate into all brain areas, but we do not rule out that other signaling pathways other than the PI3K/Akt signaling pathway involved also contribute to the effect of d-fenfluramine on FoxOs.

Unlike d-fenfluramine, which has a selective dual action on serotonin by blocking reuptake and stimulating release, the antidepressant imipramine inhibits monoamine reuptake to enhance serotonergic and noradrenergic activity in the brain. Interestingly, chronic, but not
acute imipramine treatment significantly increased phosphorylation of FoxO1 and FoxO3a, as well as moderately increased phosphorylation of Akt. Additional to modulating monoamine neurotransmission, chronic antidepressant treatment, including imipramine, is known to increase brain BDNF (67, 68), a neurotrophin that also phosphorylates and inactivates FoxOs (48, 56). Thus, imipramine may have increased FoxO phosphorylation by enhancing serotonin action and increasing brain BDNF. Since the therapeutic effect of an antidepressant typically requires prolonged treatment, the observation from this study links FoxO1 and FoxO3a to a clinically relevant treatment for anxiety and depression. We examined this role of FoxOs by taking advantage of mice with genetically modified FoxO1 and FoxO3a.

Unlike the systemic FoxO1 knockout mice, mice lacking brain FoxO1 survive through adulthood, thus nestin-directed brain FoxO1 deletion provides a useful model to identify behaviors that involve complex neuronal circuits. Mice lacking brain FoxO1 consistently displayed an anxiolytic behavioral phenotype, which in males only required a partial depletion of FoxO1, indicating that FoxO1 has a prominent role in regulating anxiety-like behaviors. This interesting finding is in accordance with the well-known effect of serotonin on anxiety and the anxiolytic effect of most monoamine reuptake inhibitor antidepressants (69). A somewhat surprising observation from this study is that mice lacking brain FoxO1 appear to have a depression-like behavioral phenotype. A significant difference from the matching wild-type mice suggests that the results represent a FoxO1-specific behavioral alteration. Since mood is a complex behavior regulated diversely in brain by monoamines and neurotrophins in a temporal and spatial-dependent manner (70, 71), we speculate that several factors may contribute to this differential mood/anxiety-related effect.
of FoxO1. Noticeably, unlike FoxO3a, the distribution of FoxO1 in the brain is mostly confined to a few brain areas, including dentate gyrus and ventral CA3 area of the hippocampus, the amygdalohippocampal region, the piriform cortex, the striatum, the caudate putamen, and the nucleus accumbens (64). Serotonin regulates mood and anxiety by activating, as well as desensitizing many types of serotonin receptors located in different brain regions (72, 73). BDNF, on the other hand, differentially regulates mood and anxiety in selective brain regions (71, 74, 75). Serotonin and BDNF also interact with each other to achieve enhanced regulation of mood and anxiety (76, 77). The localization of FoxO1 may determine its selective regulation by either BDNF or serotonin in a brain region-specific manner. However, a conclusion cannot be reached until the regulation of brain FoxO1 by neuromodulators is further investigated in detail. On the other hand, the findings in this study may also suggest that maintaining FoxO1 transcriptional activity in brain is necessary to stabilize mood and prevent depression developed under environmental changes, such as stress. A limitation of this study was that the brain FoxO1 knockout mice were generated originally from a mouse line containing floxed alleles of FoxO1, 3a, and 4. During selection for behavior tests, no homozygous FoxO3a knockout mouse was included in the tests. Since FoxO4 expression was limited in brain (62, 63), no FoxO4 genotype was excluded during selection, and there was no significant behavioral difference noticed between Nestin-Cre:FoxO4-floxed and wild type mice (data not shown). To avoid this potential limitation, we tested FoxO3a behavior phenotypes in another line of mice that were only lack of FoxO3a.

In contrast to FoxO1 knockout mice, reducing anxiety was not a prominent feature in mice systemically deficient of FoxO3a. Perhaps more interestingly, FoxO3a-deficient mice
had significantly less immobility in the FST. The non-responsiveness to chronic imipramine treatment in FoxO3a-deficient mice provides evidence that FoxO3a may be a transcriptional mediator of the antidepressant action of imipramine. The increased phosphorylation of Akt and FoxO3a and the decreased nuclear FoxO3a after chronic imipramine treatment also suggest that the Akt signaling pathway may contribute to the delayed antidepressant effect of imipramine in which transcriptional regulation of gene expression is critical for sustained therapeutic effect (78). Furthermore, our group previously reported that glycogen synthase kinase 3 (GSK3), another major phosphorylation substrate of Akt, can be inhibited by serotonin and antidepressants that increase its inhibitory serine phosphorylation (60), and Beaulieu et. al. (79) reported that the depressive and anxiogenic behaviors of serotonin-deficient mice were blunted when GSK3 was semi-deleted. Those studies, in agreement with our findings in this study, indicate that Akt signaling pathway have an important impact in 5HT-regulated behaviors, and a coordinated regulation of substrates of the Akt-dependent signaling pathway by antidepressants may contribute to their therapeutic effects.

As has been observed in studies in other systems, FoxO1 and FoxO3a have divergent physiological effects despite their physical and functional relatedness (45-47). Findings in this study provides new evidence that the two major FoxO subtypes also exhibit distinct physiological effects in brain, where they preferentially regulate behaviors related to anxiety and mood. Detailed studies in the context of FoxO brain distribution, preferential regulation by serotonin and neurotrophins, and selective molecular targets of each FoxO may be useful in addressing their differential roles in brain. On the other hand, the common regulation of FoxOs by serotonin and antidepressant may add a level of complexity to the physiological consequence when all subtypes of FoxOs are regulated simultaneously by a neuromodulator.
Using genetic mouse model that carries more than one FoxO mutant may help further identify the common as well as the divergent functions of FoxOs in brain.
Disclosure / Conflict of Interest / Acknowledgment

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References


Table 1. The Effect of PI3K Inhibitor LY294002 on d-Fenfluramine-Induced Phosphorylation of Akt and FoxOs

<table>
<thead>
<tr>
<th>Brain Regions</th>
<th>LY294002+d-fenfluramine / d-fenfluramine (mean ± SEM)</th>
<th>Sample size n=6</th>
<th>Phospho-Thr³⁰⁸-Akt</th>
<th>p-value</th>
<th>Phospho-Ser²⁵³-FoxO3a</th>
<th>p-value</th>
<th>Phospho-Ser²⁵⁶-FoxO1</th>
<th>p-value</th>
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<td>Cerebral Cortex</td>
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<td>0.55± 0.19</td>
<td>0.06</td>
<td>0.76 ± 0.07</td>
<td>0.02</td>
<td>0.66 ± 0.12</td>
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<td>Hippocampus</td>
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<td>0.72 ± 0.10</td>
<td>0.06</td>
<td>0.78 ± 0.10</td>
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Table 2. Acute Imipramine Treatment

<table>
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<th>Brain Regions</th>
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<th>Phopho-Ser253-FoxO3a</th>
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<td>p-value (n=9)</td>
<td>Mean ± SEM (% saline)</td>
<td>p-value (n=9)</td>
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<td>Cerebral Cortex</td>
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Figure 1. Regulation of FoxOs by d-fenfluramine. C57BL/6 mice were treated with dfenfluramine before FoxO proteins from homogenates of the cerebral cortex (CTX), hippocampus (HIP), and striatum (STR) were examined by immunoblots. (a) Immunoblots of brain phospho-Ser256-FoxO1 and total FoxO1 after saline (0 min) or d-fenfluramine treatment (50 mg/kg, i.p.). (b) Quantification of phospho-Ser256-FoxO1 (ttest; CTX: t=-4.324, p=0.003; HIP: t=-3.205, p=0.013; STR: t=-2.559, p=0.043; n=4-5 per group) and total FoxO1 after saline or d-fenfluramine (d-Fen) treatment for 2 hr. (c) Immunoblots of brain phospho-Ser253-FoxO3a, phospho-Thr32-FoxO3a, and total FoxO3a after d-fenfluramine treatment. (d) Quantification of phospho-Ser253-FoxO3a (ttest; CTX: t=-2.805, p=0.023; HIP: t=-4.356, p=0.005; STR: t=-4.660, p=0.003; n=4-5 per group), phospho-Thr32-FoxO3a (t-test; CTX: t=-2.842, p=0.030; HIP: t=-2.993, p=0.040; STR: t=-4.110, p=0.015; n=3-4 per group), and total FoxO3a after saline or d-fenfluramine treatment for 2 hr. Data are mean ± SEM. *p<0.05 when values are compared to saline treatment in Student’s t-test. (e) Dose-dependent response of FoxO1 and FoxO3a to d-fenfluramine in the cerebral cortex and hippocampus. Values are expressed as % saline treatment (Control). (f) Immunoblots of nuclear and cytosolic FoxO1 and FoxO3a from the cerebral cortex of mice treated with saline (0 min) or dfenfluramine for indicated times (left panel) or 1 hr (middle panel). Data from nuclear FoxO1 and FoxO3a were quantified and calculated as % saline treatment. *p<0.01 when values are compared to saline treatment (t-test; FoxO1: t=5.765, p=0.001; FoxO3a: t=3.911, p=0.006; n=5 per group). (g) Immunohistochemical detection of brain FoxO1 and FoxO3a. C57BL/6 mice were treated with saline- or d-fenfluramine. Coronal section of mouse brain at Bregma -1.5 mm position was photographed under a 10x objective (left) with the mid cortical region squared for capturing Immuno-fluorescent images of FoxO1 and FoxO3a under a 100x objective. In the colored fluorescent photographs, red color shows FoxO1 and FoxO3a, respectively, and blue color shows nuclear stain. For negative controls, brain sections from the FoxO1 knockout mouse and the FoxO3a-deficient mouse were immuno-stained with anti-FoxO1 and anti-FoxO3a antibodies, respectively.
Figure 2. Regulation of Akt signaling pathway by d-fenfluramine. (a) C57BL/6 mice were treated with d-fenfluramine (50 mg/kg, i.p.) before phospho-Thr\textsuperscript{308}-Akt, phospho-Ser\textsuperscript{473}-Akt and total Akt were examined in brain homogenates. (b) Quantification of phospho-Thr\textsuperscript{308}-Akt (t-test; CTX: $t$=-2.873, $p$=0.021; HIP: $t$=-7.842, $p$=0.001; STR: $t$=-4.208, $p$=0.014; n=3-5 per group), phospho-Ser\textsuperscript{473}-Akt (t-test; CTX: $t$=-2.887, $p$=0.020; HIP: $t$=-2.848, $p$=0.047; STR: $t$=-3.588, $p$=0.023; n=3-5 per group), and total Akt after saline and d-fenfluramine (d-Fen) treatment for 2 hr. Data are mean ± SEM. *$p$<0.05 when values are compared to saline treatment in Student’s t-test. (c) Mice received unilateral intracerebroventricular (i.c.v.) injection of either LY294002 (5 nM in 1 ml) or DMSO (vehicle, 1 ml) 1.5 hr prior to receiving saline or d-fenfluramine treatment (2 hr). Immunoblots of phosphorylated and total Akt, FoxO1, and FoxO3a are representatives of five separate experiments.
Figure 3. Regulation of FoxOs and Akt by imipramine. C57BL/6 mice were treated with imipramine (20 mg/kg/d, i.p.) for 23 days before the FST, followed by immunoblotting brain FoxOs and Akt at the end of imipramine treatment (28 days). (a) Mice were subjected to FST 30 min after the 23rd day of saline or imipramine treatment. The immobility (resting time) was recorded during the last 4 min of a 6-min test. Data are mean ± SEM. *p<0.04 (t=2.194, n=10 per group) when values are compared to saline treatment in Student’s t-test. (b) Immunoblots of brain FoxO1, FoxO3a, and Akt after saline (−) or imipramine treatment. (c) Quantification of phospho-Ser256-FoxO1 (t-test; CTX: t=-2.628, p=0.020; HIP: t=-2.226, p=0.044; STR: t=-2.229, p=0.041), phospho- Ser253-FoxO3a (t-test; CTX: t=-2.209, p=0.044; HIP: t=-2.456, p=0.027; STR: t=-3.674, p=0.002), and phospho-T308-Akt (t-test; CTX: t=-2.617, p=0.019; HIP: t=-2.425, p=0.027; STR: t=-2.746, p=0.015). Protein levels in imipramine treated samples are calculated as % saline treatment. Data are mean ± SEM. *p<0.05 (n=7-9 per group) when values are compared to saline treatment in Student’s t-test.
A  EPMT - males

Time in Open Arms  
Time in Closed Arms  
Entries to Closed Arms  

B  EPMT - females

Time in Open Arms  
Time in Closed Arms  
Entries to Closed Arms

C  Forced Swim Test

Males  
Females

D  Tail Suspension Test - Males

Time before intubation (s)

E  Baseline OFT

Total Travel Distance  
Arena Velocity

OFT after d-Amphetamine

Total Travel Distance  
Arena Velocity
Figure 4. Behavior tests in FoxO1 knockout mice. Homozygous (-/-) and heterozygous (+/-) FoxO1 knockout and matching wild-type (+/+ ) mice were subjected to the EPMT in (a) male mice (ANOVA; time in open arm: F{genotype}(2,48)=4.119, p=0.022; entries to closed arms: F{genotype}(2,47)=3.411, p=0.041); (b) female mice (ANOVA; entries to closed arms: F{genotype}(2,39)=11.616, p<0.001); (c) the FST in male (ANOVA; F{genotype}(2,46)=4.350, p=0.019) and female (ANOVA; F{genotype}(2,30)=2.608, p=0.045) mice; and (d) the TST in male mice (t-test; t=-3.115, P=0.005). (e) The OFT was performed in male mice at baseline (no drug treatment) and after d-amphetamine treatment (4 mg/kg, i.p., 30 min). Values after d-amphetamine treatment were calculated as % baseline. Data are expressed as mean ± SEM (male: n=18; female: n=16 for each test). Statistical analysis is conducted using one-way ANOVA with pair-wise comparisons of sample means via the Tukey HSD test or Student’s t-test (d). *p<0.05 when the testing group is compared to the wild-type mice.
Figure 5. Behavior tests in FoxO3a-deficient mice. Homozygous (trap/trap) and heterozygous (+/trap) FoxO3a-deficient and matching wild-type (WT) mice were subjected to behavioral tests (male: n=10; female: n=9 for each test). (a) The EPMT in male and female mice. (b) Baseline OFT in male mice. (c) The FST in male and female mice. Data are expressed as mean ± SEM. Statistical analysis is conducted using one-way ANOVA (male: $F_{\text{genotype}(2,30) }=4.647$, $p=0.017$; female: $F_{\text{genotype}(2,15) }=5.959$, $p=0.012$) with pair-wise comparisons of sample means via the Tukey HSD test. *$p<0.05$ when the testing group was compared to the wild-type mice, and **$p<0.05$ when heterozygous were compared to homozygous FoxO3a-deficient mice. (d) The level of total FoxO3a in the cerebral cortex of FoxO3a-deficient (trap/trap) and wild-type (WT) mice (left panel), and in the cytosolic and nuclear fractions of the cerebral cortex of wild-type mice treated with saline or imipramine for 28 days (right panel). (e) Male FoxO3a-deficient (trap/trap) and littermate wild-type (WT) mice were treated with either saline or imipramine (20 mg/kg/d, i.p.) for 23 days before the FST. Data are mean ± SEM. Statistical analysis is conducted using one-way ANOVA ($F_{\text{genotype} \times \text{treatment}(3,25)=8.543}$, $p<0.001$) with post hoc multiple group comparisons (n=6-8). Since this analysis involved 6 comparisons, all $p$ values less than 0.008 were considered significant. *$p<0.005$ when data from each testing group was compared to the saline-treated wild type group.
Supplementary Materials and Methods

*FoxO1 and FoxO3a mouse strains*

Homozygous FoxO1/3/4-flox/flox mice on a FVB background (1, 2) were cross-bred with Nestin-Cre mice on a C57BL/6 background (Jackson Laboratory, Bar Harbor, Maine) to generate homozygous Nestin-Cre:FoxO1-flox/flox (Brain-targeted FoxO1 knockout) mice. Genotype was confirmed by PCR using primers 5’-GCTTAGAGCAGAGATGTTCTCACATT, 5’-CCAGAGTCTTTGTATCAGGCAAATAA, and 5’-CAAGTCCATTAATTCAACGACATTGA for FoxO1 (2), and 5’-CGAGTGATGAGGTTCGCAAGAACC and 5’-TCCATGAGTGAACGAACCTGGTCG for Cre. Littermates with Nestin-Cre negative or FoxO1 wild type genotypes were used as FoxO1 controls. Homozygous FoxO3a-deficient and littermate wild-type mice were generated from the heterozygous FoxO3a-trap mice on a C57BL/6 background (3). Genotyping of each mouse was confirmed at 30 days of age as previously described (3).

*Behavior Tests*

Forced Swim Test (FST) (4) was conducted using an automated apparatus (Kinder Scientific, Poway, CA). Movements were continuously monitored by computer for 6 min with data recorded using the Motor Monitor Software. The resting time (representing immobility) during the last 4 min of testing time was analyzed (5). Tail Suspension Test (TST) (6) was conducted using an automated TST system (Med Associates Inc, St. Albans, VT). Mouse tail was attached to a strain gauge that detects any movements of a mouse and movement was tested for 6 min. The duration of immobility during the last 4 min of the test was recorded by the computer software and calculated as the time the movement force was below a preset threshold (7).
For the Elevated Plus Maze Test (EPMT) (8), each mouse was placed on the central platform of the maze (San Diego Instruments, San Diego, CA), and movements were observed for 4 min with an Ethovision camera driven tracker system (Noldus, The Netherlands). Entries into (number of entries) and time spent (seconds) in each arm were recorded via computer during the 4-min testing time and data were analyzed using the Ethovision software.

For the Open Field Test (OFT) (9), each mouse was placed into an arena of 42 cm$^2$ with 20 cm (Coulburn Instruments, Whitehall, PA), and activity was monitored with the Ethovision system for 4 min. The locomotor activity was recorded via computer as total travel distance (cm) and travel velocity (cm/sec), and data were analyzed using the Ethovision software. Some mice received d-amphetamine (4 mg/kg, i.p.) treatment for 30 min before testing. The d-amphetamine-induced locomotor activity in each mouse was recorded and calculated as the % of its own baseline activity.

Results

A line of nestin-directed FoxO1 knockout mice was generated by cross-breeding FoxO1/3/4-flox/flox mice (2, 10) with Nestin-Cre mice carrying Cre recombinase under the control of a nestin promoter to preferentially knockout brain FoxO1 (11) (Figure S1a). The PCR products from tail and brain DNAs in the Cre+ FoxO1-flox/flox mice confirmed a specific brain FoxO1 knockout pattern represented by a 149bp fragment in the tail DNA and a 190bp fragment in the brain DNA (Figure S1b). RT-PCR detection of FoxO1 mRNA in the cerebral cortex was lower in the Cre+ FoxO1-flox/flox mice than in littermate controls (which contain intact FoxO1) (Figure S1c). Immunoblots of FoxO1 in the cerebral cortex,
hippocampus, and striatum revealed almost complete deletion of FoxO1 protein in the FoxO1 knockout mice, whereas FoxO1 is detectable in the lungs (Figure S1d).


**Figure S1.** Generation of brain FoxO1 knockout mice. FoxO1/3/4-flox/flox mice were crossbred with Nestin-Cre mice. (a) Representative PCR genotyping results from tail DNA show different genotype combinations of Cre, FoxO1, FoxO3a, and FoxO4. The maps of the floxed FoxO constructs, the relative primer positions, and size of DNA fragments are as previously reported (1, 2). (b) PCR of tail and brain DNA from wild type (Cre-), heterozygous (Cre+:FoxO1-fl/+), and homozygous (Cre+:FoxO1-fl/fl) FoxO1 knockout mice. (c) RT-PCR of brain FoxO1 mRNA from homozygous and heterozygous FoxO1 knockout and matching wild type mice. (d) Representative immunoblots of the levels of FoxO1 protein in the cerebral cortex, hippocampus, striatum, and lung in homozygous FoxO1 knockout and matching wild-type mice.
Regulation of Hippocampal Glycogen Synthase Kinase 3 by 5-HT1A Receptors

by

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Abstract

Serotonin is a monoamine neurotransmitter that plays important roles in regulating physiology and behavior. Among the many serotonin receptor subtypes, 5-HT1A receptors are among the most widely studied for their regulation of anxiety, mood, and cognition, and are important in the treatment of disorders related to these behaviors. Increasing evidence suggests that 5-HT1A receptors regulate other signaling pathways, such as those traditionally regulated by growth factors. We previously reported that 5-HT1A receptors regulate glycogen synthase Kinase 3 (GSK3), a serine/threonine protein kinase that is enriched in the brain and regulates a variety of neuronal functions. Emerging evidence suggests that multiple classes of treatments for psychiatric disorders converge on inhibition of GSK3 as a common step, and that dysregulation of GSK3 can lead to mood-related behavioral abnormalities. In this study, we further elucidate the specific signaling pathway responsible for regulation of GSK3α and GSK3β isoforms by 5-HT1A receptors in the hippocampus and the role of this regulation in hippocampal-dependent behavioral effect of 5-HT1A receptors. We find that systemic treatment with a 5-HT1A receptor agonist increases inhibitory N-terminal serine phosphorylation of both GSK3α and GSK3β in the hippocampus through activation of the PI3K/Akt pathway. Additionally, genetically modified GSK3 knock-in mice with disabled inhibitory N-terminal serine phosphorylation have significantly reduced response to 8-OH-DPAT-induced inhibition of hippocampal-dependent contextual fear learning. In summary, these data suggest that 5-HT1A receptors regulate hippocampal GSK3 through activation of the PI3K/Akt cascade, and that inactivation of GSK3 is an important mechanism in the hippocampal-dependent behavioral effect of 5-HT1A receptors.
Introduction

Serotonin (5-HT) is a crucial neurotransmitter involved in regulation of a number of physiological and behavioral states, including anxiety, mood, cognition, sleep, sexual behavior, and food intake. Serotonergic neurons arise in the dorsal and median raphe nucleus of the brain stem, and their projections reach throughout the brain. Serotonin is generally released in a paracrine manner [1], allowing one serotonergic fiber to affect the function of numerous cells in the forebrain. Serotonin exerts its effects through a large and still growing family of receptors, the majority of which are members of the g-protein coupled receptor superfamily [1]. Of these, 5-HT1A receptors have been particularly implicated in pathological states such as depression [2] and anxiety [3], as well as ongoing non-pathological processes such as cognition [4].

5-HT1A receptors are widely distributed throughout the brain [5, 6] with highest expression in the dorsal raphe nucleus, hippocampus, lateral septum, several hypothalamic nuclei, and entorhinal cortex. Within the hippocampus, 5-HT1A receptors are highly prevalent in the stratum radiatum and stratum oriens layers of the CA1 subfield as well as in the molecular layer and granule cell layer of the dentate gyrus. More moderate expression occurs in stratum radiatum and stratum lacunosum moleculare of the CA3 subfield. The robust expression of these receptors in the hippocampus strongly suggests that they play an important role in hippocampal function.

Indeed, a wide body of literature suggests that 5-HT1A receptors play an important role in hippocampus-dependent cognitive tasks [4]. Notably, activation of 5-HT1A receptors has repeatedly been shown to inhibit contextual fear conditioning [7, 8]. 5-HT1A receptors have also been strongly implicated in both physiological and pathological control of anxiety.
[9] and mood [2], both of which can be modulated by the hippocampus [10, 11]. Activation of 5-HT1A receptors has an anxiolytic effect in preclinical models of anxiety [3, 12], and 5-HT1A receptor knock-out mice have severe anxiety [13, 14]. Activation of 5-HT1A receptors also has an antidepressant effect in animal models of depression [12]. Thus, 5-HT1A receptors play an important role in regulating hippocampal mediated behavior.

One missing link in the study of 5-HT1A receptors and their control of physiology and behavior is the signaling that connects these receptors to their downstream functions. 5-HT1A receptors activate a number of signaling pathways [12]. 5-HT1A receptors classically couple to inhibitory G proteins. Through this pathway, 5-HT1A receptors couple to inhibition of adenylyl cyclase and decreased cAMP production [15, 16]. 5-HT1A receptors have been shown to couple to activation of Extracellular Signaling Related kinase (ERK) in cultured cells [17, 18], but in the hippocampus 5-HT1A receptor activation leads to decreased ERK activation [19-22]. Additionally, increasing evidence shows that 5-HT1A receptors are capable of activating the Phospho-inositol-3-Kinase (PI3K) cascade. In transfected cells and primary cultured hippocampal neurons, activation of 5-HT1A receptors increases activation of Akt in a PI3K dependent fashion [23-26]. Although activation of Akt by 5-HT1A receptors has not been directly shown in the rodent brain, indirect evidence suggests that this may be the case. Increasing serotonin signaling with d-Fenfluramine increases active Akt in the rodent hippocampus, as well as increasing Akt-mediated inhibition of FoxO transcription factors [27]. 5-HT1A receptor agonist treatment also increases phosphorylation of the Akt target GSK3 in the rodent brain, although the mechanism of this increase is unclear [28]. Thus, 5-HT1A receptors are capable of activating a number of signaling
pathways, but it remains unclear what role these pathways play in the effects of 5-HT1A receptors.

One of the most intriguing targets of 5-HT1A receptors is GSK3, a serine/threonine protein kinase that has been implicated in a wide range of functions in the nervous system [29]. There are two highly similar isoforms of GSK3, α and β [30], and these isoforms are expressed ubiquitously throughout the body and enriched in the brain [31, 32]. GSK3 is active at basal state, but can be strongly inhibited by phosphorylation on an N-terminal serine (S21 and S9 on GSK3α and β respectively) [33, 34] mediated by upstream kinases such as Protein Kinase C [35], Protein Kinase A [36], and Akt [37]. GSK3 has been shown to play an important role in a number of neuronal functions, including regulation of gene expression [29], neuronal development [38], apoptosis [39], neuronal structure and polarity [29], and synaptic plasticity [40]. GSK3 has been shown to play a particularly important role in the hippocampus, regulating long-term depression and potentiation [40] and adult hippocampal neurogenesis [41].

Given the importance of GSK3 in the hippocampus, we hypothesized that GSK3 may be a mediator of the effects of 5-HT1A receptors on the hippocampus. To investigate this, we first sought to elucidate the mechanism by which 5-HT1A receptor activation results in increased phosphorylation of GSK3. We also investigated the necessity of this phosphorylation for 5-HT1A receptor mediated inhibition of contextual fear learning. We find that 5-HT1A receptors increase the phosphorylation of GSK3 through activation of the PI3K/Akt pathway, and that regulation of GSK3β, but not GSK3α is necessary for 5-HT1A receptors’ effects on contextual fear conditioning.
Materials and Methods

Animals

The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham approved all experimental protocols. Adult male C57BL/6 mice (8-12 weeks of age, Fredrick) were used for pharmacological treatments. Knock-in mice bearing S21A mutations in GSK3α (αKI) or S9A mutations in GSK3β (βKI) were derived from GSK3α/β S21/9A knock-in mice crossed with C57BL/6 [42]. Mice with the desired mutation were crossed with C57BL/6 mice through 10 generations to generate a line of mice with a 99% pure C57BL/6 background. For all behavioral experiments, genetically modified mice were tested with their wild-type litter mates.

Pharmacological treatment

After a one-week accommodation in the university animal facility, mice were treated with intraperitoneal (i.p.) injections of 8-OH-DPAT (Sigma, St. Louis, MO), or 0.9% Saline (vehicle). All drugs and vehicle for i.p. injections were administered at a volume of 5 μl drug/g body weight. For intrahippocampal (IH) injection, mice were first outfitted with guide cannulae (Plastic One, Roanoke, VA) to give access to the dorsal hippocampus. Mice were anesthetized with a mixture of ketamine and xylazine (100 mg/kg:10 mg/kg) and placed in a stereotaxic frame. Bilateral burr holes were drilled 2.0 mm posterior to and 1.5 mm lateral to Bregma. A guide cannula (projection length 1.8 mm) was attached to the skull with dental cement. Mice were allowed to recover for 5-7 days after implantation of cannulae. Following this time, mice were lightly anesthetized with isofluorane and placed in the stereotaxic frame. An injector projecting 0.5 mm past the guide was inserted into the guide cannula and LY294002 (2.5 nM in 0.5 μL of 10% DMSO) or vehicle were infused into the left ventricle over 2 minutes. At the end of treatment, mice were rapidly decapitated. Half of each brain
was immediately immersed in Bouin’s solution and fixed overnight at 4°C. The hippocampus was rapidly dissected from the remaining hemisphere and homogenized.

Protein preparation and immunoblotting

Proteins from brain homogenate was prepared as previously described[28]. Proteins were resolved in 10% SDS-polyacrylamide gels, and immunoblotted with antibodies to phospho-Ser\(^{21}\)-GSK3\(\alpha\), phospho-Ser\(^{9}\)-GSK3\(\beta\), phospho-Thr\(^{308}\)-Akt, phospho-Ser\(^{473}\)-Akt, Akt (Cell Signaling Technologies, Danvers, MA), and total GSK3\(\alpha\beta\), (Upstate Biotech, Lake Placid, NY). Following a reaction with horseradish peroxidase-conjugated anti-mouse or goat anti-rabbit IgG (Bio-Rad), the immunoreactions were detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ), and protein bands were quantified with densitometry software.

Immunohistochemistry

The immunohistochemistry method was as described previously [27, 43]. Fixed brains were processed in paraffin, and 7 μm brain sections were prepared on a microtome. Deparaffinized sections were incubated with antibodies to phosphorylated GSK3 or Akt, labeled with horseradish peroxidase-conjugated anti-rabbit IgG, and developed with a TSA-Plus kit (Perkin-Elmer, Waltham, MA). Sections were counter-stained with Hoechst 33,258 (Sigma, St. Louis, MO). Immuno-fluorescence in brain sections was viewed with an Olympus BX-51 fluorescence microscope, and fluorescence intensity was measured using MicroBrightField Stereo Investigator Software (MBF Bioscience, Williston, VT).

Fear Conditioning

Mice were placed in an operant chamber inside an sound-attenuating cubicle to allow exploring the operant chamber for two minutes. After this acclimation, they were
administered three rounds of a 15-second, 75-db white noise tone each followed immediately by a 0.5 mA foot shock. Twenty-four hours after this training, mice were tested for the contextual memory by placing them back into the same operant chamber for 5 minutes. Freezing was monitored by Med Associates Video Freeze software. Three hours after contextual testing, the cued memory was tested by placing mice in a novel context for 3 minutes (pre-tone), followed by a 75-dB white noise tone. Freezing was again monitored using Video Freeze software.

Statistics

All data are presented as mean ±SEM. Quantified immunoblot data is shown as percentage of control, with data from each animal being calculated as a percentage of the average of the saline treated animals in the same experimental cohort. Statistical analyses were completed using SigmaStat software. Statistical significance was determined by Student’s t-test for comparisons between two groups and one-way ANOVA followed by post-hoc testing for experiments with multiple treatments. Studies examining treatment effects in different genotypes were analyzed using two-way ANOVA followed by post-hoc testing.

Results

5-HT1A receptors regulate hippocampal GSK3 α and β

We previously reported that 5-HT1A receptor activation with 8-OH-DPAT dose-dependently increased the inhibitory serine-9 phosphorylation of GSK3β in several brain regions [28]. To better understand the physiological significance of this regulation, we sought to identify the 5-HT1A receptor-regulated serine phosphorylation of GSK3 in the
hippocampus. In this experiment, mice received systemic injection of a low dose of the 5-HT1A receptor agonist 8-OH-DPAT (1 mg/kg, i.p.) for 30 min before serine phosphorylation of GSK3α and GSK3β was detected in the hippocampus (Figure 1). Immunoblot analysis of GSK3 in the hippocampal protein lysate showed that 8-OH-DPAT significantly increased the levels of phospho-Ser21-GSK3α (169.57±17.24% control, p<0.005) and phospho-Ser9-GSK3β (353.69±83.65% control, p<0.05), (Figure 1A-B) but did not alter the levels of total GSK3α (95.32±7.52% control) or GSK3β (105.7±6.8% control). Without 8-OH-DPAT treatment, a low level of phospho-Ser9-GSK3β was detected in the cell bodies of CA1 pyramidal neurons and in the cell bodies and stratum radiatum dendrites of CA3 pyramidal neurons, and a moderate level of GSK3 phosphorylation was seen in cell bodies in the hilus of dentate gyrus (Figure 1C). In response to 8-OH-DPAT, Phospho-Ser9-GSK3β robustly increased in the dendrites and cell bodies of CA3 and moderately in the dendrites of CA1 (Figure 1C). In the dentate gyrus, Phospho-Ser9-GSK3β was also increased in the cell bodies and projections of dentate granule cells (Figure 1C). We quantified luminescence of phospho-GSK3β in the subfields of the hippocampus. Significant increases were found in the stratum pyramidale (treated 6.28 ±0.07 vs untreated 2.14±0.46 pGSK3β/Hoescht 33342 luminescence, p<0.0005) and stratum radiatum/stratum lucidum of CA3 (treated 5.03 ±0.84 vs untreated 1.89±0.19 pGSK3β/Hoescht 33342 luminescence, p<0.01) as well as in the Hilus of the dentate gyrus (treated 3.84 ±0.27 vs untreated 1.76±0.27 pGSK3β/Hoescht 33342 luminescence, p<0.005). Additionally, a near-significant increase was seen in the granule cell layer of the dentate gyrus (treated 2.29 ±0.56 vs untreated 1.234±0.41 pGSK3β/Hoescht 33342 luminescence, p=0.09). No significant changes were observed in stratum pyramidale (treated 2.81 ±0.79 vs untreated 1.74±0.64 pGSK3β/Hoescht 33342
luminescence) or stratum radiatum (treated 3.23 ±1.23 vs untreated 1.60 ±0.23 pGSK3β/Hoescht 33342 luminescence), although a trend towards an increase was seen. Thus, increases in phospho-Ser9-GSK3β induced by activation of 5-HT1A receptors are strongest in CA3 and the dentate gyrus of the hippocampus. Immunohistochemistry of total GSK3β revealed that GSK3β was ubiquitously expressed in the hippocampus [32], with high levels of immunoreactivity in neuronal cell bodies and dendritic processes (Figure 1C). Localization of phospho-Ser21-GSK3α was unable to be studied because the available antibody for phospho-Ser21-GSk3a is not suitable for immunohistochemistry.

Regulation of hippocampal GSK3 by 5-HT1A receptor is mediated by Akt

Serine phosphorylation of GSK3 is often induced by activation of upstream kinases, one of the foremost of which is Akt [37]. To test if 5-HT1A receptor agonist activates Akt in the hippocampus, protein lysates from the hippocampus were immunoblotted for the active Thr308- and Ser473-phosphorylated Akt and total Akt Treatment with 8-OH-DPAT significantly increased the level of phospho-Thr308-Akt (211.24±37.87% control, p<0.05) (Figure 2A-B). A small but significant increase was also seen in levels of phospho-Ser473-Akt (119.68±6.29 control, p<0.05), while total Akt was unchanged (93.56± 5.33% control). The distribution of Akt in the hippocampus was then examined by immunohistochemistry. Without 8-OH-DPAT treatment, phospho-Thr308-Akt was detected at moderate levels throughout the hippocampus, primarily in the cell bodies and dendrites of pyramidal and dentate granule neurons. 8-OH-DPAT treatment caused an increase in phospho-Thr308-Akt in the cell bodies and dendrites of CA3 pyramidal cells and dentate granule cells, and a moderate increase in the cell bodies and dendrites of CA1 pyramidal cells (Figure 2C). Total
Akt was found to distribute widely throughout the cell bodies and dendrites of pyramidal and granule neurons of the hippocampus (Figure 2C).

In response to 8-OH-DPAT treatment, the increase in phospho-Thr308-Akt and Phospho-Ser9-GSK3 strongly co-localized with each other, particularly in CA3 and the dentate gyrus. In CA3, co-localization was primarily seen in the cell bodies of pyramidal neurons, and to a lesser extent in the pyramidal cell basal dendrites (Figure 3A-B). In the dentate gyrus, co-localization occurred mostly in hilar cell bodies, with lesser co-localization in the dendrites of granule cells. This suggests that 5-HT1A receptor activation leads to phosphorylation of GSK3 through activation of Akt.

To test if the PI3K/Akt pathway mediates 5-HT1A receptor-induced increase in phospho-Ser-GSK3, mice received bilateral intrahippocampal infusion of LY294002 (2.5 nMoles/side), followed by a systemic injection of 8-OH-DPAT. The phosphorylation of Akt and GSK3 were measured by immunoblotting (Figure 3C-E). Levels of phospho-Ser21-GSK3α (F3,14=8.630), phospho-Ser9-GSK3β (F3,14=5.769), and phospho-Thr308-Akt (F3,14=6.758) were all significantly different among treatment groups. As previously shown, 8-OH-DPAT significantly increased phospho-Ser21-GSK3α (180.51±35.89% control), phospho-Ser9-GSK3β (161.06±26.72% control), and phospho-Thr308-Akt (139.16±13.15% control, p<0.05). Treatment with LY294002 alone did not significantly alter the level of phospho-Thr308-Akt (114.96±0.76% control), nor were there any changes in the levels of phospho-Ser21-GSK3α (89.56±10.37% control) and phospho-Ser9-GSK3β (91.9±5.1% control). Infusion of LY294002 completely blocked 8-OH-DPAT-induced increase in phospho-Thr308-Akt (81.71±7.91% control) of the effect induced by 8-OH-DPAT. Infusion of LY 294002 also blocked 8-OH-DPAT-induced increases in phospho-Ser21-GSK3α.
(72.73±5.87 % control) and phospho-Ser9-GSK3β (63.05±12.34 % control). We therefore conclude that 5-HT1A receptor activation leads to inhibition of GSK3 through activation of PI3K and Akt.

**GSK3β is a behavioral mediator of 5-HT1A receptor in the hippocampus.**

To determine if inhibition of GSK3 in the hippocampus by 5-HT1A receptor has physiological significance, we tested the expression of contextual and cued fear memories, since these are hippocampus-dependent behaviors that can be modulated by 5-HT1A receptor [7, 8]. The behavior test was conducted in wild type mice and GSK3α and GSK3β knock-in mice derived from the S21A/S9A-GSK3α/β knock-in mice [42]. GSK3α knock-in mice express normal levels of total GSK3α, GSK3β, and phospho-Ser9-GSK3β but no Ser21 phosphorylation of GSK3α (Figure 4A). GSK3β knock-in mice express normal levels of total GSK3α, GSK3β, but are deficient in Ser9-phosphorylated GSK3β and exhibit increased phospho-Ser21-GSK3α (Figure 4A). These mice were tested in a standard fear conditioning protocol (Figure 4B) and were treated with 8-OH-DPAT or Saline prior to contextual or cued fear conditioning.

Wild-type mice returned to the training context twenty-four hours later exhibited a significant increase in freezing compared to freezing during training (53.6±5.6% of time freezing in context vs 19.18±2.7% of time freezing during training, p<0.005), an effect that was lost when mice were treated with 8-OH-DPAT (1 mg/kg) 30 minutes prior to the contextual test (24.9±4.5% of time freezing in context vs 23.17±2.35% of time freezing during training). In cued fear conditioning, wild type mice exhibited minimal freezing in a novel environment, but freezing significantly increased in response to the conditioned tone (9.6± 2.71 % time freezing pretone vs 63.5±5.89% of time freezing posttone, p<0.05).
Systemic administration of 8-OH-DPAT 30 min prior to testing prevented the increase in freezing induced by the conditioned tone (25.78±8.14% of time freezing pretone vs 29.6±7.68% of time freezing posttone p<0.05).

Similarly to wild-type mice, GSK3α knock-in mice a significant increase in freezing during contextual fear testing (25.75±2.64% of time freezing during training vs 48.12±6.11% of time freezing during contextual test p<0.05), and cued fear testing (12.75±3.45 % of time freezing pretone vs 69.25±8.33% of time freezing post-tone, p<0.05) (Fig. 3D). Similarly to wild type mice, both contextual (27.88±4.2% of time freezing during training vs 17.4±3.9% during contextual test) and cued (14.7± 4.8% of time freezing pretone vs 29.6±6.67% freezing posttone) freezing was significantly reduced by systemic 8-OH-DPAT treatment. Therefore, serine phosphorylation of GSK3α is not involved in the effect of 5-HT1A receptor in fear conditioning.

Similarly to GSK3α knock-in mice, GSK3β knock-in mice exhibited a significant increase in freezing in the contextual test (23.25± 2.80% of time freezing during training vs 50.25±6.93% freezing during contextual test, p<0.05) and cued test (5.25± 2.8% of time freezing pretone vs 70.2±4.83% freezing posttone, p<0.05) (Fig. 4E). However, in contrast to wild type and GSK3α knock-in mice, 8-OH-DPAT had no significant effect in reducing the context freezing (23.29±2.60% of time freezing during training vs 40.75±7.77% freezing during contextual testing, p<0.05), suggesting that serine phosphorylation of GSK3β is necessary for 5-HT1A receptor-mediated inhibition of contextual fear learning. Mediating the effect of 5-HT1A receptor in context fear learning appears to be a specific function of GSK3β on hippocampal-dependent contextual memory, since 8-OH-DPAT was able to block cued fear memory in GSK3 knock-in mice (12.0± 6.4% of time freezing pretone vs
19.25±8.23% freezing posttone, p<0.05). Thus, GSK3β plays a unique role in 5-HT1A receptor mediated regulation of contextual fear memory.

Discussion

In this study, we have demonstrated that 5-HT1A receptors strongly regulate Akt and GSK3 in the hippocampus in a PI3K dependent fashion and that regulation of GSK3β, but not GSK3α is necessary for inhibition of the expression of fear based contextual memories in mice. We focused on regulation of GSK3 in the hippocampus as this region is important in cognitive and emotional function, and because those functions can be strongly regulated by 5-HT1A receptors. We demonstrate that 5-HT1A receptor mediated regulation of GSK3 is due to activation of the PI3K, and considering the close co-localization of activated Akt and inhibited GSK3 is likely due to a phosphorylation by Akt. This regulation occurs primarily in the principal neurons of the hippocampus, suggesting that inhibition of GSK3 by 5-HT1A receptors has an effect on the primary circuitry of the hippocampus and that regulation of GSK3 may play an important role in manipulation of hippocampal-dependent behavior.

This is the first study showing that 5-HT1A receptors activate Akt in the mammalian hippocampus. This is in accordance with previous studies demonstrating 5-HT1A receptor mediated activation of Akt in transfected cells and primary hippocampal neurons [24-26]. Furthermore, similarly to our previous studies showing PI3K-dependent activation of Akt through serotonergic antidepressants [27], activation of Akt in the rodent brain in response to a 5-HT1A receptor agonist is blocked by an inhibitor of PI3K. The mechanism of 5-HT1A receptor mediated activation of PI3K in the brain remains unclear. In transfected CHO cells, 5-HT1A receptors can activate PI3K through Giβγ subunit dependent activation of ras/raf
signaling [25], but it remains to be seen if this occurs in the mammalian brain. Alternatively, 5-HT1A receptors may lead to activation of PI3K through an indirect mechanism. 5-HT1A receptor activation results in changes in serotonergic neurotransmission [44] as well as hyperpolarization [45] and alterations in post-synaptic responses [46] of glutamatergic and GABA-ergic hippocampal neurons. Thus, 5-HT1A receptor activation may affect GSK3 phosphorylation through alterations in neuronal activity rather than direction activation of signaling by 5-HT1A receptors. This mechanism is supported by our immunohistochemical findings that 5-HT1A receptor activation leads to the greatest changes in phosphorylation of GSK3 in CA3 region of the hippocampus, despite 5-HT1A receptors being expressed at significantly lower levels in CA3 than in CA1 or the dentate gyrus [5].

5-HT1A receptors regulate a number of behavioral functions. Notably, 5-HT1A receptors can inhibit both contextual and cued fear conditioning [4]. The precise mechanism of how this occurs is unknown, but we have presented evidence here that it involves phosphorylation of GSK3β, but not GSK3α. The necessity of GSK3β, but not GSK3α phosphorylation is in accordance with our results showing a greater degree of inhibition of GSK3β than GSK3α after treatment with 8-OH-DPAT. The difference in effect between GSK3α and GSK3β is intriguing, as the high degree of similarity between the two isoforms has hindered identification of distinct fuctions of each isoform. Nonetheless, several isoform-specific targets have been identified [47, 48].

Interestingly, phosphorylation of GSK3β is required for 5-HT1A receptor inhibition of hippocampal-dependent contextual fear memories, but not cued fear memories, which are independent of the hippocampus. This is in accordance with previous studies showing that
inhibition of GSK3 with lithium promotes, rather than inhibits, cued fear conditioning [49]. Thus, GSK3β phosphorylation is an important step specifically in the regulation of hippocampal dependent behavior by 5-HT1A receptors. As 5-HT1A receptors are thought to play a role in mood [11] and anxiety [3] disorders, understanding the signaling pathways underlying 5-HT1A mediated behaviors gives crucial information about the processes that may be involved in the pathogenesis and treatment of these diseases.
Acknowledgements

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REFERENCES


A

- **Hippocampus**
  - P-S21-GSK3α
  - P-S9-GSK3β
  - GSK3α/β
  - DPAT 1 mg/kg - +

B

- **GSK3**
  - pGSK3α
  - pGSK3β
  - GSK3α
  - GSK3β

C

- 4X HIP
  - Saline
  - p-S9-GSK3β
  - DPAT

- 20X CA1
  - Saline
  - p-S9-GSK3β
  - DPAT

- 20X CA3
  - Saline
  - p-S9-GSK3β
  - DPAT

- 20X DG
  - Saline
  - p-S9-GSK3β
  - DPAT

D

- **pGSK3β luminescence**
  - CA1-SP
  - CA1-SR
  - CA3-SP
  - CA3-SR/SL
  - DG-GL
  - DG Hilus

- **%Control**
  - Saline
  - DPAT

- **Statistical Significance**
  - *
Figure 1. Regulation of GSK3 by 5-HT1A receptor activation. Mice were treated with the 5-HT1A receptor agonist 8-OH-DPAT (DPAT, 1 mg/kg 30 minutes) and levels of phosphorylated and total GSK3 were determined by immunoblotting and immunohistochemistry. (A) Representative immunoblots and (B) quantified data of phospho-Ser21-GSK3α, phospho-Ser9-GSK3β, and total GSK3α and GSK3β. * p<0.05, Student’s t-test, n=12-16 (C) Immunohistochemistry of phospho-Ser9-GSK3β and total GSK3β in the hippocampus (4X) and hippocampal subfields (20X). Red, Phospho-Ser9-GSK3β, Blue Hoescht 33342 nuclear marker (D) Quantified immunofluorescence of phospho-Ser90GSK3β in hippocampal subregions, expressed as ratio of phospho-Ser9-GSK3β to Hoescht 33342. *p<0.05, Student’s t-test, n=3-4 animals. SP, stratum pyramidale; SR, stratum radiatum; SL, stratum lacunosum; GL, Granule Cell Layer.
Figure 2. Regulation of Akt by 5-HT1A receptor activation. Mice were treated with the 5-HT1A receptor agonist 8-OH-DPAT (DPAT, 1 mg/kg 30 minutes) and levels of phosphorylated and total GSK3 were determined by immunoblotting and immunohistochemistry. (A) Representative immunoblots and (B) quantified data of phospho-Thr308-Akt, phospho-Ser473-Akt and total Akt. (C) Immunohistochemistry of phospho-Thr308-Akt and total Akt in the hippocampus (4X) and hippocampal subfields (20X). * p<0.05, Student’s t-test, n=12-16
Figure 3. 5-HT1A receptors increase GSK3 through the PI3K/Akt pathway. (A) 4X images of the hippocampus and (B) 20x images of the hippocampal subfields showing colocalization of phospho-Thr308-Akt (Green) and phospho-Ser9-GSK3β (red). Nuclear marker Hoechst 33342 is in blue. (C) Experimental timeline for LY294002 (LY) and 8-OH-DPAT (DPAT) experiments. (D) Representative immunoblots and (E) quantified data of phospho-Ser21-GSK3α, phospho-Ser9-GSK3β, total GSK3α and β, phospho-Thr308-Akt, phospho-Ser473-Akt, and total Akt. * p<0.05, 1-way ANOVA followed by Holm-Sidak comparison, n=4-6.
A. Western blots showing the expression of GSK3α and GSK3β in S21A GSK3α KI and S9A GSK3β KI mice. The genotypes are WT (wild-type) and αKI or βKI (knock-in). The blots show the levels of pS21-GSK3α, pS9-GSK3β, and GSK3α/β.

B. Timeline of the experiments: Training, Tone/shock x3, DPAT, Context Test, DPAT, Cued Test. The time points are marked as 24 hours, 30 minutes, 4 hours, and 30 minutes.

C. Contextual Fear Conditioning:
- WT Veh: % Freezing during Training and Context.
- WT DPAT: % Freezing during Pretone and Tone.

D. Contextual Fear Conditioning:
- alpha Ki Veh: % Freezing during Training and Context.
- alpha Ki DPAT: % Freezing during Pretone and Tone.

E. Contextual Fear Conditioning:
- beta Ki veh: % Freezing during Training and Context.
- beta Ki DPAT: % Freezing during Pretone and Tone.
**Figure 4.** Phosphorylation of GSK3β is required for 5-HT1A receptor mediated regulation of fear conditioning. (A) Immunoblots of phospho-Ser21-GSK3α, phospho-Ser9-GSK3β and total GSK3α and β in GSK3α knock-in (αKI), GSK3β knock-in (βKI) and wild-type (WT) mice. (B) Experimental timeline (C) Contextual and cued fear conditioning in WT mice. (D) Contextual and cued fear conditioning in αKI mice. (E) Contextual and cued fear conditioning in βKI mice. * p<0.05, 2-way ANOVA followed by Holm-Sidak comparison. # p<0.05, Student’s t-test.
Beta-Arrestin2–Mediated Regulation of GSK3 by 5-HT2A Receptors and Antidepressants

by

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Abstract

5-HT2A receptors are important mediators of serotonin signaling that have been shown to play a role in mood disorders. Antagonism of 5-HT2A receptors has been shown to be a promising strategy for enhancing the effects of Serotonin Reuptake Inhibitors in the treatment of depression. Although much is known about the effects of 5-HT2A on brain and behavior, the signaling pathways that mediate these effects are largely unknown. One known target of 5-HT2A receptors is Glycogen Synthase Kinase, a multi-faceted kinase shown to play an important role in the regulation of mood. In this study, we investigated the role of GSK3 in 5-HT2A receptor and antidepressant signaling. We find that a 5-HT2A receptor antagonist increases phosphorylated GSK3 through activation of Akt in a β-arrestin2-independent fashion, while a 5-HT2A receptor agonist increases phosphorylated GSK3 only in the absence of β-arrestin2. Furthermore, in the absence of β-arrestin2, the antidepressant Fluoxetine has both a regionally-specific enhanced increase of phosphorylation of GSK3 and an enhanced anti-depressant-like behavioral effect. Finally we demonstrate that the acute behavioral effect of fluoxetine is dependent on phosphorylation of GSK3, as mice lacking this phosphorylation exhibit no response to fluoxetine. These studies indicate that GSK3 phosphorylation is dynamically regulated by 5-HT2A receptors and antidepressants, and that this phosphorylation is crucial for the response to antidepressants.
**Introduction**

Serotonin is a monoaminergic neurotransmitter that plays an important role in many physiological and pathological functions. Of the numerous receptors responsible for serotonergic signaling [1], 5-HT2A receptors are among the most well studied. These receptors are expressed throughout the brain [2], particularly in the prefrontal cortex, where they are located on dendrites and axons of pyramidal neurons and interneurons, as well as on monoaminergic axons [2], where they can enhance the release of dopamine [3].

5-HT2A receptors play an increasing role in the treatment of psychiatric illnesses. Atypical antipsychotics are combined 5-HT2A and dopamine D2 receptors. In addition to their use as treatments for schizophrenia and bipolar disorder, atypical antipsychotics are increasingly used as augmentative treatments for patients who do not respond to a Serotonin Reuptake Inhibitor (SRI) [4-6]. SRI antidepressants induce a general increase in synaptic levels of serotonin, presumably enhancing activation of the many different subtypes of serotonin receptors in the brain. While some of these receptors, such as the 5-HT1A receptor, have been shown to be crucial for an antidepressant response [7], others may have negative consequences. 5-HT2A receptors have been shown via post-mortem studies and imaging studies to be upregulated in the cortex of depressed patients [8]. Furthermore, 5-HT2A receptors are frequently expressed in the same cells as crucial 5-HT1A receptors [9] and these receptors have been shown to have opposing effects on neurons [10, 11]. Thus, activation of 5-HT2A receptors may counter the actions of antidepressants, and antagonism of these receptors may enhance antidepressant responses.

5-HT2A receptors classically couple through the G<sub>q</sub> coupled signaling pathway to activate the Phospholipase C (PLC)/ Protein Kinase C (PKC) pathway [12-15]. In addition
to this traditional pathway, 5-HT2A receptors have also been shown to activate ERK [16-19] in cultured cells and in the mammalian brain as well as Akt [16], and Jak/Stat [20] in cultured cells. 5-HT2A receptor regulation of intracellular signaling pathways has increasingly been associated with intracellular scaffolding proteins. For example, 5-HT2A receptor activation of ERK in the mammalian brain depends on PSD-95 [21] and in response to some ligands, β-arrestin 2 [19]. Furthermore, there is increasing evidence that some signaling pathways are regulated by antagonism of 5-HT2A receptors. Our previous work has shown that antagonism of 5-HT2 receptors increases inhibitory phosphorylation of Glycogen Synthase Kinase 3 (GSK3) [22], although it is not clear which of the three 5-HT2 receptor subtypes is responsible for this increase. Thus, there are number of potential mediators of the downstream effects of 5-HT2A receptors.

GSK3 is a target of particular interest due to significant evidence suggesting that it is a key molecule in mood disorders [23]. GSK3 is a serine/threonine protein kinase that plays an important role in a variety of neuronal functions including neurogenesis and development [24], synaptic plasticity [25], receptor regulation [26-28], apoptosis and cell survival [29] and gene expression [30]. Activity of both isoforms of GSK3, GSK3α and GSK3β are tightly regulated by inhibitory phosphorylation on an N-terminal serine residue (serine 21 and serine 9, respectively) mediated by upstream kinases such as Akt and PKC [31, 32].

GSK3 has been increasingly recognized as a potential mediator of mood disorders [23]. Overexpression of GSK3β results in hyperactivity and increased reactivity [33], and our recent results show an increase in susceptibility to both manic-like and depression-like behavior in mice bearing a S21/9A mutation in both GSK3α and β that renders them immune to upstream phosphorylation [34]. Human studies have shown an increase in GSK3 activity
in the brains of depressed suicides [35], and a decrease in serine phosphorylation of GSK3 in peripheral mononucleocytes of bipolar patients, the extent of which correlated with the severity of manic symptoms [34]. Furthermore, GSK3 is inhibited by a number of treatments for mood disorders. Lithium, a commonly used mood stabilizer, was identified as a potent direct [36, 37] and indirect [38, 39] inhibitor of GSK3 over a decade ago. More recent studies have shown that acute [22, 40, 41] and chronic [42] antidepressants, as well as atypical antipsychotics [40, 43] all increase inhibitory serine phosphorylation of GSK3. Thus, increased activity of GSK3 corresponds with aberrant states of mood, while inhibition of GSK3 appears to be a common downstream target of mood disorder therapeutics.

Our previous work has characterized regulation of GSK3 by serotonin receptors in the mammalian brain. General increase in synaptic serotonin results in increased inhibitory phosphorylation of GSK3, an effect that can be blocked with 5-HT1A receptor antagonists. Similarly, activation of 5-HT1A receptors with the specific agonist 8-OH-DPAT increases inhibitory phosphorylation of GSK3 [22]. An opposing effect was seen with 5-HT2 receptors; although the 5-HT2A/C agonist DOI has no effect on GSK3 phosphorylation, the 5-HT2 receptor antagonist LY53857 and low-dose atypical antipsychotics increase phosphorylation of GSK3 [22, 40]. Furthermore, antagonism of 5-HT2 receptors can potentiate the 5-HT1A receptor mediated increase in phosphorylation of GSK3 [22], and atypical antipsychotics can potentiate the increased phosphorylation of GSK3 induced by the antidepressants fluoxetine and imipramine [40].

The pattern of convergence of 5-HT receptor signaling on GSK3 suggests that inhibition of GSK3 may be a critical step in the behavioral effects of antidepressants. To better understand this, we sought to further define the mechanism of regulation of GSK3 by
5-HT2 receptors and antidepressants. We find that a 5-HT2A receptor antagonist increases phosphorylation of GSK3 through activation of Akt. Furthermore, coupling of 5-HT2A activation to inhibition of GSK3 is endogenously inhibited by β-arrestin 2. Genetic deletion of β-arrestin 2 results in strong inhibition of GSK3 by a 5-HT2A receptor agonist, a region-specific enhancement of the increased phosphorylation of GSK3β induced by the SRI Fluoxetine and an increased behavioral response to fluoxetine. Phosphorylation of GSK3 appears to be a critical event for the behavioral actions of fluoxetine, as GSK3β knock-in mice with an S9A mutation that renders them immune to phosphorylation do not respond to acute fluoxetine treatment.

Materials and Methods

Animals and treatment

The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham approved all experimental protocols. Adult male C57BL/6 mice (8-12 weeks of age, Frederick Cancer Research, MD) were used for pharmacological treatments. β-arrestin2 knockout (KO) mice on a C57BL/6 background were provided by Robert Lefkowitz (Duke University) and were continuously bred at UAB. β-arrestin2 knockout (KO) mice were paired with age-matched C57BL/6 controls. Knock-in mice bearing S9A mutations in GSK3β (βKI) were derived from GSK3α/β S21/9A knock-in mice crossed with C57BL/6 [44]. Mice with the desired mutation were crossed with C57BL/6 mice through 10 generations to generate a line of mice with a 99% pure C57BL/6 background. Mice were treated with intraperitoneal (i.p.) injections of DOI (Sigma, St. Louis, MO), 8-OH-DPAT (Sigma, St. Louis, MO), MDL 11939 (Tocris, Ellsville, MO), Fluoxetine (NIMH Chemical Synthesis and Drug Supply Program), RS (Tocris) and SB (Tocris) or vehicle. Physiological
saline was used as vehicle for all drugs except MDL 11939, which was dissolved in 10% ethanol. All drugs and vehicles for i.p. injections were administered at a volume of 5 μl/g body weight, with the exception of fluoxetine, which was administered at a volume of 10 μl/g body weight due to solubility limitations. Animals were sacrificed 30 or 60 minutes after treatment, according to experiment. At the end of treatment, mice were rapidly decapitated and the brain removed. The hippocampus, cortex, and striatum were dissected in ice-cold saline and homogenized in NP-40 lysis buffer containing protease and phosphotase inhibitors.

Protein preparation and immunoblotting

Proteins from brain homogenate were prepared as previously described [40]. Proteins were resolved in 10% SDS-polyacrylamide gels, and immunoblotted with antibodies to phospho-Ser21-GSK3α, phospho-Ser9-GSK3β, phospho-Thr308-Akt, phospho-Ser473-Akt, Akt (Cell Signaling Technologies, Danvers, MA), and total GSK3αβ, (Upstate Biotech, Lake Placid, NY). Following a reaction with horseradish peroxidase-conjugated anti-mouse or goat anti-rabbit IgG (Bio-Rad, Hercules, CA), the immunoreactions were detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ), and protein bands were quantified with densitometry software.

Forced Swim Test

Mice were placed in an automated apparatus consisting of clear Plexiglas cylinders containing distilled water (23-25°C) and outfitted with 2 rings of photo-beam detectors (Kinder Scientific, Poway, CA) [45]. Movements were continuously monitored by computer for 6 minutes. Data were recorded using Motor Monitor Software (Kinder Scientific, Poway,
CA) and transferred into Microsoft Excel. The immobility time was represented by recording the resting time (seconds without beam breaks,) during the last 4 minutes of testing.

Statistics

All data are presented as mean ±SEM. In order to control for day-to-day variation, quantified immunoblot data is shown as percentage of control, with data from each animal being calculated as a percentage of the average of the saline treated animals of the same genotype in the same experimental cohort. Statistical significance was determined by one-way ANOVA followed by post-hoc testing for experiments with multiple treatments and by two-way ANOVA followed by post-hoc testing for experiments comparing response to treatments across genotypes.

Results

Antagonism of 5-HT2A, but not 5-HT2B or 5-HT2C receptors inhibits GSK3

We previously reported that acute treatment with a non-selective 5-HT2 receptor antagonist LY53857 [22] increased phospho-ser-9 GSK3β in the frontal cortex, hippocampus, and striatum of the mouse brain. In this study, we further tested the selectivity of specific antagonists of 5-HT2A, 5-HT2B, and 5-HT2C receptors in regulating GSK3. Treatment of mice with the 5-HT2A receptor antagonist MDL11939 (4 mg/kg, i.p.) for 60 minutes significantly increased phospho-Ser21-GSK3α in the cerebral cortex (175±29 % of control, p<0.01), and the striatum (259±58 % of control, p<0.01), but its effect on GSK3α in the hippocampus was not significant. MDL 11939 also significantly increased phospho-Ser9-GSK3β in the cerebral cortex (218±32% of control, p<0.001), the hippocampus (164±29 % of control, p<0.05), and the striatum (208±29% of control, p<0.001) (Figure 1A
and B). Blocking 5-HT2A receptors by MDL 11939 did not cause significant change in the level of total GSK3. In contrast, treatment with the 5-HT2B receptor antagonist SB 204741 (2 mg/kg, i.p., 120 minutes) or the 5-HT2C receptor antagonist RS102221 (3 mg/kg, i.p. 120 minutes) had no effect on serine phosphorylation or total level of GSK3 (Figure 1A and 1B). These data suggest that antagonism of 5-HT2A receptors, but not 5-HT2B or 5-HT2C, receptors can robustly increase serine phosphorylation of GSK3 in several brain regions.

Akt, but not β-arrestin2 mediates the effect of 5-HT2A receptor antagonist on GSK3

N-terminal serine phosphorylation of GSK3 can be regulated by Akt[32]. To test if the increase in phospho-Ser-GSK3 induced by the 5-HT2A receptor antagonist was mediated by Akt, phosphorylation of two residues that indicate active Akt, phospho-ser473-Akt, and phospho-thr308-Akt, was measured in brain regions where MDL 11939 increased phospho-Ser-GSK3. MDL 11939 also significantly increased phospho-thr308-Akt in the cortex (180±38 % of control, p<0.05), the hippocampus (134±13 % of control, p<0.05), and the striatum (154±20 % of control, p<0.05) (Figure 2A), whereas phospho-ser473-Akt was increased by MDL 11939 in the striatum (154±11 % of control, p<0.001) but not in the cortex (125±16 % of control, p=0.17) or the hippocampus (112±14 % of control, p=0.45). No change was seen in total levels of Akt. These results suggest that blocking 5-HT2A receptor by MDL 11939 can activate Akt, which may mediate the effect of MDL 11939 on phospho-Ser-GSK3.

Beta-arrestin has been shown to participate in the regulation of Akt and GSK3 by dopamine D2 receptor [46]. To test if β-arrestin2 is involved in regulation of GSK3 by 5-HT2A receptor antagonist, we treated WT and β-arrestin 2 KO mice with MDL 11939. The MDL 11939-induced increase in phospho-Ser21-GSK3α was equivalent in WT and b-
arrestin2-KO mice in the cortex (WT 175±29% vs KO 166±27% control), the hippocampus (WT 134±20% vs KO 141±14% control) and the striatum (WT 259±59% vs KO 244±57% control) (Figure 2B). Likewise, MDL 11939 also increased phospho-Ser9-GSK3β equivalently in WT and b-arrestin2-KO mice in the cortex (WT 218±31% vs KO 180±50% control), the hippocampus (WT 167±32% vs KO 158±24% control) and the striatum (WT 208±29% vs KO 198±42% control) (FIGURE 2B). Therefore, the increased phospho-Ser-GSK3 induced by blocking 5-HT2A receptors occurs independently of β-arrestin 2.

*Beta-arrestin2 determines the response of GSK3 to 5-HT2AR agonist-regulated GSK3*

Although β-arrestin2 did not affect 5-HT2A receptor antagonist-induced serine phosphorylation of GSK3, it was found to be a determinant of GSK3 regulation by a 5-HT2A receptor agonist. In WT mice, treatment with the 5-HT2A receptor agonist DOI (20 mg/kg, 60 min) had minimal effect on phospho-Ser21-GSK3α in the cortex (151±20% control), the hippocampus (130±15% control) and the striatum (121±30% control). In contrast, DOI strongly increased phospho-Ser21-GSK3α in the cortex (216±21% control), the hippocampus (221±29% control), and the striatum (220±43% control) of β-arrestin2-KO mice (Figure 3). This effect of DOI in β-arrestin2-KO mice was significantly different from the WT mice, especially in the hippocampus (F1,27=6.465, p<0.05), (Cortex F1,27=3.3, p=0.07;Striatum F1,27=2.6, p=0.11) (Figure 3A-B). As we reported previously [22], treatment with DOI had no significant effect in phospho-Ser9-GSK3β in the cortex (153±27% control), the hippocampus (116±16% control), or the striatum (105±25% control) of WT mice. In β-arrestin2-KO mice, however, DOI induced a significant increase in phospho-ser9-GSK3β in the cortex (308±49% control, p<0.005), the hippocampus (220±29% control, p<0.005), and the striatum (220±43% control, p<0.05), an effect that is significantly different from the wild type mice.
(Cortex $F_{1,27}=6.102$, $p<0.05$; Hippocampus $F_{1,27}=6.352$, $p<0.05$; Striatum $F_{1,27}=6.105$, $p<0.05$) (Figure 3A-B). In both WT and β-arrestin2-KO mice, DOI did not change the level of total GSK3α or GSK3β. These results suggest that β-arrestin2 functions as a gate keeper to defer 5-HT2A receptor coupling to inhibition of GSK3. In contrast to 5-HT2A receptor antagonist-induced serine phosphorylation of GSK3, which is mediated by activation of Akt, DOI did not significantly increase phospho-Thr308-Akt and phospho-Ser473-Akt in either wild type mice or β-arrestin2-KO mice (Table 1). Thus, the β-arrestin2–dependent regulation of GSK3 by agonism of 5-HT2A receptors is not mediated by Akt.

*The role of β-arrestin2 in fluoxetine-regulated GSK3*

As 5-HT2A receptor activation induces the inhibitory serine phosphorylation of GSK3 in β-arrestin2 KO mice, we hypothesized that deleting β-arrestin 2 may potentiate the inhibitory effect of serotonin-reuptake inhibitor on GSK3 [22, 40]. To test this, we treated WT and β-arrestin2 KO mice with the SRI antidepressant fluoxetine (20 mg/kg) for 30 minutes and examined the levels of serine phosphorylated and total GSK3 in the brain. Fluoxetine caused a mild increase in phospho-ser21-GSK3α in the cortex of WT mice and β-arrestin2-KO mice (WT 129.7±15.7% vs KO 144.4±8.7 % control) (Figure 4A and B). The treatment in both genotypes were significant ($F_{1,45}=12.971$, $p<0.01$), but the effect was not different between wild type and b-arrestin-KO mice (Figure 4B). In the hippocampus, fluoxetine treatment modestly increased phospho-ser21-GSK3α in both WT and β-arrestin2-KO mice (WT 169.78±20.9% vs KO 144.92±14.4% control), but the effect in the two genotypes was not different (Figure 4B). Fluoxetine had little effect in phospho-Ser21-GSK3α in the striatum of WT mice (132.77±29.6%), but it caused a moderate increase in phospho-Se21-GSK3α in β-arrestin2-KO mice (189.72±15.67 % control) (Figure 4B).
A 30-min treatment with fluoxetine did not change the level of phospho-Ser9-GSK3β in cortex of WT mice, but this same treatment significantly increased in β-arrestin2 KOs (WT 128.55±17.22% vs KO 231.36±29.56% control). This effect of fluoxetine in β-arrestin2-KO mice was significantly different from WT mice (F_{1,45}=9.757, p<0.005). In contrast to the cortex, fluoxetine treatment increased phospho-ser9-GSK3β in the hippocampus of both WT and KO mice (WT 190.31±23.84% vs KO 210.13±29.48% control, p<0.001) (Figure 4B), and the effect was not different between wild type and b-arrestin2-KO mice. The effect of fluoxetine in the striatum was similar as seen in the cortex, it had minimal effect in phospho-Ser9-GSK3β in WT mice but a significant increase in β-arrestin2-knockout mice (WT 149.9±23.32% vs KO 205.29±31.13 % control) (Figure 4B).

Fluoxetine treatment of WT and b-arrestin2-KO mice did not cause significant changes in total GSK3α or β in the tested brain regions (Figure 4C and 4D). Together, these data suggest that fluoxetine preferentially regulates GSK3b with minimal effect on GSK3a. The effect of fluoxetine on serine phosphorylation of GSK3b in the hippocampus is independent on β-arrestin2, whereas its effect in the cortex and the striatum can be enhanced by deletion of β-arrestin2.

The Role of GSK3 in the antidepressant effect of fluoxetine

GSK3 is thought to be a crucial mediator of mood-related behavior. To test if selective regulation of GSK3b by fluoxetine and the influence of β-arrestin2 in its effect, the antidepressant-like effect of fluoxetine was tested in forced swim test, a well established model of antidepressant activity [47]. We first sought to determine if Ser9 phosphorylation of GSK3β was necessary for the behavioral effects of fluoxetine, using knock-in animals with an S9A mutation preventing Ser9 phosphorylation (GSK3β-KI. Wild-type and GSK3β-
KI mice were treated with either saline or fluoxetine (20 mg/kg) 30 minutes before they were subjected to the forced swim test (Figure 5A). No significant difference in immobility was seen between WT and GSK3β-KI animals (107.65±1.21 vs 91.14±13.38 seconds of immobility, p>0.05). Two-way ANOVA showed a significant effect of genotype on the response to fluoxetine (F_{1,39}=5.543, p<0.05). In WT animals, fluoxetine treatment significantly reduced immobility to 57.25% of that of saline treated animals (107.65±1.21 vs 61.64±8.21 seconds of immobility, p>0.005). In contrast, fluoxetine had no effect on immobility in GSK3β-KI mice (91.14±13.38 vs 82.20±15.15 seconds of immobility, p>0.05), demonstrating that phosphorylation of GSK3β is necessary for the antidepressant-like effects of fluoxetine in the FST.

In the next experiment, WT and β-arrestin2-KO mice were treated with either saline or fluoxetine (20 mg/kg) 30 minutes before they were subjected to the forced swim test (Figure 5B). Saline treated WT and β-arrestin2-KO mice exhibited similar levels of immobility (WT 109.67±9.62 seconds vs KO 108.44±9.62 seconds). Fluoxetine caused a significant reduction in immobility in wild type mice (62.79±8.91 sec, p<0.001), and a significant greater reduction in immobility in β-arrestin2-KO mice (23.54±9.25 sec, p<0.001, F_{1,47}=4.130). Thus, deletion of β-arrestin2 not only potentiates SRI-mediated inhibition of GSK3, but also facilitates the behavioral effect of acute SRI treatment in mice.

Discussion

5-HT2A receptors are important receptors that have been suggested to be hyperfunctional in pathological conditions such as depression [8]. In this study, we extend our previous work showing regulation of GSK3 by 5-HT2 receptors to show specific
regulation of GSK3 by 5-HT2A receptors. We show that 5-HT2A receptors inhibit GSK3 through activation of Akt. Furthermore, we show that β-arrestin2 acts as an endogenous brake on signaling from 5-HT2A receptors to GSK3β, and that when this is reversed the signaling of the SRI Fluoxetine to GSK3 is potentiated, which correlates with a potentiation of the behavioral effect of Fluoxetine.

Our previous studies indicated that a general 5-HT2 antagonist dramatically increases serine phosphorylation of GSK3β [22]. We here report that a highly specific 5-HT2A antagonist also increases serine phosphorylation of GSK3β as well as GSK3α. This fits well with studies showing that atypical antipsychotics, which are 5-HT2A receptor antagonists as well as D2 receptor antagonists, also increase serine phosphorylation of GSK3 [40, 43, 48]. This increase occurs alongside an increase in activated Akt. Given that Akt is a primary regulator of GSK3 [32], it is likely that 5-HT2A receptor antagonism inhibits GSK3 through activation of Akt.

The precise mechanism of regulation of Akt and GSK3 by antagonizing 5-HT2A receptors is unknown. Activation of Akt and subsequent inactivation of GSK3 likely occurs through either interruption of constitutive activity of 5-HT2A signaling that promotes inhibition of Akt, or through changes in scaffolding proteins induced by ligand binding to the receptor. Regulation of Akt signaling by GPCRs is often mediated by the scaffolding protein β-arrestin2 [46] and β-arrestin2 has previously been shown to direct signaling of 5-HT2A receptors to ERK [19]. In our results, however, we find that regulation of Akt and GSK3 by 5-HT2A receptor antagonists is β-arrestin2 independent. This leaves several additional possibilities for activation of Akt by 5-HT2A receptor antagonists. Additional scaffolding proteins, such as PSD-95, may be responsible for increasing Akt activation in response to 5-
HT2A receptor antagonists, as PSD-95 has been shown to interact with 5-HT2A receptors and alter signaling [49]. Another possibility is that activation of Akt comes through disruption of constitutive 5-HT2A receptor signaling, such as modulation of synaptic transmission or activation of protein phosphatases which dephosphorylate and inactivate Akt.

In contrast to the 5-HT2A antagonist, the effect of the 5-HT2A agonist DOI is dependent on β-arrestin2. While we see no increase in serine phosphorylated GSK3 in wild-type animals in response to DOI, in β-arrestin2 knock-out animals DOI induces a dramatic increase in serine phosphorylated GSK3. This is not accompanied by an increase in phosphorylated Akt, so it is unlikely that this increase is mediated by Akt. As 5-HT2A receptors activate PKC [15], and PKC can phosphorylate and inhibit GSK3 [31], it is possible that 5-HT2A receptors inhibit GSK3 through activation of PKC. β-arrestin2 thus serves as an endogenous brake on the inhibition of GSK3 by 5-HT2A.

In wild type animals, our data suggests that a general increase in serotonin such as that induced by an SRI would induce phosphorylation of GSK3 primarily through activation of 5-HT1A receptors, and that this would be unaffected or mildly inhibited by activation of 5-HT2A receptor. In the absence of β-arrestin2, 5-HT2A receptor coupling to GSK3 is uninhibited, and this leads mice have enhanced serine phosphorylation of GSK3 in response to fluoxetine. Interestingly, no significant enhancement of phosphorylated GSK3β was seen in the hippocampus of β-arrestin2 knock-out mice. Although this could be due to regional differences in β-arrestin2’s role in signaling from 5-HT2A receptors to GSK3, DOI induces a robust increase in phosphorylated GSK3 in the hippocampus of β-arrestin2 knock-out mice. This suggests the effect of β-arrestin2 on 5-HT2A receptor mediated signal transduction is
similar in the hippocampus and the cortex. It is more likely that serine phosphorylation of GSK3 in the hippocampus is saturated due to the strong effect of 5-HT1A receptors there.

Given that fluoxetine mediated inhibition of GSK3 is enhanced in β-arrestin2 knockout mice, we hypothesized that if inhibition of GSK3 is important for the behavioral effects of fluoxetine, we should see an increase in this response in these animals. In line with this, we find that β-arrestin 2 knock-out mice have a significantly greater degree of immobility in the forced swim test, indicating an enhanced antidepressant effect. To confirm the importance of GSK3β phosphorylation in the behavioral effects of fluoxetine, we tested GSK3β knock-in animals in the forced swim test. These animals express GSK3β that cannot be phosphorylated. We found that while these mice exhibited no baseline changes in immobility from wild-type, they showed no behavioral response to fluoxetine. Thus, phosphorylation of GSK3β is necessary for the antidepressant effect of fluoxetine in an animal model of depression. The necessity of GSK3β phosphorylation for the antidepressant effects of fluoxetine is in line with previous studies suggesting that inhibition of GSK3 is sufficient to induce an antidepressant response [41, 50-53].

The opposing effects of 5-HT1A and 5-HT2A receptors on GSK3 are in accordance with several studies showing that these two receptors are often in opposition to each other [54]. Indeed, this oppositional effect has been suggested to underlie some of less-than optimal efficacy of antidepressants, as a general increase in serotonin will activate both receptors. The results of our studies suggest that GSK3 may be a crucial molecule that is opposingly regulated by 5-HT1A and 5-HT2A receptors. Furthermore, β-arrestin2 serves as an endogenous brake on 5-HT2A signaling, preventing it from coupling to inhibition of GSK3. Knock-out of β-arrestin2 removes this barrier and allows greater inhibition of GSK3.
This raises the interesting possibility that inhibitors of interactions between β-arrestin2 and GSK3 may serve as useful adjunctive therapy for depressed patients, and may speed the onset of recovery. Interestingly, loss of β-arrestin2 has been shown to reduce the actions of chronic antidepressant treatment [55]. This divergence in the effect of loss of β-arrestin2 suggests that phosphorylation of GSK3 may be an early mediator of the effects of antidepressants. Thus, we have shown that GSK3 is dynamically regulated by serotonin receptors in the mammalian brain, and this regulation plays an important role in the behavioral effects of serotonergic agents.
Acknowledgements

This work was supported by NIH grants MH64555, MH73723, MH86622 and a NARSAD independent investigator award (XL) and NS61788 (Training Program in the Neurobiology of Cognition and Cognitive Disorders, awardee AP). The authors thank Dario Alessi for the original strain of GSK3α/β knock-in mice and Robert Lefkowitz for the β-arrestin2 knock-out mice. We thank Dr. Richard S. Jope for scientific advice and resources.
References


**Table 1. DOI-induced phosphorylation of Akt**

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<th>Phospho-Ser473-Akt Mean% control ±SEM</th>
<th>Phospho-Thr308-Akt Mean% control ±SEM</th>
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<tr>
<td>WT</td>
<td>122±19</td>
<td>108±14</td>
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<td>KO</td>
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<td>105±12</td>
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<td><strong>Striatum</strong></td>
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<tr>
<td>WT</td>
<td>97±14</td>
<td>122±18</td>
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<tr>
<td>KO</td>
<td>92±10</td>
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Figure 1. Antagonism of 5-HT2A, but not 5-HT2B or 5-HT2C receptors, increases inhibitory phosphorylation of GSK3. Mice were treated with MDL 11939 (MDL, 4 mg/kg, 1 hr, n=11-13), SB204741 (SB, 2 mg/kg, 2 hours), or RS 10221 (RS, 3 mg/kg, 2 hours) and levels of were determined by immunoblotting. (A) Representative immunoblots of phospho-Ser21-GSK3α, phospho-Ser9-GSK3β, and total GSK3 in the hippocampus, striatum, and cortex of treated animals. (B) Quantification of phospho-Ser21- GSK3α, phospho-Ser9- GSK3β, total GSK3α and total GSK3β. One-way ANOVA showed a significant difference between treatments on the levels of phospho-Ser21-GSK3α in the cortex and striatum (Cortex F₃,₃₈=4.005, p<0.01; Striatum F₃,₃₈=6.126, p<0.005) One-way ANOVA also showed significant differences between treatments on serine-9 phosphorylation of GSK3β in all three regions tested (Cortex F₃,₃₈=11.86, p<0.001; Hippocampus F₃,₄₁=3.794, p<0.05; Striatum F₃,₃₈=3.741, p<0.005). *p<0.05, *p>0.005, one-way ANOVA followed by Holm-Sidak comparison.
**Figure 2.** MDL11939-mediated inhibition of GSK3 involves activation of Akt. Wild-type (WT) and β-arrestin2-KO (KO) mice were treated with MDL 11939 (MDL, 4 mg/kg, 1 hr, n=11-13) and protein was analyzed by immunoblotting. (A) Representative blots of phospho-Thr308-Akt, Phospho-Ser473-Akt, and total Akt in the cortex, hippocampus, and striatum of WT mice. (B) Quantification of phospho-Thr308-Akt and phospho-Ser473-Akt in the cortex, hippocampus, and striatum of WT mice. (C) WT and β-arrestin2-KO mice were treated with MDL 11939 and levels of phosphorylated and total GSK3 α and β were determined by immunoblotting. Representative immunoblots of phospho-Ser21-GSK3α, phospho-Ser9-GSK3β, and total GSK3 in the cortex, hippocampus, and striatum of MDL treated animals. (D) Quantification of phospho-Ser21-GSK3α, phospho-Ser9-GSK3β, and total GSK3 in the hippocampus, striatum, and cortex of MDL treated animals. Two-way ANOVA performed for each brain region revealed a significant effect of treatment (Cortex $F_{1,40}=10.72$, $p<0.005$; Hippocampus $F_{1,43}=7.487$, $p<0.01$; Striatum $F_{1,32}=10.825$, $p<0.05$) but no significant effect of genotype and no significant interaction between the two factors on phospho-Ser21-GSK3α. Two way ANOVA performed for each brain region revealed a significant effect of treatment (Cortex $F_{1,40}=13.04$, $p<0.001$; Hippocampus $F_{1,43}=9.682$, $p<0.05$; Striatum $F_{1,32}=14.645$, $p<0.001$), but no significant effect of genotype and no significant interaction between the two groups on phospho-Ser9-GSK3β. * $p<0.05$, Student’s t-test ns $p>0.05$
Figure 3. WT and β-arrestin2-KO (KO) mice were treated with DOI (20 mg/kg, 1 hr, n=10-12) and levels of phosphorylated and total GSK3 were determined by immunoblotting. (A) Representative immunoblots of phospho-Ser21-GSK3α, phospho-Ser9-GSK3β, and total GSK3 in the cortex, hippocampus, and striatum of DOI treated animals. (B) Quantification of phospho-Ser21-GSK3α, phospho-Ser9-GSK3β, and total GSK3 in the hippocampus, striatum, and cortex of DOI treated animals. ** p<0.05, ns p>0.05, 2 way ANOVA followed by Holm-Sidak comparison, *p<0.05 Student’s t-test
**Figure 4.** Loss of β-arrestin 2 potentiates antidepressant induced increase in GSK3 phosphorylation. WT and β-arrestin2-KO mice were treated with Fluoxetine (FLX, 20 mg/kg, 30 minutes, n=11-14) and levels of phosphorylated and total GSK3 α and β were determined by immunoblotting. (A) Representative immunoblots of phospho-Ser21-GSK3α, phospho-Ser9-GSK3β and total GSK3 α and β. (B) Quantification of phospho-Ser21-GSK3α, phospho-Ser9-GSK3β and total GSK3 α and β. **p<0.05, 2 way ANOVA followed by Holm-Sidak comparison, *p<0.05, Student’s t-test.
FIGURE 5 Loss of β-arrestin 2 potentiates the behavioral response to antidepressants, while loss of serine-phosphorylation of GSK3 prevents it. A) FST in GSK3-KI mice treated with fluoxetine (Flx, 20 mg/kg 30 minutes, n=8-12) (B) Forced Swim Test in WT and β-arrestin 2 KO mice treated with fluoxetine (Flx, 20 mg/kg 30 minutes, n=8-10) *p<0.05, **p<0.005, ns p>0.05 2-way ANOVA followed by Student-Newman Keul’s or Holm-Sidak comparison.
Deficiency in the Inhibitory Serine-Phosphorylation of Glycogen Synthase Kinase-3 Increases Sensitivity to Mood Disturbances

by

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Format adapted for dissertation
Abstract

Bipolar disorder, characterized by extreme manic and depressive moods, is a prevalent debilitating disease of unknown etiology. Because mood stabilizers, antipsychotics, antidepressants, and mood-regulating neuromodulators increase the inhibitory serine phosphorylation of glycogen synthase kinase-3 (GSK3), we hypothesized that deficient GSK3 serine-phosphorylation may increase vulnerability to mood-related behavioral disturbances. This was tested by measuring behavioral characteristics of GSK3 / 21A/21A/9A/9A knockin mice with serine-to-alanine mutations to block inhibitory serine-phosphorylation of GSK3. GSK3 knockin mice displayed increased susceptibility to amphetamine-induced hyperactivity and to stress-induced depressive-like behaviors. Furthermore, serine phosphorylation of GSK3 was reduced during both mood-related behavioral responses in wild-type mouse brain and in blood cells from patients with bipolar disorder. Therefore, proper control of GSK3 by serine-phosphorylation, which is targeted by agents therapeutic for bipolar disorder, is an important mechanism that regulates mood stabilization, and mice with disabled GSK3 serine-phosphorylation may provide a valuable model to study bipolar disorder.
Introduction

Bipolar disorder afflicts approximately 2% of the population and is debilitating and life-threatening if untreated. The defining characteristic of bipolar disorder is uncontrollable extreme shifts in mood between mania and depression, usually triggered by environmental factors such as stress (Feder et al, 2009; Martinowich et al, 2009). The pathophysiological mechanisms underlying bipolar disorder are unknown, but may include genetic variations, abnormal development of neural networks, and dysregulation of neurotransmitter, neuroendocrine, and signaling activities (Gold and Chrousos, 2002; Martinowich et al, 2009; Pittenger and Duman, 2008). Therefore, mechanistic insights gained by identifying key mood-regulating molecules and animal models that present heightened sensitivity to both manic-like and depressive-like behaviors are critical for deciphering the pathophysiology of bipolar disorder and developing new therapeutic interventions (Gould and Einat, 2007a).

Glycogen synthase kinase-3 (GSK3) is a highly conserved serine/threonine protein kinase. Two isoforms of GSK3, GSK3α and GSK3β, share 84% sequence homology but are encoded by different genes (Woodgett, 1990). GSK3 is constitutively partially active, and is predominantly regulated in an inhibitory manner by phosphorylation of serines in the N-terminal regions of its two isoforms, serine-9 in GSK3 and serine-21 in GSK3 (Woodgett, 1990). This phosphorylation transforms the N-terminus into a pseudo-substrate that blocks access of substrates to GSK3 (Frame et al, 2001). The inhibitory mechanism of GSK3 serine phosphorylation is used by many neuromodulators and neurotransmitter receptors to regulate GSK3 (Beaulieu et al, 2004; Li et al, 2004; Mai et al, 2002).

GSK3 has been suspected of contributing to bipolar disorder since the discovery that lithium, an effective mood stabilizer used therapeutically for bipolar disorder, is a selective
inhibitor of GSK3 (Klein and Melton, 1996; Stambolic et al, 1996). Besides directly inhibiting GSK3, lithium also increases the inhibitory serine-phosphorylation of GSK3 (De Sarno et al, 2002), as do other neuromodulators implicated in mood disorders, such as serotonin (Li et al, 2004) and brain-derived neurotrophic factor (BDNF) (Mai et al, 2002), raising the possibility that this action is critical for regulating mood (Beaulieu et al, 2009; Phiel and Klein, 2001; Rowe et al, 2007). Several studies provided links between GSK3 and manic- or depressive-like behaviors in animals or humans. Evidence that GSK3 contributes to manic-like behavior include the findings that GSK3 overexpressing transgenic mice exhibit hyperactivity (Prickaerts et al, 2006), amphetamine-induced hyperactivity concurs with reduced serine-dephosphorylation of GSK3 (Beaulieu et al, 2004), and both lithium and selective GSK3 inhibitors reduce hyperactivity in animals (Beaulieu et al, 2004; Gould et al, 2004a). Besides lithium, the anti-manic agents valproate and antipsychotic drugs increase GSK3 serine phosphorylation or directly inhibit its activity (Chen et al, 1999; De Sarno et al, 2002; Kozlovsky et al, 2006; Li et al, 2007b). Additionally, studies of GSK3 in depressive-like behaviors have shown that mice with GSK3 haploinsufficiency, lacking one copy of the gene encoding GSK3, exhibit an anti-depressant-like behavioral phenotype (O'Brien et al, 2004), which effectively normalizes depressive behaviors induced by serotonin deficiency (Beaulieu et al, 2008). Similarly, an anti-depressant-like behavioral phenotype also occurred in GSK3 knockout mice (Kaidanovich-Beilin et al, 2009). Substantial evidence also indicates that GSK3 is a potential therapeutic target in the treatment of depression, as administration of selective GSK3 inhibitors elicits antidepressant-like behavioral effects in animals (Gould et al, 2004a; Kaidanovich-Beilin et al, 2004; Rosa et al, 2008), established antidepressants (fluoxetine and imipramine) increase the inhibitory serine phosphorylation of GSK3 in
mouse brain (Li et al., 2004), and antidepressant-induced neurogenesis (Malberg et al., 2000) is blocked in mice expressing GSK3 with disabled serine-phosphorylation (Eom and Jope, 2009). In accordance with animal studies, a postmortem examination of brain samples revealed an increase in GSK3 activity in suicide subjects with major depression (Karege et al., 2007), and GSK3 polymorphisms are associated with brain region structural changes observed in patients with major depression (Inkster et al., 2009), suggesting that abnormal GSK3 activity occurs in humans with depression.

Because GSK3 is linked to both manic-like and depressive-like behaviors, and serine phosphorylation of GSK3 is the major regulatory mechanism controlling GSK3 and is targeted by lithium and other bipolar disorder treatments, we tested the hypothesis that increased susceptibility to behavioral changes relevant to bipolar disorder may be caused by deficient serine phosphorylation of GSK3. Here we report that disabling the inhibitory serine phosphorylation of GSK3 promotes both manic-like and depressive-like behavioral disturbances in mice, and serine-phosphorylation of GSK3 is reduced during each of these behavioral disturbances in both mice and bipolar disorder patients, establishing this mode of GSK3 regulation as a key modulator of both polarities of mood.

Materials and methods

Animals

The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham approved the experimental protocol using mice. Mice were housed 3-4 per cage with free access to food and water in a 12 hr light/dark cycle animal facility. Male homozygous GSK3 \(21A/21A/9A/9A\) knockin (GSK3 KI) mice (12-20 weeks of age) were used
along with strain-, age-, and generation-matched wild-type (WT) mice (both were kindly provided by D.R. Alessi, Dundee, Scotland) (McManus et al, 2005) for all experiments. Where indicated, mice were injected intraperitoneally with the indicated dose of d-amphetamine in saline, 0.83 mg/kg LPS (PBS, 0.1% DMSO), or vehicle. Lithium carbonate was administrated at 0.2% in mouse chow for 4 weeks, supplemented with water and saline. Serum corticosterone levels were measured by ELISA according to the manufacturer’s instructions (Cayman Chemical).

Behavior Tests

All behavior tests were conducted between 10:00 AM – 3:00 PM.

Home Cage Activity

Each mouse was individually placed in a standard micro-isolator cage (29 cm L x 19 cm W x 13 cm H) with sufficient food and water for 6 days. The spontaneous horizontal and vertical locomotor activities of mice were continuously monitored by an infrared beam activity monitor system, in which a total of eight beams spanned the width of each cage. Six lower beams measuring the horizontal activity are evenly spaced (4.5 cm part, 2.5 cm from cage floor), and would be broken by mice normally walking the length of the cage. Two upper beams measuring the vertical activity are located near the cage ends (1.8 cm from each cage wall and 7.0 cm above cage floor), and could be broken by rearing or climbing upside-down on the cage rack, but not by food or water intake. Data were recorded by a computer programmed to store data at 2 min intervals for each of the beams. Data from the first day and night of accommodation were discarded to allow a 24 hr acclimation to the fresh cage. Data are expressed as the beam breaks during 12 hr light and dark cycles over a 5 day period.
Open Field Test

Mice were placed in a plexiglass open field (Med Associates, St. Albans, VT) outfitted with photobeam detectors, and activity was monitored using the activity monitoring software (Med Associates, St. Albans VT). Mice were allowed to habituate in the open field for 15 min, and activity during this period was recorded as baseline activity, including ambulatory distance and center ambulation. Following this 15 min time, mice were given a single intraperitoneal injection of d-amphetamine (Sigma, St. Louis, MO) and immediately returned to the open field to be monitored for an additional 30 min. Travel distance during each 5 min block and total distance travelled during the 15 min preamphetamine and the 30 min post-amphetamine time periods were recorded.

Learned Helplessness

Mice were placed in one side of a Gemini Avoidance system shuttle box (San Diego Instruments, San Diego, CA) with the gate between chambers closed, and 180 inescapable foot shocks were delivered at an amplitude of 0.3 mA, a duration of 3-5 sec per shock, and a randomized inter-shock interval of 5-45 sec (Duman et al, 2007). In a modified inescapable shock protocol, referred to as the reduced inescapable shock protocol, mice were given 180 foot shocks with amplitude of 0.3 mA and fixed 3 sec shock duration, and a randomized inter-shock interval of 5-25 sec (Supplemental Table 1). Mice exposed to the same chamber for one hr without shocks were used as controls. Twenty-four hr after inescapable shocks, mice were returned to the shuttle box and the escape task was tested by giving 30 escape trials with each trial preceded by a foot shock at amplitude of 0.3 mA for a maximum duration of 24 sec. The door of the chamber opens at the beginning of the foot shock administration to allow mice to escape. Latency to escape the shock was recorded using
Gemini software, and trials in which the animal did not escape within the 24 sec time limit were counted as escape failures. Mice with greater than 15 escape failures were defined as helpless.

**Shock Response and Pain Response Threshold**

Mice were placed in an operant chamber (Med Associates Inc, St. Albans, VT) and administered a series of 10 foot shocks with consecutive increases in amplitude from 0.1 to 1 mA at 0.1 mA increments and with a 20 sec interval between shocks. At the onset of each shock, mice were observed for flinching, jumping, and vocalization. The minimum shock intensity required to elicit these responses was recorded for each mouse. As a measure of sensitivity to pain, mice were placed on a hot plate maintained at 52°C. Latency of mice to lift or lick a paw was recorded manually.

**Forced Swim Test**

Mice were placed in an automated apparatus consisting of clear Plexiglas cylinders containing distilled water (23-25°C) and outfitted with 2 rings of photo-beam detectors (Kinder Scientific, Poway, CA). Movements were continuously monitored by computer for 6 min. Data were recorded using Motor Monitor Software (Kinder Scientific, Poway, CA) and transferred into Microsoft Excel. The immobility time was represented by recording the resting time (sec without beam breaks) during the last 4 min of testing (Kurtuncu et al., 2005).

**Tail Suspension Test**

Mice were tested using an automated testing system (Med Associates Inc, St. Albans, VT) consisting of an open-fronted testing chamber with a hanging vertical metal bar attached to a strain gauge that detects any movements by a mouse. The mouse tail is attached to the apparatus and movement is measured for 6 min with Med Associates software, and the
immobility time was calculated as the time the force of movement was below a preset threshold during the last four min of testing (Crowley et al, 2004).

**Elevated Plus Maze**

Mice were placed on the central platform of an elevated plus maze that consists of a platform of 40 cm in height and four arms of 31 x 5 cm in length, with 15 cm high sides of opaque material in the two closed arms (San Diego Instruments, San Diego, CA). Entries of mice to each arm were monitored with an Ethovision camera-driven tracking system (Noldus, The Netherlands). The number of entries and time spent in each arm during the 4 min test were recorded via computer and data were analyzed using Ethovision software.

**Fear Conditioning**

Mice were placed in an operant chamber inside an isolation box to allow exploring the operant chamber for 2 min. After this acclimation, they were administered three series of a 15 sec, 75 decibel white noise tone each followed immediately by a 0.5 mA foot shock. Twenty-four hr after training, mice were tested for contextual memory by placing them in the same operant chamber for 5 min. Freezing was monitored by Med Associates Video Freeze software. Three hr after contextual testing, the cued memory was tested by placing mice in a novel context for 3 min (pretone), followed by a 75 decibel white noise tone. Freezing was monitored using Video Freeze software.

**Electrophysiology**

Hippocampal slices for electrophysiology were prepared from 8-12-week old mice using standard methods (Scheiderer et al, 2004). Mice were rapidly decapitated, and brains were removed and briefly rinsed in artificial cerebrospinal fluid (aCSF) containing 119 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl$_2$, 1.3 mM MgSO$_4$, 1 mM NaH$_2$PO$_4$, 26 mM NaHCO$_3$, and
10 mM glucose. Coronal slices (400 m) were cut from the ventral pole of the hippocampus using a Vibratome (Vibratome, St. Louis, MO) in aCSF with continuous 95%O2/5%CO2. Slices were allowed to rest for one hr in oxygenated aCSF before electrophysiological recording. For all experiments, slices were placed in a submersion recording chamber and continuously bathed in oxygenated aCSF at 28-30°C. A bipolar tungsten stimulating electrode (FHC, Bowdoinham, ME) was placed in the stratum radiatum of CA3 to stimulate the axons of the Schaeffer collaterals. Extracellular dendritic field excitatory post-synaptic potentials (fEPSPs) were recorded from stratum radiatum of CA1 with a glass microelectrode filled with aCSF using AxoPatch 2B (Molecular Devices, Sunnyvale, CA). Baseline synaptic transmission was elicited using paired pulses of electrical stimuli (0.1 Hz, 100 μs duration, 20 ms apart). Stimulation intensity was adjusted to elicit fEPSPs between 0.6 and 0.8 mV in amplitude. At least 20 min of a stable baseline was acquired before induction of long term potentiation (LTP) or long term depression (LTD). To induce LTP, high frequency stimulation was applied to slices with 4 trains of 50 pulses at 100 Hz, separated by 20 sec. Stimulation intensity was escalated to 1.5 x baseline stimulation during tetanus and returned to baseline immediately after the course of stimulation. To induce NMDA receptor-dependent LTD by low frequency stimulation, slices were stimulated with single pulses at a frequency of 1 Hz for 15 min. Stimulus-response curves and paired pulse data were collected after a stable 20 min baseline. Slices were stimulated at a range of intensities from 0 to 30 A for 10 sweeps and the fEPSP slope was recorded. Two stimulus-response curves were collected and averaged from each slice, and only slices in which the two curves differed by <10% were included in the final average. The paired pulse ratio was tested over a range of 10-160 ms for ten sets of pulses, and the ratio of the second fEPSP to the first was recorded.
Data were filtered at 3 kHz and acquired using National Instruments acquisition software. Data from each experiment was normalized to its own baseline. The normalized slope of the fEPSP was plotted against time, with each point representing an average of five data points.

**Immunoblotting and ELISA**

Mice were decapitated, and brain regions were rapidly dissected and homogenized in ice-cold lysis buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 10 g/ml leupeptin, 10 g/ml aprotinin, 5 g/ml pepstatin, 0.1 mM β-glycerophosphate, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium vanadate, 50 mM NaF, and 100 nM okadaic acid. The lysates were centrifuged at 20,800 g for 15 min. Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL). Samples were mixed with Laemmli sample buffer (2% SDS), placed in a boiling water bath for 5 min, proteins were resolved in SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with antibodies to phospho-Ser9-GSK3β, phospho-Ser21-GSK3α, phospho-Ser473-Akt, phospho-Thr308-Akt, Akt, phospho-Thr202/Tyr204-ERK1/2, ERK1/2, phospho-Ser380-PTEN, PTEN, phospho-Thr183/Tyr185-JNK, JNK, phospho-Thr180/Tyr182-p38, p38 (Cell Signaling, Danvers, MA), total GSK3α/β (Upstate Biotechnology Inc., Lake Placid, NY), phospho-Tyr279/216GSK3, GSK3 (Millipore), and β-actin (Sigma).

Immunoblots were developed using horseradish peroxidase-conjugated goat antimouse, or goat anti-rabbit IgG, followed by detection with enhanced chemiluminescence, and the protein bands were quantitated with a densitometer. IL-6 levels were measured by ELISA from 100 g protein of hippocampal extracts according to the manufacturer’s instructions (eBioscience).
Study with human subjects was approved by the University of Alabama at Birmingham Institutional Review Board and was conducted at the University of Alabama at Birmingham (UAB). Written informed consent was obtained from all subjects after receiving a complete description of the study. Men and women, aged 19–65 years, from any racial and ethnic group were eligible for study screen. Bipolar disorder patients were recruited from the UAB psychiatric outpatient clinic or the Center for Psychiatric Medicine in-patient service. The inclusion criteria include: 1) a primary DSM-IV™ diagnosis of bipolar disorder, type I or type II; 2) currently in a 12 symptomatic episode, either manic/hypomanic with a minimal score of ≥ 20 on Young Manic Rating Scale (YMRS), or depressive with a minimal score of ≥ 25 on Montgomery-Asberg Depression Rating Scale (MADRS); 3) not taking psychotropic medications for at least 3 days prior to blood collection; and 4) no major unstable medical illnesses for 3 months prior to blood collection. Subjects were excluded if they concomitantly met criteria for any other major DSM-IV Axis I diagnosis, such as psychotic disorder, major depressive disorder, anxiety disorder, or alcohol and/or other substance dependence. A total of eighteen bipolar disorder patients met inclusion criteria and donated blood, among them 12 were in a manic/hypomanic episode and 6 were in a depressive episode. Additionally, eleven healthy control subjects were also enrolled for comparative studies (Supplemental Table 2). Psychiatric and medical histories along with vital signs, a physical examination, and a neurological examination, were obtained prior to blood collection. The most recent (within 2 week) laboratory data on fluid balance, blood glucose, CBC with differential, thyroid function, and liver function, were reviewed to assure that these parameters were within normal range. Clinical symptoms were assessed using the YMRS for mania, the
MADRS for depression, and the Clinical Global Impression for Bipolar Disorder-severity (BD-CGI-S). All assessments were completed on the day of blood collection.

**Human PBMC preparation and GSK3 measurements**

Once inclusion criteria were confirmed at screen visit, approximately 30 ml blood from each subject was immediately collected by venipuncture. PBMCs were extracted from whole blood as described previously (Li et al, 2007a), and protein lysate from PBMCs was prepared and frozen. For immunoblotting, each group of up to seven protein samples, from at least one bipolar disorder patient and one healthy control subject that were collected within a three-month period, were processed. An aliquot of the same protein lysate from SH-SY5Y cells was included in each immunoblot to adjust the intensity of immunoblot exposure. Protein bands were quantified with a densitometer.

**Statistical Analysis**

All data are presented as mean ± SEM. Statistical analyses were conducted using SigmaStat 10.0. All data were checked for assumptions of normal distribution and homogeneity of variances in study samples, and any unexplained outlier value greater than ±2 standard deviations from the mean of the group was excluded. For a two-group comparison, statistical analysis was performed using unpaired Student’s t-test. For comparison of more than three experimental groups, one- or two-way analysis of variance (ANOVA) was used to test for significant differences (p≤0.05) between groups. Any significant difference detected by ANOVA was followed by post hoc or inter-group comparisons using Tukey’s test, Holm- Sidak comparison, or Tamhane’s test. Correlation of two sets of data from the same group of
samples was tested using Pearson’s correlation analysis. No attempt was made for multiple comparisons due to the number of parameters that were examined. The results presented are those that may be clinically worth investigating in future studies.

Results

To evaluate the role of GSK3 serine-phosphorylation in mood regulation, we used GSK3 KI mice expressing GSK3 and GSK3 with the regulatory N-terminal serines mutated to alanines (McManus et al, 2005). GSK3 KI mice reproduce and develop normally, display no overt physical or behavioral phenotype (McManus et al, 2005), and express both isoforms of GSK3 in brain at levels identical to WT mice, but without GSK3 serine-phosphorylation (Supplemental Fig. 1).

GSK3 KI mice displayed increased susceptibility to hyperactivity, a behavior often used to evaluate manic-like disturbances. When home cage activity was assessed, there were no significant differences between GSK3 KI and WT mice during the light or dark cycles, suggesting normal baseline activity (Fig. 1A). However, when placed in an open field, GSK3 KI mice initially displayed modest hyperactivity (Fig. 1B), but they habituated to WT levels of activity upon repeated testing in the same open field (Fig. 1C), suggesting that GSK3 KI mice have a heightened response to a novel environment. After habituation in the open field, administration of amphetamine (2 mg/kg), often used to model manic-like hyperactivity (Hasler et al, 2006), caused over 2.5-fold greater hyperactivity in GSK3 KI mice than WT mice during a 30 min testing period, with a total ambulatory activity of 16798±1720 cm in GSK3 KI mice compared to 6037±839 cm in WT mice (Fig. 2A). Examination of the dose response to amphetamine demonstrated a lower threshold for hyperactivity in GSK3 KI mice,
as administration of 1 mg/kg amphetamine caused significantly greater activity of GSK3 KI mice than WT mice (Fig. 2B). This heightened response of GSK3 KI mice to amphetamine shows that the serine-phosphorylation state of GSK3 directly affects the magnitude of hyperactivity, which extends a previous report that amphetamine reduced serine phosphorylation of GSK3 (Beaulieu et al, 2004). Additionally, GSK3 KI mice displayed 19% and 59% increases in hyperactivity after two and three weekly treatments with 1 mg/kg amphetamine, whereas WT mice did not display sensitization to this once-a-week low dose repeated amphetamine administration (Fig. 2C). These results indicate that serine dephosphorylation of GSK3 not only mediates hyperactivity caused by amphetamine (Beaulieu et al, 2004), but also increases the sensitivity for the response. Similar to its effect on amphetamine-induced hyperactivity in WT mice (Gould et al, 2007b), chronic lithium administration to GSK3 KI mice partially reduced amphetamine-induced hyperactivity to the level equivalent to WT mice receiving amphetamine treatment (Fig. 2D). The partial effect of lithium is likely due to its direct inhibition of GSK3 (Klein et al, 1996; Stambolic et al, 1996), since its additional inhibitory effect on GSK3 by increasing serine-phosphorylation is eliminated in GSK3 KI mice.

Besides being sensitive to hyperactivity, GSK3 KI mice were highly susceptible to stress-induced depressive-like behavior. The "learned helplessness" model of depression was examined by conditioning mice with random, mild, inescapable shocks. Measurements of responses to escapable foot shocks the next day using a standard protocol (Duman et al, 2007) (Supplemental Table 1) showed that both WT and GSK3 KI mice failed to escape (Fig. 3A,B), a learned helplessness behavior that models stress-induced depression. To test if GSK3 KI mice have increased vulnerability to stress-induced depressive-like behavior, the
intensity of the inescapable foot shock paradigm was reduced (Supplemental Table 1) to a level that did not cause failure to escape (defined as greater than 15 escape failures in 30 trials) in most WT mice (Fig. 3B). However, with this lower level of aversive stimuli, 91% of GSK3 KI mice still displayed failure to escape. Analysis of the escape latency over each five trial block demonstrated that WT mice had an average of 18±2 sec escape latency during the first five trials, but this helpless behavior gradually diminished over repeated trials, with only 8±3 sec average escape latency during the last five trials (Fig. 3C). In contrast, GSK3 KI mice displayed an average escape latency of 23±1 sec and the helpless behavior persisted throughout the 30 trials. Remarkably, even without prior conditioning by inescapable foot shocks, 36% of the GSK3 KI mice failed to escape, whereas no WT mice displayed this behavior (Fig. 3B). GSK3 KI mice that exhibited significant helpless behavior during escape trials displayed increasing escape latency to more than 21 sec by the third five-trial block, whereas those without this behavior had escape latencies similar to WT mice (Fig. 3D). Failure of GSK3 KI mice to escape was not due to a sensory deficit since both WT and GSK3 KI mice displayed equivalent sensitivities to foot shock and pain (Supplementary Fig. 2). These results indicate that blocking serine-phosphorylation of GSK3 strongly increases susceptibility to learned helpless behavior.

The heightened susceptibility to stress-induced depressive-like behavior was also evident in two despair-based behavioral tests, the forced swim test (Cryan et al, 2002) and the tail suspension test (Cryan et al, 2005). In both tests, GSK3 KI mice spent more time immobile than WT mice (29% and 27% increases in the forced swim test and tail suspension test, respectively) (Fig. 4A,B), confirming that the serine phosphorylation of GSK3 sets the threshold for depressive-like behaviors in response to stressful conditions. To further test if
serine phosphorylation of GSK3 affects susceptibility to depressive-like behavior, a combination of aversive stimuli was applied before the tail suspension test. In some mouse strains, administration of a very low dose of lipopolysaccharide (LPS), a gram-negative bacteria cell wall component that induces an inflammatory response, can cause subtle expression of depressive-like behaviors (Dantzer et al, 2008). Administration of 0.83 mg/kg LPS did not change the already heightened immobility time of GSK3 KI mice compared with WT mice (Fig. 4B). The immobility time after inescapable foot shocks was not different between GSK3 KI and WT mice, but applying LPS 24 hr after this aversive stress caused an additional increased in the immobility time in GSK3 KI mice. The combined aversive stimuli-induced depressive-like behavior in GSK3 KI mice was accompanied by increased serum levels of IL-6 (Fig 4C), one of many inflammatory mediators that can influence mood relevant behaviors (Dantzer et al, 2008). These findings demonstrated that serine phosphorylation of GSK3 is an important determinant of the susceptibility of mice to acquire depressive-like behavior when exposed to aversive stimuli.

This absence of adaptability of the GSK3 KI mice to stress may involve anxiety, which often co-exists with depression. GSK3 KI mice displayed mild-anxious behavior in the elevated plus maze, spending 44% less time than WT mice in the open arms of the maze, and a significantly longer time in the closed arms (Fig. 4D). However, when the anxiety-like behavior of thigmotaxis was tested for 15 min, GSK3 KI mice were slightly more anxious only during the first 5 min of testing, whereas the overall difference between GSK3 KI and WT mice throughout the 15 min test was not significant (Fig. 4E), suggesting that GSK3 KI mice may only display mild anxiety-like behavior when exposed to a new environment.
To test if increased sensitivity of GSK3 KI mice to mild stress was associated with altered hippocampal function, a stress-vulnerable mood regulating region of the brain (Duman et al., 1997), fear conditioning was examined. In the fear conditioning paradigm, baseline freezing times for WT and GSK3 KI mice were equivalent during training to associate both a context and a cue (tone) with a mild aversive stimuli (foot shocks) (Fig. 5A). The freezing time of GSK3 KI mice was significantly 28% higher than WT mice upon subjection 24 hr later to the contextual test, a function of both hippocampus and amygdala, suggesting that GSK3 KI mice display an enhanced emotional association with a stressful stimulus that likely involves either hippocampal or amygdalar function. Upon exposure to a novel context (pretone cue test), GSK3 KI mice initially exhibited 50% less freezing than WT mice, which is consistent with increased activity by GSK3 KI mice when exposed to a novel environment (Fig. 1). However, when exposed to a tone paired with the new context, a function primarily dependent on the amygdala (LeDoux, 2000), both GSK3 KI and WT mice displayed equivalent high freezing times, suggesting that amygdalar function is likely intact.

To further test if neuronal activity in the hippocampus is abnormal in GSK3 KI mice, synaptic plasticity at the Schaffer collateral CA3-CA1 synapse in the ventral hippocampus was measured. The baseline synaptic transmission and paired pulse ratio revealed no significant differences between WT and GSK3 KI mice (Supplemental Fig. 3). High frequency stimulation robustly induced LTP in the CA1 to a similar magnitude in WT and GSK3 KI mice (Fig. 5B). However, NMDA receptor-dependent LTD induced by low frequency stimulation was converted to a slow onset LTP-like response in GSK3 KI mice (Fig. 5C), a conversion previously noted to be induced by stress in WT rats (Maggio and Segal, 2009). These findings indicate that impaired serine-phosphorylation of GSK3 in
GSK3 KI mice establishes a stressed state that alters responses to aversive stimuli. Since GSK3 KI mice display heightened responses to stress, we tested if they produce higher levels of corticosterone, a leading candidate to mediate stress-induced episodes of mood disorders. Basal levels of serum corticosterone were similar in GSK3 KI and WT mice, but immediately following a session of inescapable foot shocks, the serum corticosterone was greater in GSK3 KI mice than in WT mice, which was reduced to WT levels by lithium pretreatment (Supplemental Fig. 4A). Immediately after exposure of GSK3 KI mice to escapable foot shocks the serum corticosterone levels were increased above basal levels, but not as much as after exposure to inescapable foot shocks (Supplemental Fig. 4B), and there was a tendency (p=0.06) for the corticosterone levels to be modestly higher in helpless than in non-helpless GSK3 KI mice after the escapable foot shock treatment.

Since the absence of serine-phosphorylation of GSK3 is associated with increased susceptibility to hyperactivity and depressive-like behaviors, we examined if dephosphorylation of the inhibitory serines in GSK3 occurs in WT mice that display amphetamine-induced hyperactivity or develop learned helplessness after inescapable foot shocks. As previously reported (Beaulieu et al., 2004), amphetamine (2 mg/kg) treatment reduced the inhibitory serine phosphorylation of GSK3 in the striatum of WT mice (Supplemental Fig. 5). There were also dramatic decreases in the inhibitory serine-phosphorylation of both GSK3 isoforms in the hippocampus and cerebral cortex of WT mice in the learned helpless state 24 hr after inescapable foot shocks (Fig. 6A). Foot shock treatments not causing learned helplessness did not change serine-phosphorylation of GSK3, demonstrating that the treatment did not alter GSK3 unless it was associated with learned helplessness. Learned helplessness-induced decreases in serine-phosphorylated GSK3 were
associated with decreased phosphorylation of Akt, while there were no changes in the activity-regulating phosphorylation of several other intracellular signaling kinases (Fig. 6B; Supplemental Fig. 6), indicating selective reduction in the activity of Akt, a predominant kinase that phosphorylates the regulatory serines of GSK3. Basal phosphorylation of Akt was not different between GSK3 KI and WT mice (Fig. 6C), but when assessed after escapable foot shocks, lower Akt phosphorylation was found in hippocampal extracts of helpless GSK3 KI mice than in WT mice (Fig. 6D). Akt phosphorylation was also significantly lower in hippocampal extracts of helpless GSK3 KI mice than in non-helpless GSK3 KI mice when the two groups were differentiated by responses to escapable foot shocks (Fig. 6E), demonstrating that the decreased Akt phosphorylation was associated with depressive-like behavior, but not the shock response in GSK3 KI mice. Thus, reduced Akt activation occurs in conjunction with decreased inhibitory serine-phosphorylation of GSK3 during depressive-like states.

To examine if the diminished serine-phosphorylation of GSK3 observed in mice with manic-like and depressive-like behaviors is evident in patients with bipolar disorder, serine-phosphorylated GSK3 was measured in peripheral blood mononuclear cells (PBMCs) from symptomatic, drug-free bipolar disorder patients and matched healthy controls (Supplemental Table 2). Phospho-Ser21-GSK3 and phospho-Ser9-GSK3 were each approximately 35% lower in PBMCs from bipolar disorder patients than healthy controls (Fig. 7A-C). Total levels of each GSK3 isoform in PBMCs were equivalent in bipolar disorder patients and healthy controls (Fig. 7A), indicating that lower levels of serine-phosphorylated GSK3 in patients was not due to reduced expression of GSK3. Remarkably, reductions in phospho-Ser21-GSK3α and phospho-Ser9-GSK3β significantly correlated with severity of manic
symptoms evaluated by the Young Mania Rating scale (Fig. 7D), and reduced phospho-
Ser21-GSK3α also significantly correlated with severity of depressive symptoms evaluated
by the Montgomery-Asberg Depression Rating scale (Fig. 7E). Therefore, decreased
inhibitory serine phosphorylation of GSK3 also occurs in symptomatic bipolar disorder
patients. Along with the findings from hyperactive and stressed mice, this result provides
additional evidence that dysregulation of serine phosphorylation of GSK3 contributes to the
etiology of mood disturbances in bipolar disorder.

Discussion

This study identified a single molecular mechanism, reduced serine-phosphorylation
of GSK3, that causes behavioral alterations in mice resembling the mood symptoms in
human bipolar disorder. Not only does blocking serine-phosphorylation of GSK3 increase
susceptibility to both stimulus-induced hyperactivity and stress-induced depressive-like
behaviors in mice, but as might be predicted if GSK3 serine-phosphorylation is critical in
regulating mood-related behaviors, serine-phosphorylation of GSK3 also is reduced during
both hyperactive and depressive-like states in mice. Importantly, in agreement with findings
in mice, GSK3 serine-phosphorylation also is reduced in PBMCs from symptomatic bipolar
disorder patients and the reduction is correlated with severity of symptoms. Taken together,
all of these results indicate that insufficient serine-phosphorylation of GSK3 may play a
critical pathological role in behavioral disturbances that occur in bipolar disorder.

Mice expressing normal levels of GSK3 but with the inhibitory serine-
phosphorylation ablated were studied because serine-phosphorylation of GSK3 is a target of
the mood stabilizer lithium and several other mood disorder treatments, but the role of this
regulatory mechanism had not been studied in behavioral responses that may be related to mood disturbances. We hypothesized that ablation of the inhibitory serines in GSK3α/β would cause behavioral changes opposite to the effects of bipolar disorder treatments. This was confirmed, as prominent features of GSK3 KI mice are heightened sensitivity to amphetamine-induced hyperactivity and increased vulnerability to stress-induced depressive-like behavior. This extends previous studies of animal models that exhibit either hyperactivity (Gould et al., 2004b; Roybal et al., 2007) or depressive-like behavior (Kalueff et al., 2007; Pittenger et al., 2008), and should facilitate identification of factors that contribute to bipolar disorder and that can be used to test new therapeutic strategies, such as targeting pathways converging on the serine phosphorylation of GSK3 or abnormally regulated substrates of GSK3. This study used the GSK3 and GSK3 double KI mice because both GSK3 isoforms may contribute to regulating mood-related behaviors (Kaidanovich-Beilin et al., 2009; O’Brien et al., 2004; Prickaerts et al., 2006), and all chemical inhibitors of GSK3 inhibit both GSK3 and GSK3 equally. Although some studies have identified GSK3 isoform-selective actions (Force and Woodgett, 2009; Hoeflich et al., 2000; Liang and Chuang, 2006, 2007; Phiel et al., 2003; Wang et al., 1994), there is no published information delineating a predominant role of either isoform of GSK3 on mood-related behaviors.

Importantly, decreased serine-phosphorylation of GSK3 during both manic-like and depressive-like states were also found in PMBCs from bipolar disorder patients, providing further support that abnormal regulation of GSK3 serine-phosphorylation is critically altered during mood disturbances in bipolar disorder. We used PBMCs as a surrogate system since lymphocytes in PBMCs express receptors, intracellular signaling proteins, and enzymes that are regulated by many of the same genetic and environmental influences as neurons in the
brain (Gladkevich et al., 2004; Tsuang et al., 2005). The expression levels of many classes of biologically relevant processes are similar in PBMCs and brain (Sullivan et al., 2006), as may be the case with GSK3 and its regulatory signaling pathways (Castri et al., 2007; Li et al., 2007a), making PBMCs a valuable human tissue to study signaling systems that also occur in human brain. However, it is clear that further studies are needed to clarify the relationships between the activities of signaling systems in PBMCs and the brain. Although our data provide clinical evidence validating the findings of the animal behavioral studies and in mouse brain that dysregulation of GSK3 serine-phosphorylation facilitates behavioral disturbances, a larger sample size of bipolar disorder patients is required to confirm the clinical relevance. Nevertheless, the significant reduction of serine-phosphorylation of GSK3 in mice during hyperactivity or depressive-like behavioral disturbances and in symptomatic bipolar patients, as well as a significant relationship between the severity of mood symptoms in bipolar patients and dysregulated GSK3 in PBMCs, suggest that a mood state-dependent change of GSK3 serine-phosphorylation exists during mood-related behavioral disturbances.

Ablation of the regulatory serines in GSK3 sensitized GSK3 KI mice to stress, which was evident in their behavioral responses to all types of stressful conditions that were studied. The increased sensitivity to both extremes of behavioral disturbances in GSK3 KI mice could be indicative of a lack of proper regulation of GSK3 serine phosphorylation in bipolar disorder, which could result from altered signaling to GSK3 by abnormalities in mood-regulating neuromodulators. Increased dopaminergic activity is associated with hyperactivity, a behavior that often occurs in bipolar disorder, and enhanced dopamine D2 receptor activity has been reported to dephosphorylate the regulatory serines in GSK3 (Beaulieu et al., 2004). Since one effect of amphetamine is to increase dopamine release, the
increased vulnerability of GSK3 KI mice to amphetamine-induced hyperactivity suggests that the dephosphorylation of GSK3 contributes to setting the threshold for hyperactivity induced by dopaminergic neurotransmission. Similarly, serotonin- and BDNF-induced signaling normally increase serine-phosphorylation of GSK3 (Beaulieu et al, 2004; Li et al, 2004; Mai et al, 2002), thus lacking regulation in GSK3 KI mice by these modulators likely models the states of serotonin and BDNF deficiency that may be associated with susceptibility to depression. Consistent with altered responses to stress, we found an abnormal LTD in GSK3 KI mice in the ventral hippocampus, the septotemporal portion of the hippocampus that is primarily associated with emotional memories (Bannerman et al, 2003). This finding may be related to a recent report (Maggio et al, 2009) in which an almost identical conversion of LTD to a low amplitude LTP in the ventral hippocampus occurred in WT rats exposed to acute swim stress. The induction of depressive-like learned helplessness in GSK3 KI mice is also associated with an upstream reduced phosphorylation of Akt. This modulation of Akt suggests a feedback loop of Akt regulation by GSK3, which could involve regulation of signaling of receptors to Akt or other regulators that respond to stress.

Altogether, these findings demonstrate a critical role of the serine phosphorylation of GSK3 in the signaling pathways associated with manic-like and depressive-like behaviors and indicate that impaired serine-phosphorylation of GSK3 establishes a state of increased susceptibility to stress-induced behavioral disturbances. The targets affected by deletion of serine-phosphorylation of GSK3 that mediate increased vulnerability to mood disturbances may include changes in neurotransmission, neurotrophins, activity of the HPA axis, stress-induced inflammatory responses, or other mediators.
Disclosure

The authors declare no conflict of interest.

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References


**Figure 1.** GSK3 KI mice display hyperactivity in novel, but not familiar, settings. (A) Home cage activity over a five-day period recorded from the light cycle of day-2 (L2) to the dark cycle of day-6 (D6) was not significantly different for GSK3 KI and WT mice (n=10). (B) GSK3 KI mice traveled a significant 56% longer distance than WT mice (2735±205 cm in GSK3 KI mice vs. 1838±183 cm in WT mice) during a 15 min testing period (n=40 WT, 61 KI mice, *p<0.05, Student’s t-test). (C) Distance traveled on each weekly exposure (week 1, 2, and 3) to the open field. On the second and third exposures to the open field, there were no significant differences in activity between GSK3 KI and WT mice (n=14 mice/group, *p<0.05 compared to WT (F_{genotype}=23.95, F_{exposure}=22.91, F_{interaction}=4.91, two way ANOVA).
Figure 2. GSK3 KI mice display heightened manic-like hyperactivity after amphetamine administration. (A) Time course of response to 2 mg/kg amphetamine (n=20 WT, 21 KI mice, *p<0.05 compared to WT, F\text{genotype}=5.775, F\text{block}=25.450, F\text{interaction}=2.295, two-way repeated measure ANOVA, in which factors include genotypes = WT + GSK3 KI, block = 5-trial testing blocks, and interaction = genotypes x blocks). (B) Dose-response to amphetamine during a 30-min test (0.5, 1, and 2 mg/kg) (n=15-21 mice/group, *p<0.05 compared to WT, F\text{genotype}=23.95, F\text{exposure}=22.91, F\text{interaction}=4.91, two-way ANOVA). (C) Mice received repeated amphetamine injection (1 mg/kg) once a week for three weeks. Distance traveled in the open field after each amphetamine treatment was normalized to the initial response in the same mouse, data are represented as group averages of normalized responses (n=14/group, *p<0.05 compared to WT, F\text{genotype}=7.365, F\text{dose number}=0.234, F\text{interaction}=4.635, two way ANOVA). (D) Lithium was administered to GSK3 KI and WT mice for 4 weeks, followed by amphetamine (2 mg/kg) administration. Ambulatory distance traveled by GSK3 KI mice is plotted against each 5-trial blocks (left panel) (n=10 without lithium (control), 13 with lithium, *p<0.05 compared to lithium-treated mice at the same time block, F\text{treatment}=3.395, F\text{block}=13.448, F\text{interaction}=2.59, two-way repeated measure ANOVA). Total distance traveled in GSK3 KI mice is also compared with wild type mice treated with amphetamine (n=8) (right panel). *p<0.05 compared to amphetamine-treated GSK3 KI mice without lithium treatment, one way ANOVA.
Figure 3. GSK3 KI mice display increased sensitivity to stress-related learned helplessness. (A) WT and GSK3 KI mice received standard inescapable foot shocks (IES), and average escape latency during the entire escape trial period was calculated (n=9-10/group, *p<0.05 in one way ANOVA). (B) Escape failures (defined as escape failure >15 of 30 trials) after standard IES (IES), reduced IES (rIES), and no IES, tested by escapable foot shocks (ES). With rIES only 36% of WT mice exhibited learned helplessness defined as greater than 15 escape failures, whereas 91% of GSK3 KI mice displayed learned helplessness. Each diamond represents total escape failures for each individual mouse, bars represent group means (n=9-15 mice/group, *p<0.05 compared to WT mice that received the same shock treatment. (C) Average escape latency during 5-trial blocks after reduced IES. WT mice had an average of 18±2 sec escape latency during the first 5 trials, but the helpless behavior was gradually reduced over repeated trials, with only 8±2 sec average escape latency during the last 5 trials. GSK3 KI mice displayed an average escape latency of 23±1 sec and the helpless behavior persisted throughout the 30 trials. (n=15/group, *p<0.05 compared to WT control, F\text{group}=31.361, F\text{block}=7.096, F\text{interaction}=5.322 two-way repeated measure ANOVA). (D) Average escape latency over the 5-trial blocks in WT and GSK3 KI mice without IES, with “escaped” defined as 0-15 escape failures over 30 trials, and “failed to escape” defined as >15 escape failures (n=9-15 mice/group, *p<0.05 compared to WT, **p<0.05 compared to both WT and escaped GSK3 KI, F\text{group}=23.581, F\text{block}=79.048, F\text{interaction}=6.782, two-way repeated measure ANOVA).
Figure 4. Depressive-like and anxiety-like behaviors in GSK3 KI mice. (A) In the forced swim test, over the last 4 min of a 6 min test, GSK3 KI mice spent longer times immobile than matched WT mice (135±8 sec and 104±9 sec, respectively), which was a significant 29% increase in immobility (n=20/group). (B) In the tail suspension test the average immobility time of GSK3 KI mice was 27% higher than the matched WT mice (148±5 sec and 117±11 sec, respectively) (n=18/group). WT and GSK3 KI mice were also treated with LPS (0.83 mg/kg) or vehicle (PBS, 0.1% DMSO), the standard inescapable shock protocol (IES), or IES followed 24 hr later by treatment with LPS (0.83 mg/kg) or vehicle, and 24 hr later immobility time in the tail suspension test was measured. n=4; Means ± SEM; *p<0.05 Student's t-test compared with equivalently treated WT mice. (C) Hippocampal IL-6 levels were measured in WT and GSK3 KI mice that were subjected to IES followed 24 hr later by treatment with LPS (0.83 mg/kg) or vehicle. Basal IL-6 levels in untreated WT and GSK3 KI mice were 0.58 ± 0.17 and 0.57 ± 0.20 ng/ml, respectively (n=7 per group). (D) In the elevated plus maze during a 4 min testing period, GSK3 KI mice spent 44% less time in the open arms of the maze than matched WT mice (24±3 sec vs. 42±6 sec), and exhibited a significant increase in time spent in the closed arms (194±6 sec in GSK3 KI vs. 170±6 sec in WT mice) (n=18/group). (E) When the anxiety-like behavior was re-tested in the open field for thigmotaxis for 15 min, GSK3 KI mice exhibited a trend towards increased anxiety only during the first five min of testing, whereas the overall difference between GSK3 KI and WT mice throughout the 15 min test was not significantly different. Data are means ± SEM, *p<0.05, **p<0.01 by Student's t-test.
A

\[ \text{% Time Freezing} \]

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B

\[ \text{LTP} \]

\[ \text{Normalized EPSP slope} \]

\[ \text{Time (min)} \]

C

\[ \text{LTD} \]

\[ \text{Normalized EPSP slope} \]

\[ \text{1 Hz} \]

\[ \text{Time (min)} \]
**Figure 5.** Altered hippocampal responses to stress in GSK3 KI mice. (A) Contextual and cued fear conditioning. Twenty-four hr after training, GSK3 KI mice spent significantly more time freezing (79±5% and 61±4% in GSK3 KI and WT mice, respectively) in the contextual test. When exposed to novel context (pre-tone), GSK3 KI mice displayed significantly less freezing (41±4% and 17±4% in GSK3 KI and WT mice, respectively), but response to tone was equivalent in both WT and GSK3 KI mice. n=14 mice/group, *p<0.05 compared to WT, contextual: F_{genotype}=5.137, F_{condition}=36.233, F_{interaction}=5.319, cued: F_{genotype}=4.593, F_{condition}=108.935, F_{interaction}=8.202, two-way ANOVA. (B) LTP. Plasticity induced by high frequency stimulation was recorded in the CA1 region of ventral hippocampal slices from WT and GSK3 KI mice. Waveforms are representative averages of 10 fEPSPs from 5 min before (solid line) and 30 minutes after (dotted line) tetanus. Bar graph represents potentiation 30 min after tetanus. (C) LTD. Plasticity induced by low frequency stimulation. Waveforms are representative averages of 10 fEPSPs from 5 minutes before (solid line) and 30 min after (dotted line) low frequency stimulation. Bar graph represents changes from baseline (dotted line) 30 min after stimulation. *p<0.05, Student's t test, n=6-7 per group.
Figure 6. GSK3 serine-phosphorylation is low in brains of learned helpless mice. (A) Cerebral cortical and hippocampal extracts from WT mice were immunoblotted for the indicated proteins. Abbreviations for treatments are: NN=No inescapable shock, No escapable shock (control condition); S0=inescapable shock, sacrifice immediately (0 min); NS=No inescapable shock, followed by escapable shock, sacrifice immediately; SS=learned helplessness paradigm with inescapable shock followed by escapable Shock. Means±SEM, n=4-7, *p<0.05 vs NN. (B) Hippocampal extracts from WT mice were immunoblotted for phospho-Thr308-Akt, phospho-Ser473-Akt, total Akt, phospho-Thr202/Tyr204-ERK1/2 (PERK1/2), total ERK1/2, phospho-Thr183/Tyr185-JNK (P-JNK), total JNK, phospho-Thr180/Tyr182-p38 (P-p38), total p38, phospho-Ser380-PTEN (P-PTEN), total PTEN, and β-actin. (C) Hippocampal extracts from untreated WT and GSK3 KI mice, (D) after exposing WT and GSK3 KI mice to escapable shock, and (E) with the GSK3 KI mice differentiated into non-helpless (NH) or helpless (HL) groups, were immunoblotted for the indicated proteins. n=4-7; Means±SEM; *p<0.05 by Student’s t-test.
Figure 7. GSK3 serine phosphorylation in PBMCs of bipolar disorder patients. (A) Representative immunoblots of phospho-Ser21-GSK3 phospho-Ser9-GSK3 and total GSK3 in PBMCs from healthy controls (HC) and bipolar disorder (BD) patients. (B,C) quantification of phospho-Ser21-GSK3 and phospho-Ser9-GSK3 Values are phospho-Ser-GSK3 as % of total GSK3 in each individual subject. n=10 (HC) and 17 (BD), Means±SEM; *p<0.05 by Student’s t-test. (D) Pearson correlation analysis between PBMC phospho-Ser21-GSK3 or phospho-Ser9-GSK3 and total Young Mania Rating Scale (YMRS) scores in manic/hypomanic patients. (E) Pearson correlation analysis between PBMC phospho-Ser21-GSK3 or phospho-Ser9-GSK3 and total Montgomery-Asberg Depression Rating Scale (MADRS) scores in depressed patients. n=11 in mania; n=6 in depression.
Supplemental Table 1. Inescapable foot shock protocols for learned helplessness paradigms.

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Supplemental Table 2. Demographic and clinical information of human subjects

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*Means ± SD  
YMRS: Young Manic Rating Scale  
MADRS: Montgomery-Asberg Depression Rating Scale  
BD-CGI-S: Clinical Global Impression-Severity for Bipolar Disorder
**Supplemental Figure 1.** GSK3 in WT and GSK3 KI mouse brains. Immunoblots showing that serine phosphorylation (PS) of GSK3 and GSK3 is absent in the striatum, cerebral cortex, hippocampus, and cerebellum of GSK3 KI mice, whereas tyrosine-phosphorylated (PT) and total levels of GSK3 and GSK3 are equal in GSK3 KI and WT mice.
Supplemental Figure 2. Sensory tests in GSK3 KI and WT mice. (A) Shock response thresholds evaluated for flinching, jumping, and vocalization were equivalent for GSK3 KI and WT mice. (B) There was not a significant difference in the latencies to lift a foot from a hot plate for the GSK3 KI and WT mice.
Supplemental Figure 3. Baseline synaptic transmission is equivalent in WT and GSK3 KI mice. (A) Baseline synaptic transmission and (B) paired pulse ratio were recorded in the CA1 region of ventral hippocampal slices.
Supplemental Figure 4. Plasma corticosterone levels after learned helplessness in GSK3 KI and WT mice. (A) Mice were subjected to inescapable shock (IES) with or without chronic lithium pretreatment. Plasma corticosterone levels were significantly higher in GSK3 KI mice than WT mice after IES, and were lowered in GSK3 KI mice by lithium pretreatment. (B) GSK3 KI mice were separated into non-helpless (NH) and helpless (HL) by escapable shock test (ES). Plasma corticosterone levels in HL had a tendency to be higher than in NH mice. *p=0.06.
Supplemental Figure 5. Amphetamine reduced serine phosphorylation of GSK3 in WT mice. Amphetamine (Amph; 2 mg/kg; 90 min) treatment decreased the inhibitory serine phosphorylation of both isoforms of GSK3 in the striatum of WT mice. Total levels of GSK3 (α and β) were unaltered by amphetamine administration. n=4; Means±SEM; *p<0.05 compared with saline (Sal)-treated mice by Student’s t-test.
**Supplemental Figure 6.** Quantitation of immunoblot results shown in Figure 6B. Immunoblots for phospho-Thr202/Tyr204-ERK1/2 (P-ERK1/2), total ERK1/2, phospho-Thr183/Tyr185-JNK (P-JNK), total JNK, phospho-Thr180/Tyr182-p38 (P-p38), total p38, phospho-Ser380-PTEN (P-PTEN), and total PTEN are quantified. n=3; Means±SEM.
CONCLUSIONS

GSK3 is a multifaceted protein kinase that has received considerable attention recently for its role in cellular processes such as gene expression, metabolism, and synaptic plasticity, and several pathological conditions including mood disorders. In the brain, as GSK3 is dynamically regulated by a number of neurotransmitters and neurotrophic factors, it is uniquely poised to play an important role in regulation of mood (Li and Jope 2010). Regulation of GSK3 often occurs through alteration of N-terminal inhibitory phosphorylation of Serine 21/9 on the α and β isoforms, respectively. While numerous studies have investigated the role of general increases or decreases in GSK3α and β activity in mood regulation using pharmacological inhibitors, gene knock-out, and transgenic overexpression of GSK3, fewer have looked specifically at the role serine phosphorylation of GSK3 plays in either the treatment or the underlying pathophysiology of mood disorders. In this study, we sought to address these questions.

Several previous studies have shown that pharmacological treatments of mood disorders, including lithium (Chalecka-Franaszek and Chuang 1999; De Sarno et al 2002; Li et al 2007a), antidepressants (Beaulieu et al 2008b; Li et al 2007b; Li et al 2004; Okamoto et al 2010), and atypical antipsychotics (Alimohamad et al 2005; Li et al 2007b) increase serine phosphorylation of GSK3, suggesting that this is an important mechanism in mood regulation. First, we investigated the signaling pathway responsible for regulation of GSK3 by antidepressants and increased synaptic serotonin. Secondly, we investigated the mechanism of GSK3 regulation by two serotonin receptors that are crucial to antidepressant and atypical antipsychotic responses, 5-HT1A and 5-HT2A receptors. Finally, we used genetically modified mice that are immune to N-terminal serine phosphorylation to
investigate the requirement of this phosphorylation for behaviors controlled by serotonin receptors.

Previous studies by our and other groups have indicated that acute increases in serotonin induce Ser9/21-phosphorylation of GSK3 in the rodent brain (Li et al 2004). One major unanswered question about serotonin mediated inactivation of GSK3 is the upstream kinase responsible for the regulation. Numerous protein kinases have been shown to regulate GSK3 (Fang et al 2000; Goode et al 1992; Stambolic and Woodgett 1994), but one of the most notable of these is Akt (Cross et al 1995). Akt is primarily associated with growth factor signaling (Sale and Sale 2008), which is activated by phosphorylation through Phosphoinositide-3-Kinase (PI3K). Increasing evidence suggests that Akt is also regulated by neurotransmitter receptors via either traditional PI3K-dependent and β-arrestin2-dependent mechanisms (Beaulieu et al 2007). Considerable work has shown that Akt and subsequent regulation of GSK3 is a major mediator of dopamine receptor signaling (Beaulieu et al 2007), but little is known about the role of Akt in serotonin signaling. Several studies in C. elegans (Liang et al 2006) and cultured cells (Cowen et al 2005; Hsiung et al 2005; Hsiung et al 2008) have shown that serotonin receptors are capable of activating Akt signaling. To test if serotonin could activate Akt in the mammalian brain, we treated mice with d-Fenfluramine, an agent that increases synaptic serotonin through inhibiting its reuptake and potentiating its release. We found that serotonin increased phosphorylation of Akt in the hippocampus, cortex, and striatum of the mammalian brain, the same regions that we have previously reported increases in Ser9-phosphorylation of GSK3β by d-Fenfluramine (Li et al 2004). Thus, serotonin increases Ser9/21-phosphorylation of GSK3 and activates its upstream regulating kinase Akt in a similar temporal and spatial pattern.
To confirm the functional consequences of the serotonin-induced increase in Akt activity, we investigated inactivation of downstream targets of Akt, the FoxO transcription factors. These transcription factors have long been implicated in apoptosis and resistance to cellular stressors (Hedrick 2009; Lam et al 2006; Sedding 2008), and more recently have been suggested to play an important role in the functioning of the brain. Phosphorylation of FoxO1 and FoxO3a by Akt results in translocation of the transcription factors from the nucleus to the cytosol, preventing their binding to DNA (Brunet et al 1999). Serotonin has been shown to inhibit FoxO analogs in *C. elegans* (Liang et al 2006), but no previous studies have shown this regulation in the mammalian brain. We found that potentiating serotonin signaling with d-Fenfluramine resulted in an increase in Akt-mediated phosphorylation of FoxO1 and FoxO3a, as well as a decrease in nuclear levels of FoxOs. Thus, enhancing brain serotonergic activity results in a functional activation of Akt and alterations of its downstream targets, making it a feasible candidate to phosphorylate and inactivate GSK3. Regulation of Akt, FoxO1, and FoxO3a by serotonin is dependent on PI3K, indicating that serotonin activates Akt through the traditional growth factor coupled pathway. Serotonergic activation of Akt through the PI3K pathway has previously been demonstrated in cells (Cowen 2007), but this is the first report of it in the mammalian nervous system.

In addition to regulation by acute increases in serotonin, we have also demonstrated that chronic treatment of mice with the tricyclic antidepressant imipramine increases phosphorylation of Akt and subsequent inhibition of FoxO1 and FoxO3a in the cortex, hippocampus, and striatum. Although the specific mechanism of this action is unknown, chronic antidepressant treatment is known to cause sustained increase in serotonin release (Blier 2001), and to increase levels of the neurotrophic factor BDNF (Nibuya et al 1995),
both of which are regulators of PI3K and Akt (Johnson-Farley et al. 2006; Yamada et al. 2001). Studies in FoxO3a and FoxO1 null mice show that decreasing the activity of these molecules can have beneficial effects on mood and anxiety, suggesting that activation of the PI3K/Akt pathway plays an important role in the effects of antidepressants.

Serotonin signals through a remarkably large and diverse family of receptors to control a variety of functions (Bockaert et al. 2006). While activation of some of these receptors is necessary for the effects of antidepressants, activation of others are certainly responsible for some of the side effects of antidepressants and may in fact be detrimental towards the resolution of depression. Identification of specific pathways regulated by serotonin receptor subtypes and associated signal pathways involved in regulation of Akt and GSK3 may provide a mechanism by which to improve treatments.

5-HT1A receptors are among the best-characterized serotonin receptors, and have been strongly implicated in depression and antidepressant response (Savitz et al. 2009). Previous studies in our lab have shown that activation of 5-HT1A receptors increases inhibitory serine phosphorylation of GSK3β in the mouse brain, and that a 5-HT1A receptor antagonist prevents the increase in serine phosphorylated GSK3β induced by d-fenfluramine (Li et al. 2004). The mechanism of this regulation, however, has thus far remained unknown.

To study the mechanism of GSK3 regulation by 5-HT1A receptors, we focused on the hippocampus. 5-HT1A receptors are highly expressed in the hippocampus (Chalmers and Watson 1991), and this region has been implicated in cognition and depression, and identified as crucial for the behavioral response to antidepressants. As previously shown, 5-HT1A receptor activation increased Ser9-phosphorylation of GSK3β in the hippocampus. Activation of 5-HT1A receptors also increased Ser21-phosphorylation of GSK3α, although
to a lesser extent than the GSK3β. Thus, 5-HT1A receptors inhibit both isoforms of GSK3 in the hippocampus. 5-HT1A receptor activation also leads to activation of Akt, and 5-HT1A receptor-induced phosphorylation of Akt and both isoforms of GSK3 was blocked by intrahippocampal injection of a PI3K inhibitor. Immunohistochemistry reveals strong colocalization between activated Akt and inactivated GSK3β in the hippocampus. Therefore, 5-HT1A receptors regulate GSK3β through PI3K-dependent activation of Akt. While lack of a suitable antibody did not allow immunohistochemistry of phosphorylated GSK3α to examine colocalization between this isoform and phosphorylated Akt, this phosphorylation was inhibited by the PI3K inhibitor. It is likely that GSK3α and β are regulated by the same pathway in the hippocampus in response to 5-HT1A receptor activation.

Although PI3K is canonically associated with growth factor receptor signaling, evidence also indicates that GPCRs can activate PI3K to affect downstream signaling (Murga et al 2000). Studies in primary hippocampal neurons (Cowen et al 2005) and transfected CHO cells (Hsiung et al 2005) show that 5-HT1A receptors are capable of activating PI3K through G-protein dependent activation of ras/raf signaling. Thus, it is feasible that 5-HT1A receptors located on hippocampal neurons directly activate PI3K to lead to increases in Akt and GSK3 phosphorylation. Alternatively, 5-HT1A receptors may activate PI3K through an indirect mechanism. Through their activation of G-protein coupled inwardly rectifying potassium (GIRK) channels (Andrade and Nicoll 1987; Araneda and Andrade 1991) and inhibition of calcium channels (Penington and Kelly 1990), 5-HT1A receptors can have a profound effect on neuronal firing and post-synaptic responses. Thus, PI3K could be activated by indirect effects of 5-HT1A receptors on neurotransmission in the hippocampus. This possibility is supported by our immunohistochemistry showing that the strongest
regulation of GSK3 and Akt by 5-HT1A receptors occurs in the CA3 region of the hippocampus, despite a lower level of 5-HT1A receptor expression in this subregion than CA1 and the dentate gyrus (Chalmers and Watson 1991).

Since 5-HT1A receptors strongly inhibit GSK3α and β in the hippocampus, we next investigated whether this regulation was required for behaviors that are modified by 5-HT1A receptors. As 5-HT1A receptors are involved in regulation of fear (Klemenhagen et al 2006; Tsetsenis et al 2007), we used the common behavioral paradigm of fear conditioning. In this test, animals are trained to associate an auditory cue and a context with an aversive footshock. Cued fear conditioning is primarily dependent on the amygdala and is independent of the hippocampus, while contextual fear conditioning is primarily dependent on the hippocampus. Both cued and contextual fear conditioning are inhibited by administration of the 5-HT1A receptor agonist 8-OH-DPAT (Stiedl et al 2000). To test if serine phosphorylation of GSK3 α and β is an intermediate of 5-HT1A receptor-regulated fear memory, we utilized GSK3α and β knock-in mice. GSK3α knock-in mice have normal levels of total GSK3α and β, as well as normal levels of phospho-Ser9-GSK3β, but have an S21A mutation that prevents phosphorylation of GSK3α. Similarly, GSK3β knock-in mice have normal expression of total GSK3α and β and phospho-Ser21-GSK3α, but have a S9A mutation that prevents Ser9-phosphorylation of GSK3β. Using these animals, we find that phosphorylation of GSK3β is necessary for 5-HT1A receptor-mediated inhibition of contextual fear memories, as contextual fear memory is not inhibited by 8-OH-DPAT in GSK3β knock-in mice. It is currently unclear how inhibition of GSK3 may contribute to reduction of fear expression induced by 5-HT1A receptors. Untreated GSK3β knock-in animals express normal contextual fear conditioning, suggesting that the effect of GSK3β has
to do specifically with 5-HT1A modulation of fear memory rather than with fear memory itself. Given the role of learned fear in depression and anxiety disorders such as PTSD, this avenue of study may yield highly specific targets for treatment of these disorders.

Interestingly, the behavioral response to 8-OH-DPAT in the contextual test was identical to wild-type animals in GSK3α knock-in mice. This suggests that this isoform is not necessary for 5-HT1A receptor inhibition of fear memories, although it is possible that GSK3β is able to compensate for the function normally played by GSK3α in these animals. Furthermore, both GSK3α and GSK3β respond to 8-OH-DPAT identically to wild-type animals in the cued fear conditioning test. This is in accordance with previous work showing that inhibition of GSK3 in the amygdala promotes, rather than inhibits, the expression of cued fear memories (Maguschak and Ressler 2008). This suggests that the necessity of GSK3β for 5-HT1A receptor regulation of fear conditioning is specific to the hippocampus. While regionally specific functions of GSK3 have not been examined to date, this would be an intriguing avenue of study. Together, our studies suggest that 5-HT1A receptors strongly regulate GSK3 in the mammalian hippocampus through activation of the traditionally growth factor-regulated PI3K/Akt pathway. 5-HT1A receptor-mediated inhibition of GSK3β, but not GSK3α, is necessary for 5-HT1A receptor-mediated inhibition of contextual fear conditioning.

Another serotonin receptor involved in mood disorders and their treatment is the 5-HT2A receptor. These receptors have been shown to generally oppose 5-HT1A receptor signaling (Yuen et al 2008). Indeed, our previous results show that while activation of 5-HT2A receptors has no significant effect on GSK3, antagonism of 5-HT2 receptors resulted in a dramatic increase in serine phosphorylation of GSK3. Furthermore, 5-HT2 antagonism
in combination with a 5-HT1A agonist increases phosphorylation of GSK3 compared to either agent alone (Li et al 2004). The precise mechanism of regulation of Akt and GSK3 by antagonizing 5-HT2A receptors is unknown. As MDL 11, 939 is an antagonist of the 5-HT2A receptor, it should not initiate any G-protein related signaling. Therefore, activation of Akt likely occurs through either interruption of constitutive activity of 5-HT2A-induced inhibition of Akt and thus activation of Akt, or through changes in scaffolding proteins induced by ligand binding to the receptor. Regulation of Akt signaling by GPCRs is often mediated by the scaffolding protein β-arrestin2 (Beaulieu et al 2009), and β-arrestin2 has previously been shown to direct signaling of 5-HT2A receptors to ERK. In our results, however, we find that regulation of Akt and GSK3 by 5-HT2A receptor antagonists is β-arrestin2-independent. This leaves several additional possibilities for activation of Akt by 5-HT2A receptor antagonists. Additional scaffolding proteins, such as PSD-95, may be responsible for increasing Akt activation in response to 5-HT2A receptor antagonists, as PSD-95 has been shown to interact with 5-HT2A receptors and alter signaling (Xia et al 2003). Another possibility is that activation of Akt comes through disruption of 5-HT2A receptor activation of yet-to-be-identified protein phosphatases which dephosphorylate and inactivate Akt. Alternatively, 5-HT2A receptor activation leads to changes in excitatory and inhibitory neurotransmission (Aghajanian and Marek 1999; Marek and Aghajanian 1999; Zhou and Hablitz 1999) that may promote loss of GSK3 serine phosphorylation. Disruption of 5-HT2A receptor regulation of neurotransmission may thus lead to increases in serine phosphorylation of GSK3.

In contrast to the 5-HT2A antagonist, the effect of the 5-HT2A agonist DOI is dependent on β-arrestin2. While we see no increase in phosphorylated GSK3 in wild-type
animals in response to DOI, in β-arrestin2 knock-out animals DOI induces a dramatic increase in phosphorylated GSK3. This is not accompanied by an increase in phosphorylated Akt, so it is unlikely that this increase is mediated by Akt. As 5-HT2A receptors activate PKC (Takuwa et al 1989), and PKC can phosphorylate and inhibit GSK3 (Goode et al 1992), it is possible that 5-HT2A receptors inhibit GSK3 through activation of PKC. β-arrestin2 thus serves as an endogenous brake on the inhibition of GSK3 by 5-HT2A.

Interestingly, 5-HT2A receptor antagonist treatment has a stronger effect on GSK3β than GSK3α. Little is known about the differences between the two isoforms because there are no small molecule inhibitors that affect one isoform but not the other. Additionally genetic manipulation of one isoform may be complicated by the ability of the other to compensate for it. However, several studies have conducted head-to-head comparisons of function of GSK3α versus GSK3β (Chen et al 2007b; Hoeflich et al 2000; Liang and Chuang 2006), and it is possible that GSK3β plays a more distinct role in 5-HT2A signaling than GSK3α.

In wild type animals, our data suggests that a general increase in serotonin such as that induced by an SSRI would induce phosphorylation of GSK3 primarily through activation of 5-HT1A receptors, and that this would be mildly inhibited by activation of 5-HT2A receptors. In the absence of β-arrestin2, 5-HT2A receptors couple to inhibition of GSK3. Likewise, we find that β-arrestin2 knock-out mice show enhanced phosphorylation of GSKβ in the cortex and striatum of in response to fluoxetine. Little enhancement of GSK3α phosphorylation was seen in β-arrestin2 knock-out mice, suggesting this effect is specific to GSK3β. Interestingly, no significant enhancement of phosphorylated GSK3β was seen in the hippocampus of β-arrestin2 knock-out mice. This is unlikely due to regional differences in
β-arrestin2’s signaling from 5-HT2A receptors to GSK3 since DOI induces a robust increase in phosphorylated GSK3 in the hippocampus of β-arrestin2 knock-out mice. It is more likely that phosphorylation of GSK3 in the hippocampus is saturated due to the strong effect of 5-HT1A receptors there.

Given that fluoxetine mediated inhibition of GSK3 is enhanced in β-arrestin2 knock-out mice, we hypothesized that inhibition of GSK3 is important for the behavioral effects of fluoxetine. Our result support this hypothesis because β-arrestin 2 knock-out mice have a significant potentiation of fluoxetine-induced reduction in immobility in the forced swim test, indicating an enhanced antidepressant effect. To confirm the importance of GSK3β phosphorylation in the behavioral effects of fluoxetine, we tested GSK3β knock-in animals in the forced swim test. These animals express GSK3β that cannot be phosphorylated. We found that while these mice exhibited no baseline changes in immobility from wild-type, they showed no behavioral response to fluoxetine. Thus, phosphorylation of GSK3β is necessary for the antidepressant effect of fluoxetine in an animal model of depression. The necessity of GSK3β phosphorylation for the antidepressant effects of fluoxetine agrees with previous studies suggesting that inhibition of GSK3 is sufficient to induce an antidepressant response (Beaulieu et al 2008b; Gould et al 2004; Kaidanovich-Beilin et al 2004; O'Brien et al 2004; Rosa et al 2008).

The opposing effects of 5-HT1A and 5-HT2A receptors on GSK3 are in line with several studies showing that these two receptors are often in opposition to each other (Amargos-Bosch et al 2004; Celada et al 2004; Gudelsky et al 1986; Yuen et al 2008). Indeed, this oppositional effect has been suggested to underlie some of less-than optimal efficacy of antidepressants (Celada et al 2004), as a general increase in serotonin will activate
both receptors. Antagonism of 5-HT2A receptors has shown promise as an adjunctive therapy for treatment resistant depression (Marek et al 2003; Ostroff and Nelson 1999; Shelton et al 2001). The results of our studies suggest that GSK3 may be a crucial molecule that is regulated by 5-HT1A and 5-HT2A receptors. Furthermore, β-arrestin2 serves as an endogenous brake on 5-HT2A signaling, preventing it from coupling to inhibition of GSK3. Knock-out of β-arrestin2 removes this brake and allows greater inhibition of GSK3. This raises the interesting possibility that inhibitors of interactions between 5-HT2A receptors, β-arrestin2, and GSK3 may serve as useful adjunctive therapy for depressed patients, and may speed the onset of recovery. Although no such pharmacological agents currently exist, this is the type of highly specific target that future drug discovery will likely focus on. Interestingly, loss of β-arrestin2 has been shown to reduce the actions of chronic antidepressant treatment (David et al 2009). While our studies have only addressed acute treatments, this divergence is quite interesting. It suggests that disruption of β-arrestin2/GSK3 signaling may be beneficial in early phases of treatment, but that long-term targeting of this pathway may be detrimental. Thus, we have shown that GSK3 is dynamically regulated by serotonin receptors in the mammalian brain, and this regulation plays an important role in the behavioral effects of serotonergic agents.

While conclusive evidence seems to exist that GSK3 plays an important role in treatment of mood disorders, few studies have investigated whether dysregulation of GSK3 itself can lead to altered mood. Seeing as how alterations in levels of neurotrophic factors and monoaminergic neurotransmission may occur in the brains of mood disorder patients, this may lead to decreases in the levels of phosphorylated GSK3. In fact, our studies show decreased phosphorylation of GSK3 in response to inescapable stress or psychostimulants,
common models used to induce depressive- and manic-like states in animals. Furthermore, peripheral blood cells from untreated bipolar patients exhibit decreased phosphorylation of GSK3, a decrease which strongly correlates with the severity of symptoms. To mimic this state, we tested GSK3α/β knock-in mice in a variety of behavioral paradigms. These mice exhibit normal levels of GSK3α and GSK3β, but have N-terminal mutations replacing serine 21/9 with an alanine. This prevents phosphorylation of GSK3 by upstream kinases such as Akt (McManus et al 2005). Despite the constitutive activity of GSK3α and β, these animals exhibit no confounding overt phenotype, making them ideal for the study of behavior.

GSK3α/β knock-in mice show increased anxiety and increased susceptibility to both stress-induced depressive behavior and psychostimulant-induced manic-like behavior. Thus, phosphorylation of GSK3 is necessary for maintenance of mood, and decreases in this phosphorylation may play a role in the pathology of mood disorders. The presence of both manic and depressive-like behaviors in a single animal is rare; suggesting that phosphorylation of GSK3 may play a unique role in the pathophysiology of mood disorders. These animals have the potential to be highly useful as a model of bipolar disorder. Future studies examining deficiencies in signaling and neuronal function in these animals, and the ability to normalize behavior by rescuing these deficits will yield valuable information about the processes that underlie abnormal regulation of mood.

Bipolar disorder is frequently treated with the mood stabilizer lithium, which is a selective inhibitor of GSK3. This inhibition is both direct, through competition for a magnesium binding site (Klein and Melton 1996; Stambolic et al 1996), and indirect (Chalecka-Franaszek and Chuang 1999; De Sarno et al 2002; Li et al 2007a), through increasing phosphorylation of GSK3. Maximal direct inhibition of GSK3 by lithium occurs
at concentrations above the effective therapeutic dose, leading to speculation that the indirect
effect on GSK3 phosphorylation is necessary for the therapeutic effect of lithium. GSK3
knock-in mice provide a unique model to test this hypothesis, as they express normal levels
of GSK3 protein that is capable of being directly inhibited by lithium but lack the ability to
be indirectly regulated by phosphorylation. We found that GSK3 knock-in mice exhibited
only a partial reduction of amphetamine-induced hyperactivity in response to chronic lithium
treatment, suggesting that phosphorylation of the protein is necessary for a full therapeutic
effect.

The next step in these studies is to thoroughly investigate how GSK3 has its effects
on mood. GSK3 regulates a number of signaling pathways and functions that have been
linked to regulation of mood. One such function is regulation of gene expression. Several
transcription factors regulated by GSK3 have been shown to play a role in mood. Two
examples of this are β-catenin and CREB, transcription factors that are inhibited by GSK3.
Increased activity of both molecules has been shown to promote an antidepressant response
in animal models (Chen et al 2001; Gould et al 2007), and overexpression of β-catenin has an
antimanic effect against amphetamine induced hyperactivity (Gould et al 2007). As GSK3
negatively regulates these molecules, the antidepressant and antimanic effect of GSK3
inhibition may be due to releasing this inhibition and enhancing the function of CREB and β-
catenin.

Although clear evidence exists in favor of CREB and β-catenin playing a role in
treatment of mood, there is minimal evidence that they play a role in the pathogenesis of the
disease. Forebrain-specific down-regulation of β-catenin leads only to a mild depressive
phenotype (Gould et al 2008). Similarly, no overt depressive or manic-like phenotype is seen
in animals with reduced expression of CREB; in some brain regions loss of CREB signaling can actually induce an antidepressant response (Blendy 2006; Conti et al 2002; Newton et al 2002). Thus, regulation of CREB and β-catenin are likely candidates as downstream targets of GSK3 involved in the antidepressant and antimanic effects of GSK3 inhibition but are unlikely to be involved in the pathogenesis of mood disorders.

Another area of particular interest is the regulation of neuronal plasticity. Deficits in plasticity have been hypothesized to underlie mood disorders (Einat and Manji 2006; Krishnan and Nestler 2008), and GSK3 is a crucial mediator of synaptic plasticity in the rodent brain (Peineau et al 2008). To begin a study of this in GSK3 knock-in mice, we chose to investigate hippocampal synaptic plasticity, as the hippocampus is strongly implicated in mood disorders. We found that GSK3 knock-in mice exhibit selective deficits in NDMA-dependent long-term depression, a form of synaptic plasticity in which low-frequency stimulation of synapses results in a decrease in the amplitude of synaptic responses. In GSK3 knock-in mice, low-frequency stimulation did not lead to depression of synapses, but to a slow-onset moderate potentiation of synapses. This is similar to an effect seen in the ventral hippocampus of stressed rats (Maggio and Segal 2009), suggesting that it may be associated with the increased susceptibility to stress of the GSK3 knock-in mice. While it is unclear what role this altered plasticity plays in behavior, it demonstrates that the hippocampus of GSK3 knock-in mice exhibits inappropriate responses to certain stimuli. Future studies linking this plasticity to behavior, and examining plasticity of neurons in other mood-related brain regions, such as the ventral tegmental area and the prefrontal cortex, will provide conclusive information about the role GSK3-mediated regulation of synaptic plasticity plays in mood.
Recent studies have also indicated that GSK3 can regulate G-protein coupled serotonin receptors. Phosphorylation of the 5-HT1B receptor by GSK3β is required for receptor activity (Chen et al 2009). Although the precise function of this regulation is not yet known, 5-HT1B receptors have been strongly implicated in depression and psychostimulant addiction, and thus regulation of their activity may play an important role in regulation of manic-like and depressive-like behaviors. Furthermore, mechanisms of regulation of GPCRs are highly conserved, and thus it is likely that GSK3 can also regulate activity of a number of other receptors. Regulation of neuromodulatory GPCR receptors would give GSK3 a vast array of targets that can profoundly affect mood.

In addition to identifying substrates of GSK3 involved in mood regulation, other important questions to be answered include the temporal and spatial specificity of GSK3’s effect on mood. The GSK3 knock-in mice used in these studies express mutated GSK3 in the entirety of their body throughout development. As upstream regulators of GSK3 such as BDNF have divergent effects on mood in different brain regions (Berton et al 2006; Monteggia et al 2007), it is likely that GSK3 activity may have regionally distinct effects on mood-related behaviors. Similarly, hyperactivation of GSK3 throughout development may result in lasting alterations in circuits regulating mood. Future studies using inducible or conditional knock-in strategies to limit constitutive activity of GSK3 to distinct brain regions and developmental periods will be useful in further defining the role GSK3 plays in the pathophysiology of mood disorders.

GSK3 is uniquely poised to play an important role in mood disorders. Our studies have characterized how phosphorylation and inhibition of GSK3 is a crucial step for the actions of mood stabilizers and antidepressants, and how dysregulation of this
phosphorylation leads to profound behavioral deficits. Taken together, the data shown in these studies indicates that GSK3 is a crucial modulator of mood.
LIST OF GENERAL REFERENCES


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DATE: October 12, 2010

TO: Li, Xiaohua  
Simolian Psychiatric Clinic 213 0018  
934-1169

FROM: Judith A. Kapp, Ph.D., Chair  
Institutional Animal Care and Use Committee

SUBJECT: Title: GSK3: neuromodulators and mood disorders  
Sponsor: NIH  
Animal Project Number: 101007651

On October 12, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
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<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
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<td>Mice</td>
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Animal use is scheduled for review one year from October 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 101007651 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7662.