2014

Contribution of hyperactive glycogen synthase kinase-3 (GSK3) to impaired neurogenesis and cognition in mice

Margaret Kimbrough King
University of Alabama at Birmingham

CONTRIBUTION OF HYPERACTIVE GLYCOGEN SYNTHASE KINASE-3 (GSK3) TO IMPAIRED NEUROGENESIS AND COGNITION IN MICE

by

MARGARET KIMBROUGH KING

RICHARD S. JOPE, CO-CHAIR
RAJESH K. KANA, CO-CHAIR
MARY M. BOGGIANO
DAVID C. KNIGHT
XIAOHUA LI

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2013

CONTRIBUTION OF HYPERACTIVE GLYCOGEN SYNTHASE KINASE-3 (GSK3) TO IMPAIRED NEUROGENESIS AND COGNITION IN MICE

Margaret Kimbrough King

PSYCHOLOGY

ABSTRACT

The overall goals of this research were to examine the regulatory actions of glycogen synthase kinase-3 (GSK3) in adult mouse hippocampal neurogenesis and in mouse cognitive functions in order to gain further insight regarding the function of GSK3 in the healthy and diseased central nervous system. Focusing on differences between male and female mice, we found that hippocampal neurogenesis was impaired by hyperactive GSK3 in both sexes, but was improved by environmental enrichment in male, but not female, mice. Chronic stress reduced neurogenesis in male mice, but not in female mice. Environmental enrichment and chronic stress inhibited, and activated, respectively, GSK3 in male hippocampus but did not alter GSK3 in female hippocampus. Thus, environmental factors and GSK3 both regulate hippocampal neurogenesis, but do so differently in male and female mice.

The accumulating reports that inhibition of GSK3 using lithium or other specific GSK3 inhibitors ameliorates cognitive impairments in multiple disorders were reviewed, which was likely due to reducing several detrimental actions of GSK3 that impair cognition. We tested if GSK3 inhibitors ameliorate cognitive deficits in the mouse model of Fragile X syndrome (FXS), with deletion of the *fragile X mental retardation 1* (*Fmr1*) gene. Chronic lithium treatment during adolescence or adulthood ameliorated several cognitive impairments in *Fmr1* knockout mice. Withdrawal of lithium for four weeks reinstated the learning deficits in *Fmr1* knockout mice. To determine if the effect of

lithium on cognition was due to its inhibition of GSK3, *Fmr1* knockout mice were treated with two specific GSK3 inhibitors. We found that inhibition of GSK3, but not of metabotropic glutamate receptor-5, rescued learning in novel object detection, temporal ordering for objects, and coordinate and categorical spatial processing tasks. Thus, abnormally active GSK3 contributes to cognitive dysfunction in FXS, supporting GSK3 as a potential therapeutic target.

Overall, this project provides novel insights into the function of GSK3 in two neurologic processes and supports GSK3 as an important regulator of adult neurogenesis and of cognitive processes in FXS.

Keywords: Glycogen Synthesis Kinase 3, Lithium, Neurogenesis, Mood Disorders, Cognition, Fragile X Syndrome

ACKNOWLEDGEMENTS

I would like to acknowledge the faculty who served on my committee for their patience, guidance, support and encouragement throughout my graduate career, while I was in Birmingham and, especially, while I was in Miami: Dr. Rajesh Kana, Dr. David Knight, Dr. Mary Boggiano, and Dr. Xiaohua Li. I would like to thank Dr. Richard S. Jope for being my caring and dedicated mentor. From his knowledge of basic research to presentations to interview skills, Dr. Jope's depth and breadth of familiarity with all things pertaining to science continues to astound me. I am extremely appreciative and proud to call him my mentor and teacher. Dr. Jope is a fervent scientist, and I am grateful to him for instilling a sense of accomplishment in me.

I appreciate all of the past and present members of the Jope lab at The University of Alabama at Birmingham and at the University of Miami for all of their assistance and support throughout the years. I would like to acknowledge Dr. Ling Song for answering my seemingly endless questions and for helping me attempt to attain the "perfect Western blot" like she can so easily do. I would like to thank Dr. Eleonore Beurel for always being available to talk about experiments and techniques and for giving me words of encouragement along the way. I would especially like to thank Dr. Marjelo A. Mines for being a great co-worker, teacher, and friend who never fails to inspire confidence and fortitude in me.

I am very grateful to my family, especially my Mom, Dad, Cecilianne, and Sam, for always being excited for me and for trying to understand my research and its purpose.

Their encouragement and support have been invigorating for so many years. I would like to thank my friends for teaching me the value of time management, because these past few years would have been much harder without them. I would also like to thank Carson Moseley for his thoughtful insight, encouragement, excitement, and endurance throughout my graduate school career.

TABLE OF CONTENTS

LIST OF FIGURES

Figure *Page*

INTRODUCTION

REGULATORY DIFFERENCES IN HIPPOCAMPAL NEUROGENESIS IN ADULT MALE AND FEMALE MICE IN RESPONSE TO ENVIRONMENTAL ENRICHMENT, CHRONIC STRESS AND ACTIVE GLYCOGEN SYNTHASE KINASE-3

LIST OF TABLES

Table *Page*

REGULATORY DIFFERENCES IN HIPPOCAMPAL NEUROGENESIS IN ADULT MALE AND FEMALE MICE IN RESPONSE TO ENVIRONMENTAL ENRICHMENT, CHRONIC STRESS AND ACTIVE GLYCOGEN SYNTHASE KINASE-3

INTRODUCTION

Glycogen Synthase Kinase-3

Increasing evidence indicates that glycogen synthase kinase-3 (GSK3) is abnormally active in a number of diseases of the central nervous system (CNS), and this has raised interest in the therapeutic potential of GSK3 inhibitors (King et al., 2013). With this in mind, the overall goals of this project were to examine the potential roles of GSK3, and the effects of GSK3 inhibitors, in hippocampal neurogenesis in adult mice and in behavioral abnormalities displayed by mice with fragile X syndrome.

GSK3 is a broadly influential enzyme that regulates many cellular functions throughout the periphery and the CNS. Although it is expressed in all tissues, GSK3 levels are particularly abundant in the brain (Woodgett, 1990). GSK3 is a serine/threonine kinase that exists in two isoforms, GSK3α and GSK3ß (Woodgett, 1990). The two isoforms arise from independent genes and share nearly identical sequences in their kinase domains. GSK3ß is the predominant isoform in the CNS, as GSK3α is only expressed at ~25% the level of GSK3ß in mouse brain (Woodgett, 1990). GSK3 has more than 50 substrates, so GSK3 activity must be tightly regulated (Jope and Roh, 2006). The main way GSK3 is regulated is by phosphorylation on serine-21 of GSK3α and serine-9 of GSK3ß (Figure 1). Serine phosphorylation inhibits GSK3, reducing its activity. Several kinases are capable of mediating this modification, including Akt (also known as protein kinase B) (Cross et al., 1995), cyclic AMP-dependent protein kinase (also known as protein kinase A) (Fang et al., 2000; Li et al.,

2000), protein kinase C (Goode et al., 1992), and others, indicating that many signaling cascades converge on GSK3 to regulate its activity.

Impairments in the inhibition of GSK3 have been linked to several prevalent diseases of the CNS that may be treated with GSK3 inhibitors (King et al., 2013). These include schizophrenia, depression, bipolar mood disorder, and fragile X syndrome, which appear to exhibit alterations in signaling systems that normally regulate GSK3 (De Sarno et al., 2002; Li et al., 2004; Beaulieu et al., 2004; Karege et al., 2007; Min et al., 2009; Polter et al., 2010; Yuskaitis et al., 2010a). For example, as shown in Figure 2, the neurotransmitter serotonin (5-HT) and neurotrophins, such as brain-derived neurotrophic factor (BDNF), normally inhibit GSK3 activity (Mai et al., 2002; Li et al., 2004). There is evidence that deficient serotonin and deficient BDNF may occur in mood disorders. When serotonin and BDNF are deficient, they cannot induce signals to inhibit GSK3 sufficiently, which may contribute to the mood disorders depression and bipolar disorder. The inhibitory serine phosphorylation of both GSK3α and GSK3β is thought to be impaired in mood disorders. In vivo administration of the mood stabilizers lithium and valproate (De Sarno et al., 2002) or the antidepressants fluoxetine and imipramine (Li et al., 2004) increases inhibitory serine phosphorylation of GSK3 in mouse brain. Consistent with this, studies in human postmortem brain (Karege et al., 2007) and in brains of mice exhibiting depression-like behavior (Polter et al., 2010) have shown reduced inhibitory serine-phosphorylation of GSK3, suggesting that GSK3 is hyperactive during depression. The neurotransmitter dopamine (DA) activates GSK3. Increased dopaminergic activity may be involved in schizophrenia, and there is evidence that increased activation of GSK3 due to increased DA may be linked to schizophrenia

(Beaulieu et al., 2004). Abnormally active metabotropic glutamate receptor-5 (mGluR5) signaling, due to the absence of fragile X mental retardation protein (FMRP), may contribute to fragile X syndrome (Bear et al., 2004). Active mGluR5 signaling decreases the inhibitory phosphorylation of GSK3 and this may contribute to some of the abnormal behavioral and physiological symptoms of fragile X syndrome (Yuskaitis et al., 2010a).

Because of the potential involvement of hyperactive GSK3 in several diseases, there has been much research studying GSK3 inhibitors. The first identified inhibitor of GSK3 was lithium (Klein and Melton 1996). Lithium has been used as a primary treatment for bipolar mood disorder for over 60 years (Cade, 1949). The therapeutically relevant level of lithium is about 1 mM in human serum (Klein and Melton, 1996), and higher levels of lithium are toxic (Cade, 1949; Shorter, 2009). After Klein and Melton found that lithium inhibits GSK3 in vivo, the discovery that lithium inhibits GSK3 in vivo at therapeutically relevant levels (De Sarno et al., 2002) supported the possibility that inhibition of GSK3 might contribute to the mood-stabilizing effects of lithium (Li and Jope, 2010). Lithium directly inhibits GSK3 by competing for a magnesium binding site in the catalytic pocket of GSK3 (Ryves and Harwood, 2001), and lithium indirectly inhibits GSK3 by causing a large increase in the serine-phosphorylation of GSK3, further inhibiting GSK3 (De Sarno et al., 2002). Thus, lithium directly and indirectly inhibits GSK3, but the therapeutically relevant level of lithium only partially inhibits GSK3 (Klein and Melton, 1996). Since higher levels of lithium are toxic, higher doses cannot be used to cause greater inhibition of GSK3. Therefore, in order to test the validity of GSK3 as the therapeutic target of lithium in animal models of diseases, other selective, small molecule inhibitors of GSK3 are often used, such as TDZD-8 and VP0.7. TDZD-8 is a

highly selective ATP non-competitive inhibitor of GSK3 (Martinez et al., 2002), and it passes the blood brain barrier (Martinez, 2006; Beaulieu et al., 2008a). TDZD-8 has been used in vivo in mice, and its administration results in antidepressant-like behavior in mice (Beaulieu et al., 2008a; Beaulieu et al., 2008b; Kalinichev and Dawson, 2011; Lipina et al., 2011; Lipina et al., 2012). VP0.7 is an allosteric (not competitive with ATP or substrate) selective GSK3 inhibitor (Palomo et al., 2011) and has been used in vivo in mice (Beurel et al., 2013). Thus, both TDZD-8 and VP0.7 effectively inhibit GSK3 in rodent brain and can be used to test if they ameliorate behavioral impairments in mice similarly to lithium.

Another way to study the contribution of abnormal regulation of GSK3 to diseases is by using a GSK3 knockin mouse model. As discussed previously, the two isoforms of GSK3 are predominantly regulated by inhibitory phosphorylation on serine-21-GSK3α and serine-9-GSK3β (Figure 1). The importance of inhibitory control of GSK3 can be studied using homozygous GSK3$\alpha^{21A/21A}/\beta^{9A/9A}$ knockin mice, where the regulatory serines of both GSK3 isoforms are mutated to alanines (McManus et al., 2005). These mutations maintain GSK3 maximally active, but importantly within the physiological range since both GSK3 isoforms are expressed at normal levels. GSK3 knockin mice develop and reproduce apparently normally and show no overt phenotype. Therefore, GSK3 knockin mice and selective inhibitors of GSK3 provide tools to discern how GSK3 contributes to cellular functions and disease processes.

Adult Neurogenesis

Neurogenesis is the proliferation and differentiation of neural precursor cells (NPCs) to neurons. Although once thought to only occur during development, neurogenesis in adult mammals occurs throughout life in the hippocampus (Zhao et al., 2008). The subgranular zone of the dentate gyrus of the hippocampus harbors NPCs and provides a neurogenic niche. NPC proliferation is assessed by measuring the incorporation of 5-bromo-2'-deoxyuridine (BrdU), a synthetic analogue of thymidine, into DNA. The tissue is then fixed and stained with a BrdU antibody conjugated to a fluorophore to detect BrdU. Using a fluorescent microscope, the nuclei of cells that are labeled with BrdU are quantitated (del Rio and Soriano, 1989). Over time a subset of the new cells migrates into the granule cell layer and differentiates into neurons (Kempermann, 2002), and these neurons can be assessed by co-staining BrdU and neuronal markers. The purpose of the proliferation and differentiation of NPCs is unclear, but impaired adult hippocampal neurogenesis has been linked to several diseases of the CNS, including mood disorders.

Administration of lithium or antidepressants increases neurogenesis and this has been proposed to contribute to their therapeutic effects in mood disorders (Malberg et al., 2000; Manev et al., 2001; Czeh et al., 2001; Santarelli et al., 2003; Warner-Schmidt and Duman, 2007; David et al., 2009). As discussed above, lithium is used for the treatment of bipolar mood disorder, and it inhibits GSK3, in part by increasing the inhibitory serine-phosphorylation of GSK3. Antidepressant drugs, such as fluoxetine, a selective serotonin reuptake inhibitor, and imipramine, a tricyclic antidepressant that inhibits the reuptake of both serotonin and norepinephrine, also inhibit GSK3 by increasing its

inhibitory serine-phosphorylation in mouse brain (Li et al., 2004). Since hyperactive GSK3 may contribute to susceptibility to mood disorders, and since lithium and antidepressants increase neurogenesis (Chen et al., 2000; Malberg et al., 2000; Manev et al., 2001; Czeh et al., 2001; Hashimoto et al., 2003; Santarelli et al., 2003; Warner-Schmidt and Duman, 2007; Silva et al., 2008; Wexler et al., 2008; David et al., 2009), there may be relationships among susceptibility to mood disorders, hyperactive GSK3 and impaired neurogenesis. This was indicated by the finding that neurogenesis is impaired in GSK3 knockin mice (Eom and Jope, 2009) in which the normal inhibitory control of GSK3 is blocked. GSK3 knockin mice develop and reproduce apparently normally and show no overt phenotype, but prior research indicates that hyperactive GSK3 appears to increase vulnerability to mood disorders (Jope and Roh, 2006), which correlates with the reported decreased neurogenesis. This project extended this study to test if neurogenesis plasticity is also altered by the blocked inhibitory GSK3 serine-phosphorylation in GSK3 knockin mice, in addition to the reported reduction in basal neurogenesis (Eom and Jope, 2009).

Neurogenesis plasticity, the increase or decrease in proliferation of neuronal cells in the hippocampus, can be studied by using various experimental paradigms. Hippocampal neurogenesis is increased in male rodents by environmental enrichment (Komitova et al., 2005; Leal-Galicia et al., 2007; Zhao et al., 2008; Li et al., 2008; Hu et al., 2010; Chakrabarti et al., 2011), by certain neurotrophins (Dranovsky and Hen, 2006; Zhao et al., 2008), by exercise (Fabel et al., 2003; Stranahan et al., 2006; Glasper et al., 2010), by antidepressants (Malberg et al., 2000; Manev et al., 2001; Czeh et al., 2001; Santarelli et al., 2003; Warner-Schmidt and Duman, 2007; David et al., 2009), and by the

mood stabilizer lithium (Chen et al., 2000; Hashimoto et al., 2003; Silva et al., 2008). Environmental enrichment consists of several components that may contribute to stimulated hippocampal neurogenesis. The increased area (larger cage) and visual stimuli (toys) might increase physical activity that will result in cardiovascular stimulation, general arousal status (Brown et al., 2003), and increased expression of the neurotrophin insulin-like growth factor 1 (IGF-I), which reinforces the expression of brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) (Ding et al., 2006). Several neurotrophins, particularly IGF-1, BDNF, VEGF, and neurotrophin-3 (NT-3), promote neurogenesis (Smith et al., 1995; Dranovsky and Hen, 2006; Zhao et al., 2008). IGF-1, BDNF and VEGF also activate the signaling pathway that leads to inhibitory serine-phosphorylation of GSK3 by Akt (Farmer et al., 2004; Schmidt and Duman, 2007). Environmental enrichment and exercise also exert antidepressive-like effects in male rodents, including in the learned helplessness test (Duman et al., 2008; Greenwood and Fleshner, 2008; Salam et al., 2009), the novelty suppressed feeding task (Trejo et al., 2008; Huang et al., 2012a), the forced-swim test, the tail suspension test, and the sucrose preference paradigm (Brenes Sáenz et al. 2006; Duman et al., 2008; Green et al., 2010; Huang et al., 2012b).

Opposite to environmental enrichment, in male rodents, neurogenesis is decreased by chronic stress (Gould et al., 1992; Cameron and Gould, 1994; Duman et al., 2001; Pham et al., 2003; Falconer and Galea, 2003; Westenbroek et al., 2004; Charney and Manji, 2004; Mirescu et al., 2006; Shors et al., 2007; Koo et al., 2010; Hillerer et al., 2013). Chronic stress might decrease neurogenesis because stress activates the hypothalamic-pituitary-adrenal (HPA) axis, which elevates corticosterone, and

administration of corticosterone or dexamethasone decreases neurogenesis (Duman et al., 2001; Dranovsky and Hen, 2006). Chronic stress also increases the levels of some inflammatory cytokines in the brain that may modulate neurogenesis. Neurogenesis is impaired by several inflammatory molecules, such as the cytokines interleukin-6 (IL-6), IL-1β, and tumor necrosis factor-α (TNFα) (Monje et al., 2003; Iosif et al., 2008). Furthermore, GSK3 promotes neuroinflammation, so this action might contribute to impaired neurogenesis caused by hyperactive GSK3 (Martin et al., 2005; Beurel and Jope, 2009; Yuskaitis and Jope, 2009). Chronic stress and dysregulated glucocorticoids also increase susceptibility to depression in male rodents (Kendler et al., 1999; Murray et al., 2008), and decrease BDNF expression in the dentate gyrus (Smith et al., 1995). Deficient BDNF expression is implicated in mood disorders, and some studies report that chronic stress increases depressive-like behaviors in male rodents, such as in the learned helplessness test (Shors et al., 2007; Chiba et al., 2012), the forced-swim test, the tail suspension test, and the sucrose preference test (Kim and Han, 2006; Haenisch et al., 2009; Koo et al., 2010; Seo et al., 2012).

In sharp contrast to male mice, neither environmental enrichment nor chronic stress alters the proliferation of new cells in the hippocampus of female mice (Kempermann et al., 1997; van Praag et al., 1999; Brown et al., 2003; Falconer and Galea, 2003; Westenbroek et al., 2004; Mineur et al., 2007; Shors et al., 2007; Kobilo et al., 2011; Hillerer et al., 2013). Despite the fact that women are more likely than men to be diagnosed with depression, there are few reports of the effect of environmental enrichment or chronic stress on depressive-like behavior in female rodents. One study showed that female mice exposed to environmental enrichment do not exhibit

antidepressive-like behavior in the forced swim test or the sucrose preference test, although tail suspension test-induced hyperthermia, a physiological response to acute stress, is attenuated (Renoir et al., 2013). Another study reported that chronic stress did not increase learned helplessness depression-like behavior in female rodents (Shors et al., 2007).

In order to study the mechanisms and effects of neurogenesis plasticity, wild-type male and female mice were subjected to environmental enrichment or chronic stress. Then a potential endogenous regulator of neurogenesis, inhibitory serine-phosphorylation of GSK3, and proliferation, survival and differentiation of NPCs were measured. In order to study the effects of hyperactive GSK3 on neurogenesis plasticity, male and female GSK3 knockin mice were subjected to environmental enrichment or chronic stress. In male or female GSK3 knockin mice, a decrease, increase, or no change in neurogenesis due to environmental enrichment or chronic stress might be related to the expression of endogenous regulators of neurogenesis compared to wild-type mice. Since environmental enrichment and chronic stress oppositely regulate neurogenesis in male wild-type mice, and neurogenesis is impaired by activated GSK3, serine-phosphorylation of hippocampal GSK3 in wild-type mice might be increased by environmental enrichment and decreased by chronic stress. These studies aimed to elucidate some potential causal links between endogenous regulators of neurogenesis following environmental manipulation (enrichment or stress) in GSK3 knockin and wild-type mice.

Fragile X Syndrome

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability and the first known genetic cause of autism (Hagerman et al., 2010). The prevalence of FXS is 1 in 4000-5000 males and 1 in 2500-8000 females (Tassone et al., 2012) in the United States. The primary symptom of FXS is cognitive impairment, but several other behaviors are common, including social anxiety, attention deficit, speech and language impairments, seizures, and increased sensitivity to sensory stimuli (Berry-Kravis, 2002; Berry-Kravis and Potanos, 2004; Berry-Kravis et al., 2008b; Hessl et al., 2008; Wang et al, 2010). FXS is caused by loss of function of the *fragile X mental retardation 1* (*FMR1*) gene on the X chromosome (Pieretti et al., 1991). *FMR1* carries a CGG trinucleotide repeat in the 5' untranslated region, and normal alleles contain 5-50 CGG repeat units, premutation alleles contain 55-200 repeats, and alleles with 200 or more repeats are considered a full mutation. In FXS patients, the CGG repeat region is increased to more than 200 repeat units, which results in hypermethylation of this region and a lack of transcription of *FMR1* (Verkerk et al., 1991). The premutation can lead to fragile X associated premature ovarian insufficiency (FXPOI) in female carriers (Sherman, 2000) and adult-onset fragile X associated tremor/ataxia syndrome (FXTAS) (Berry-Kravis et al., 2007). Transcriptional silencing of *FMR1* causes the loss of the gene product, FMRP. FMRP is an mRNA binding protein that plays a regulatory role in activity-dependent mRNA functions, such as mRNA transport, stability and translation (Gross et al., 2011). The localization of FMRP is mostly cytoplasmic (Devys et al., 1993), and in neurons it is localized in dendrites and at synapses (Antar et al., 2004). FMRP has been shown to repress translation of individual target mRNAs (Zalfa et al.,

2003), but FMRP might also act as a translational activator for specific targets (Bechara et al., 2009). FMRP has been estimated to associate with up to 4% of all mRNAs in the brain (Brown et al., 2001), and these mRNA targets encode proteins important for various cellular mechanisms, including cytoskeletal regulation, synaptic structure and composition, and synaptic signal transmission (Zalfa et al., 2003). FMRP expression in the hippocampus is highest during the first week of postnatal development and is then maintained at a moderate level throughout development (Lu et al., 2004). The peak of FMRP expression coincides with a critical time point for synapse maturation, suggesting that high levels of FMRP are functionally required in translation-dependent synapse maturation (Lu et al., 2004).

Individuals with FXS display characteristic physical features, cognitive impairments, and behavioral abnormalities (Berry-Kravis, 2002; Berry-Kravis and Potanos, 2004; Berry-Kravis et al., 2008b; Hessl et al., 2008; Wang et al, 2010). Physical features are more evident in males than females and include macroorchidism, prominent ears and macrocephaly (Berry-Kravis and Potanos, 2004; Wang et al., 2010). FXS patients typically have developmental delays, particularly in speech, and hypotonia, which can affect feeding and swallowing (Berry-Kravis, 2002). Males with FXS typically exhibit intellectual disabilities, with an average IQ of 40-50 and mental age of 5-6 years in adults (Berry-Kravis, 2002). Cognitively, FXS patients have strengths in some areas, including visual memory, simultaneous processing and experiential learning, and weaknesses in areas such as auditory processing, sequential processing, working memory, and executive function and attention (Berry-Kravis et al., 2008b). Common behavioral characteristics of males with FXS include hyperactivity, impulsivity, attention

problems, anxiety, aggression, mood lability, and autistic features such as poor eye contact, shyness, hypersensitivity to sensory stimuli, and perseverative language and behavior (Berry-Kravis and Potanos, 2004; Hessl et al., 2008; Wang et al., 2010). Approximately 18-36% of males with FXS meet full criteria for autism and 43-67% of males with FXS have an autism spectrum disorder (Wang et al., 2010). FXS differs somewhat from typical autism because FXS patients have strong social interest but high levels of social anxiety and poor understanding of social cues (Berry-Kravis et al., 2010).

The *FMR1* gene is highly conserved among species (Verkerk et al., 1991), and the expression pattern of *FMR1* at the mRNA and protein level is very similar in humans and in mice in different tissues including the brain and the testes (Abitbol et al., 1993; Hinds et al., 1993), which makes the mouse a good model to study FXS. In order to study the effects of loss of transcription of the *FMR1* gene and subsequent loss of FMRP, a transgenic mouse model of FXS was generated by interrupting the *Fmr1* gene (Bakker et al., 1994). Many functions of *Fmr1* knockout (KO) mice are normal, including gait, grooming, circadian activity, swimming, feeding and mating behavior, and the mice reproduce normally (Bakker et al., 1994). Conversely, *Fmr1* KO mice display several FXS- and autism-related behaviors, including increased audiogenic seizure susceptibility, hyperactivity, abnormal social behavior, and cognitive deficits. One of the most robust phenotypes of *Fmr1* KO mice is increased susceptibility to audiogenic seizures, which models the prevalence of seizures in FXS patients (Yan et al., 2004; Bernadet and Crusio, 2006). Exposure to a high intensity sound within a specific frequency range rapidly induces seizures in 100% of *Fmr1* KO mice, but only in 20% of wild-type mice (Min et al., 2009). Status epilepticus, a sustained seizure that usually leads to respiratory arrest in

mice, follows the primary audiogenic seizure in about 80% of *Fmr1* KO mice but does not occur in wild-type mice (Min et al., 2009). Since increased audiogenic seizure susceptibility was first characterized in *Fmr1* KO mice (Musumeci et al., 1999), this characteristic has been reported by numerous laboratories (Chen and Toth, 2001; Yan et al., 2004; Qin et al., 2005; Bernadet and Crusio, 2006; Min et al., 2009).

Another robust behavioral abnormality displayed by *Fmr1* KO mice is locomotor hyperactivity in a novel open field that has also been reported by numerous laboratories (Bakker et al., 1994; O'Brien et al., 2004; Spencer et al., 2005; Min et al., 2009; Yuskaitis et al., 2010a; Liu et al., 2011). This behavior in *Fmr1* KO mice may be relevant because patients with FXS are also hyperactive. When placed in a novel open box in a lighted room, *Fmr1* KO mice display hyperactivity measured by increased total ambulatory distance compared to wild-type mice (Yuskaitis et al., 2010a).

Some social behavior deficits are apparent in *Fmr1* KO mice, compared with wild-type mice, including longer latencies to approach a novel mouse (Spencer et al., 2005; Mines et al., 2010), lower frequencies of social interactions (Mineur et al., 2006), and increased anxiety during social interactions (McNaughton et al., 2008; Liu and Smith, 2009; Mines et al., 2010), although some laboratories have not observed these social impairments (Spencer et al., 2005; Mineur et al., 2006).

Intellectual disability is a notable symptom in FXS patients, but cognitive deficits were initially difficult to identify in the *Fmr1* KO mouse model. *Fmr1* KO mice display modest cognitive deficits in several hippocampus-dependent tasks, such as the Morris water maze, radial arm maze, and operant conditioning paradigms (Bakker, 1994; Kooy et al., 1996; D'Hooge et al., 1997; Fisch et al., 1999; Paradee et al., 1999; Peier et al.,

2000; Mineur et al., 2002). *Fmr1* KO mice also exhibit deficits in fear motivated learning tasks, including passive and active avoidance behaviors, and contextual, conditioned and trace fear memory (Yan et al., 2004; Qin et al., 2005; Zhao et al., 2005; Brennan et al., 2006; Hayashi et al., 2007; Baker et al., 2010; Guo et al., 2011). Recently, severe deficits in non-aversive learning and memory tasks, including novel object recognition and context discrimination, have been identified in *Fmr1* KO mice (Pacey et al., 2011; Eadie et al., 2012; Bhattacharya and Klann, 2012).

In addition to studying *Fmr1* KO mice, another model of FXS is the *Drosophila* model that contains a mutant *dFmr1* allele and exhibits several behavioral abnormalities. Using this model, treatment with lithium, an inhibitor of GSK3, was found to rescue some aberrant behaviors in the *Drosophila* model of FXS, including alterations in courtship behavior and defects in cognition (McBride et al., 2005). This finding led this laboratory and others to investigate the regulation of GSK3 in the *Fmr1* KO mouse model. Indeed, *Fmr1* KO mice display hyperactive GSK3 because the inhibitory serine-phosphorylation of GSK3 is decreased in the *Fmr1* KO mouse striatum, hippocampus, and cortex (Min et al., 2009). This finding suggests that pharmacological therapies to decrease GSK3 activity are a potential target to rescue some behavioral phenotypes of *Fmr1* KO mice. Administration of lithium to inhibit GSK3 has been shown to increase inhibitory serine-phosphorylation of GSK3 in *Fmr1* KO mouse brain (Min et al., 2009; Yuskaitis et al., 2010a) and rescue some abnormal behaviors of *Fmr1* KO mice. Increased susceptibility to audiogenic seizures is a robust phenotype of *Fmr1* KO mice (Chen and Toth, 2001; Yan et al., 2004; Qin et al., 2005; Bernadet and Crusio, 2006; Min et al., 2009) and lithium treatment reduces susceptibility of *Fmr1* KO mice to audiogenic

seizures (Min et al., 2009). Increased susceptibility to audiogenic seizures is also normalized by administration of two other selective inhibitors of GSK3, AR-A0144018 and SB216763, indicating that inhibition of GSK3 corrects this abnormal response (Min et al., 2009). Lithium treatment also reduced locomotor hyperactivity in the open field of *Fmr1* KO mice, but did not change locomotor activity of wild-type mice (Min et al., 2009, Yuskaitis et al., 2010a), improved some of the social behavior deficits exhibited by *Fmr1* KO mice (Mines et al., 2010), and rescued the passive avoidance learning deficit in *Fmr1* KO mice (Yuskaitis et al., 2010a; Liu et al., 2011). Since these studies show promising therapeutic actions of lithium, it is critical to determine if inhibition of GSK3 is the target.

Lithium has been used to treat mood instability and aggression in FXS (Berry-Kravis and Potanos, 2004; Wang et al., 2010), but the efficacy of lithium treatment in human patients with FXS was not evaluated until a pilot 2008 clinical trial (Berry-Kravis et al., 2008a; Berry-Kravis et al., 2008b). After lithium was administered to FXS patients for two months, significant improvement in behavior was observed on clinical scales of adaptive and target behaviors, attention, and verbal memory (Berry-Kravis et al., 2008a). It is notable that lithium is the only drug that has been used in FXS patients that improved any measure of cognition. There were no major side effects of lithium treatment in these patients, but concerns about the side effects and potential toxicity of lithium hinder further clinical trials (Berry-Kravis et al., 2011).

One of the overall goals of this project was to test if reducing GSK3 activity improves impaired cognition in the mouse model of FXS in order to determine if GSK3 is a potential therapeutic target for the treatment of FXS. To do so, GSK3 was inhibited

in mice pharmacologically using chronic lithium treatment or two small molecule selective inhibitors of GSK3, TDZD-8 and VP0.7, followed by measuring behavior in four cognitive tasks: novel object detection, temporal order memory, and coordinate and categorical spatial learning. This determined if GSK3 inhibition increases learning in *Fmr1* KO mice. Because FMRP expression may be critical in early postnatal development, chronic lithium treatment was administered to adolescent and adult *Fmr1* KO mice in order to determine if cognitive impairments were abrogated in the same cognitive tasks, and experiments also addressed the question of whether improved behavior in *Fmr1* KO mice remains after withdrawal of lithium treatment.

Based on this background, the overall goals of this study were to test the participation of GSK3 in the regulation of adult hippocampal neurogenesis and in cognitive functions using impairments in *Fmr1* KO mice as a model system in order to provide greater insight into the role that GSK3 plays in the healthy and diseased CNS. My first specific aim was to test the hypothesis that GSK3 regulates the plasticity of adult hippocampal neurogenesis. To do this, I compared hippocampal neurogenesis in wild-type and GSK3 knockin mice that were untreated or were subjected to environmental enrichment or chronic stress. My second specific aim was to test the hypothesis that inhibition of GSK3 ameliorates some of the impaired cognitive behaviors exhibited by male *Fmr1* KO mice. To do this, male *Fmr1* KO and wild-type mice were chronically treated with lithium during adolescent or adult development or treated with the selective inhibitors of GSK3, VP0.7 or TDZD-8, followed by evaluation of behaviors in four cognitive tasks.

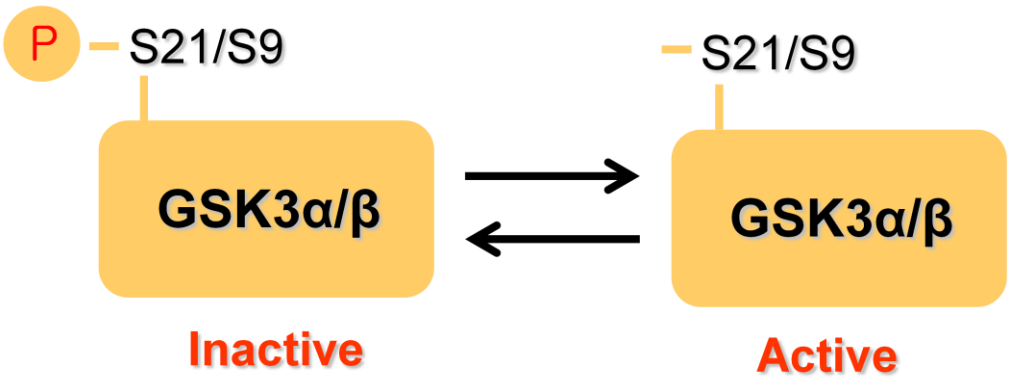


Figure 1. GSK3 regulated by phosphorylation. The predominant way that GSK3 is regulated is by phosphorylation on serine-21 of GSK3α and serine-9 of GSK3ß. Several upstream kinases have the ability to phosphorylate Serine-21 on GSK3α and Serine-9 on GSK3β, inhibiting GSK3, thus reducing its activity. GSK3 has more than 50 substrates, so GSK3 activity must be tightly regulated.

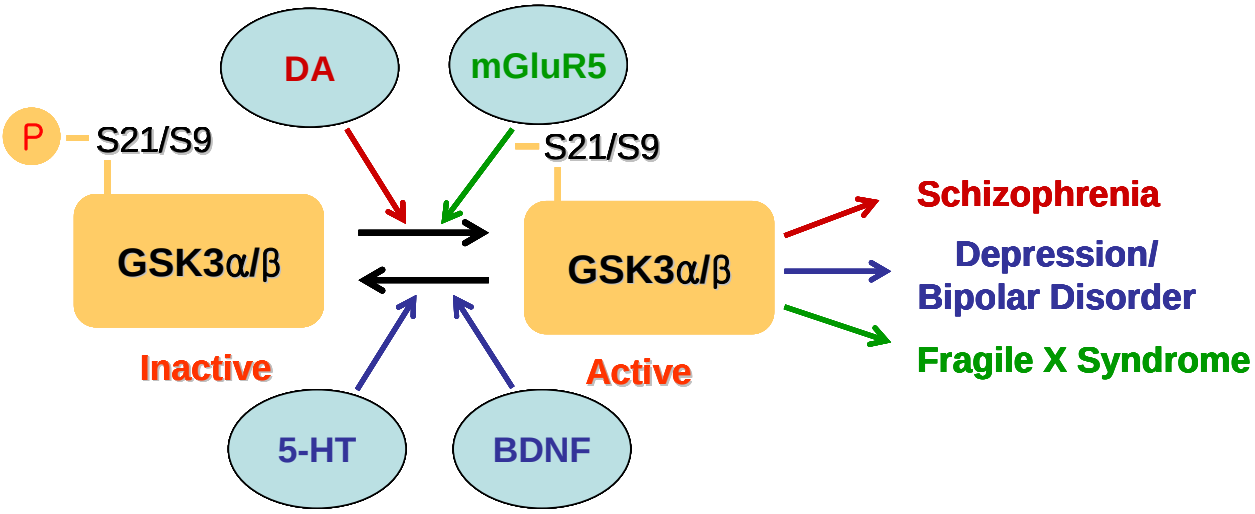


Figure 2. Impairments in the inhibition of GSK3 have been linked to several prevalent diseases of the CNS. Dysregulation of GSK3 may contribute to the cognitive and behavioral impairments in many diseases of the CNS, including schizophrenia, depression, bipolar mood disorder, and fragile X syndrome. Dopamine (DA) activates GSK3, and increased activation of GSK3 due to increased DA may be linked to schizophrenia. Serotonin (5-HT) and BDNF normally inhibit GSK3 activity, and when they are deficient, they cannot induce signals to inhibit GSK3 sufficiently, which may contribute to the mood disorders depression and bipolar disorder. Abnormally active metabotropic glutamate receptor-5 (mGluR5) signaling, due to the absence of fragile X mental retardation protein (FMRP), decreases the inhibitory phosphorylation of GSK3 and this may contribute to some of the abnormal behavioral and physiological symptoms of fragile X syndrome.

REGULATORY DIFFERENCES IN HIPPOCAMPAL NEUROGENESIS IN ADULT MALE AND FEMALE MICE IN RESPONSE TO ENVIRONMENTAL ENRICHMENT, CHRONIC STRESS AND ACTIVE GLYCOGEN SYNTHASE KINASE-3

by

MARGARET K. KING, ELEONORE BEUREL, EMMA PEREZ-COSTAS, MIGUEL MELENDEZ-FERRO, AND RICHARD S. JOPE

In preparation

Format adapted for dissertation

ABSTRACT

Mood disorders may be associated with impaired neurogenesis, which can be bolstered by drugs therapeutic for mood disorders. Abnormally active glycogen synthase kinase-3 (GSK3) impairs survival of neural precursor cells in vitro and reduces neurogenesis in adult mouse hippocampus in vivo and has been linked to susceptibility to mood disorders. These findings suggest that dysregulated GSK3 may contribute to mood disorders in part by its impairment of neurogenesis plasticity. Therefore, we examined links between GSK3 and changes in hippocampal neurogenesis induced by environmental enrichment (EE) and chronic restraint stress (CRS). Housing male wild-type mice in an enriched environment for 25 days increased the inhibitory serine-phosphorylation of GSK3 by 70% and increased the proliferation of hippocampal neural precursor cells by 150%. However, the inhibition of GSK3 was not necessary for EE-induced neurogenesis, as EE increased neurogenesis by 170% in GSK3 knockin mice in which the inhibitory serines were mutated to alanines. Two weeks of CRS decreased the inhibitory serine-phosphorylation of hippocampal GSK3 by 40% and decreased the proliferation of hippocampal neural precursor cells by 30% in male wild-type mice. CRS had no effect on the proliferation of hippocampal neural precursor cells in GSK3 knockin mice. In contrast to male wild-type mice, neither EE nor CRS altered the inhibitory serine-phosphorylation of hippocampal GSK3 or the proliferation of hippocampal neural precursor cells in female wild-type or GSK3 knockin mice, although the female GSK3 knockin mice exhibited impaired proliferation compared to male and female wild-type mice. As in male wild-type mice, EE increased NPC survival and differentiation in

female wild-type mice, and this response also occurred in female GSK3 knockin mice, which differed from the lack of response in these parameters in male GSK3 knockin mice. Thus, environmental factors and GSK3 both regulate hippocampal neurogenesis, but do so differently in male and female mice.

Introduction

The mood disorders major depressive disorder and bipolar disorder are prevalent, debilitating, and inadequately treated diseases, and their causes remain unknown. Substantial evidence indicates that hyperactive glycogen synthase kinase-3 (GSK3) promotes susceptibility to mood disorders, and that inhibition of GSK3 is an important component of the actions of therapeutic interventions, as detailed in several reviews (Manji et al., 2000; Phiel and Klein, 2001; Jope, 2011). The two GSK3 isoforms are mainly regulated by inhibitory phosphorylation on Ser21-GSK3α and Ser9-GSK3β (Jope and Johnson, 2004). This is normally maintained by signaling pathways, such as serotonergic activity (Li et al., 2004), that may be deficient in mood disorders, resulting in inadequately inhibited GSK3. The importance of inhibitory control of GSK3 can be studied using GSK3$\alpha^{21A/21A}/\beta^{9A/9A}$ knockin mice, with the regulatory serines of both GSK3 isoforms mutated to alanines (McManus et al., 2005; Eom and Jope, 2009; Polter et al., 2010). These mutations maintain GSK3 maximally active within the physiological range, since both GSK3 isoforms are expressed at normal levels.

Neurogenesis, the proliferation and differentiation of neural precursor cells (NPCs), may be impaired in mood disorders, although this link remains controversial (Lie et al., 2004; Hanson et al., 2011; Samuels and Hen, 2011). This conjecture is supported by findings that neurogenesis in mice is increased by antidepressants (Malberg et al., 2000; Manev et al., 2001; Malberg and Duman, 2003; Santarelli et al., 2003; Warner-Schmidt and Duman, 2007; David et al., 2009) and by the mood stabilizer lithium (Chen et al., 2000; Hashimoto et al., 2003; Silva et al., 2008; Wexler et al., 2008). Oppositely, chronic stress that is associated with depression-like behaviors in rodents decreases

neurogenesis (Malberg and Duman, 2003; Dranovsky and Hen, 2006; McEwen, 2008). So neurogenesis is plastic; it can be increased or decreased by environmental manipulation.

Several findings have linked GSK3 to the regulation of neurogenesis that may be involved in mood regulation. GSK3 is inhibited in vivo by both antidepressants and lithium that promote neurogenesis (De Sarno et al., 2002; Li et al., 2004), the stimulatory actions of fluoxetine and lithium on neurogenesis are blocked in GSK3 knockin mice (Eom and Jope, 2009), hyperactive GSK3 in GSK3 knockin mice impairs neurogenesis (Eom and Jope, 2009), neurogenesis is increased by GSK3 deletion (Kim et al., 2009), and GSK3 overexpression impairs, and the GSK3 inhibitor SB216763 increases, NPC proliferation that is deficient in mice with DISC1 mutations (Mao et al., 2009). These findings raise the possibility that impaired neurogenesis contributes to the greater susceptibility of GSK3 knockin mice to stress-induced depression-like behaviors (Polter et al., 2010), which may model some aspects of susceptibility to mood disorders.

Neurogenesis in adult mice is modulated by the environment. Neurogenesis is increased in male rodents exposed to environmental enrichment (EE) and exercise (Komitova et al., 2005; Leal-Galicia et al., 2007; Zhao et al., 2008; Li et al., 2008; Hu et al., 2010; Chakrabarti et al., 2011; Mustroph et al., 2012). Conversely, in male rodents, chronic stress impairs neurogenesis (Gould et al., 1992; Cameron and Gould, 1994; Duman et al., 2001; Pham et al., 2003; Falconer and Galea, 2003; Westenbroek et al., 2004; Shors et al., 2007; Koo et al., 2010; Hillerer et al., 2013). Unlike male mice, neither environmental enrichment nor chronic stress alters NPC proliferation in the hippocampus of female mice (Kempermann et al., 1997; van Praag et al., 1999; Brown et al., 2003; Falconer and Galea, 2003; Westenbroek et al., 2004; Mineur et al., 2007; Shors et al.,

2007; Kobilo et al., 2011; Hillerer et al., 2013). In the present study, we took advantage of these known regulators of neurogenesis to test if dysregulated GSK3 alters environmentally-induced changes in neurogenesis in male or female mice.

Materials and Methods

Mice and environmental manipulation

Male and female adult (8-10 weeks old) homozygous GSK3α/β$^{21A/21A/9A/9A}$ knockin mice (hereafter referred to as GSK3 knockin mice) and matched wild-type mice were used (McManus et al., 2005). GSK3 knockin mice develop and reproduce normally with no overt phenotype (McManus et al., 2005). Mice were housed in light and temperature controlled rooms and treated in accordance with NIH, the University of Miami, and the University of Alabama at Birmingham Institutional Animal Care and Use Committee regulations.

For EE, mice were housed in a large cage (55 cm x 32 cm x 22 cm) with extra wood chip bedding, nesting material, and a variety of sized, shaped, and colored toy objects for 25 days. Weekly the objects were washed and moved, and new objects were added. EE did not alter the rate of weight gain in male or female wild-type or GSK3 knockin mice.

For CRS, mice were placed in 25 ml conical tubes with breathing holes at the nose for 2 hr for 14 consecutive days. After the stress, mice were returned to their home cage until the following day. After two weeks of CRS, the body weights of male wild-type mice were 20% lower than non-stressed mice (22.4 ± 0.5 gm versus 28.0 ± 1.1 gm), and male GSK3 knockin mice body weights were 10% lower than non-stressed mice

(25.2 ± 0.8 gm versus 27.9 ± 0.9 gm), whereas the body weights of female mice were unaltered by CRS.

Administration of BrdU, immunohistochemistry, and stereology

To measure NPC proliferation in vivo, 5-bromo-2'-deoxyuridine (BrdU; 100 mg/kg; Sigma-Aldrich, St Louis, MO) was administered i.p. three times at 2 hr intervals, and mice were sacrificed 24 hr later, as we previously described (Eom and Jope 2009). To measure cell survival and differentiation, BrdU (100 mg/kg) was administered i.p. once daily for 3 consecutive days and mice were sacrificed 28 days later. Mice were deeply anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine and transcardially perfused with 0.9% sodium chloride followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were post-fixed overnight in 4% paraformaldehyde at 4°C and cryoprotected in 30% sucrose/phosphate buffered saline (PBS). Each brain was sliced coronally (30 μm) with a sliding microtome (Leica, Nuβloch, Germany) through the rostrocaudal hippocampus and stored in PBS with 0.01% sodium azide. Every sixth section was analyzed for BrdU-specific immunohistochemistry as previously described (Eom and Jope 2009). Sections were washed in Tris-Hydrochloric acid (HCl) buffer (TBS, 0.05 M, pH 7.4) and incubated in 1 N HCl on ice for 10 min, in 2 N HCl for 10 min at room temperature, and in 2 N HCl at 37°C for 20 min, washed with 1 M borate buffer, pH 8.5, on ice, and rinsed in TBS. The sections were incubated with anti-BrdU antibody (1:500; BU1/75; Abcam) in 15% normal goat serum and TBS blocking buffer (1% bovine serum albumin, 0.2% TritonX100 in TBS) for 20 hr at 4°C. Sections were washed with TBS and incubated with Alexa Fluor 488 goat anti-rat (1:200, Invitrogen) in

10% normal goat serum and TBS blocking buffer for 2 hr at room temperature in the dark. Cell nuclei were stained by incubating sections for 5 min in 0.2 μg/ml bisbenzimide (Hoechst 33258; Sigma). For double labeling, anti-neuronal nuclei (NeuN) (1:1000; Millipore, Billerica, MA) was added to the primary antibody solution, and Alexa Fluor 594 goat anti-mouse (1:200, Invitrogen) was added to the secondary antibody solution. BrdU positive cells in the granule cell layer of the dentate gyrus and the subgranular zone were counted in each section and analyzed by unbiased stereology using the StereoInvestigator system (MicroBrightField, Williston, VT). To distinguish single cells within clusters, all counts were performed using a 60× oil immersion objective (Olympus BX-51), omitting cells in the outermost focal plane. The total number of BrdU-labeled cells per section was determined and multiplied by 6 to obtain the total number of cells per dentate gyrus.

Immunoblot analysis

The hippocampus was rapidly removed and homogenized in ice-cold lysis buffer containing 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 μg/ml leupeptin, 1 μg/ml aprotinine, 1 μg/ml pepstatin A, 1 mM phenylmethanesulfonyl fluoride, 2 mM sodium vanadate, 50 mM sodium fluoride, and 100 nM okadaic acid. The lysates were centrifuged at 14,000 rpm for 10 min to remove insoluble debris. Protein concentrations in the lysate were determined in duplicate using the Bradford protein assay. Extracts were mixed with Laemmli sample buffer (2% SDS) and placed in a boiling water bath for 5 min. Proteins (10 μg) were resolved in SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with primary antibodies

to phospho-Ser9-GSK3β (1:2000), phospho-Ser21-GSK3α (1:2000), (Cell Signaling Technology, Beverly, MA), total GSK3α/β (1:2000; Millipore, Bedford, MA), and β-actin (1:10,000; Sigma, St Louis, MO). Immunoblots were developed using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (1:4000; Bio-Rad Laboratories, Hercules, CA), followed by detection with enhanced chemiluminescence, and quantitation by densitometry.

Statistical analyses

All results were analyzed by one-way or two-way ANOVA followed by Bonferroni's multiple comparison tests or by Student's t-tests.

Results

Neurogenesis is impaired in GSK3 knockin male and female mice

Immunohistochemical analysis of cell proliferation in the hippocampus measured 24 hr after three injections of BrdU (100 mg/kg) given at 2 hr intervals showed that BrdU-labeled mitotic cells were predominantly located in the subgranular zone of the dentate gyrus in both wild-type and GSK3 knockin mice (Figure 1A). We previously reported that wild-type and GSK3 knockin mouse brains displayed equivalent morphological features, hippocampal volumes, and staining for neuronal nuclei (NeuN) and glial fibrillary acidic protein (GFAP) (Eom and Jope, 2009). As reported previously (Eom and Jope, 2009), quantitative unbiased stereology analysis revealed that the number of BrdU-labeled cells within the dentate gyrus in male GSK3 knockin mice was significantly 40% lower ($t=3.42$, $p<0.05$) than in matched wild-type mice (Figure 1B). Here, the analysis

was extended to female mice, which revealed a 40% deficit ($t=2.28$, $p<0.05$) in NPC proliferation in female GSK3 knockin mice compared with female wild-type mice. There were no differences between wild-type male and female mice ($t=0.23$, $p>0.05$) or between GSK3 knockin male and female mice ($t=0.04$, $p>0.05$). Thus, NPC proliferation was significantly decreased by hyperactive GSK3 (one-way ANOVA (genotype x sex), $F(3,34)=5.50$, $p<0.05$).

The survival and differentiation of NPCs in male wild-type and GSK3 knockin mice was not significantly different (survival: $t=0.46$, $p>0.05$; differentiation: $t=0.35$, $p>0.05$) (Figures 2A and 2B), as reported previously (Eom and Jope, 2009). In wild-type mice, the females exhibited greater survival of NPCs ($t=3.81$, $p<0.05$) and differentiation to neurons ($t=3.69$, $p<0.05$) compared to wild-type male mice. This difference was not evident in female compared to male GSK3 knockin mice ($t=0.20$, $p>0.05$), and GSK3 knockin female mice revealed an impairment in survival ($t=5.01$, $p<0.05$) and differentiation ($t=3.30$, $p<0.05$) of NPCs compared to wild-type female mice. One-way ANOVA (genotype x sex) revealed a significant difference in survival of NPCs ($F(3,18)=7.56$, $p<0.01$) and differentiation to neurons ($F(3,18)=5.36$, $p<0.01$) among male and female wild-type and GSK3 knockin mice.

Modulation of neurogenesis by housing in an enriched environment (EE)

We tested if EE influenced the inhibitory serine-phosphorylation of GSK3 in male and female wild-type mice, which is abolished in GSK3 knockin mice. Immunoblots of hippocampal extracts from male wild-type mice revealed that EE increased the inhibitory serine-phosphorylation of GSK3α by 80% ($t=3.246$, $p<0.05$) and GSK3β by 60% ($t=2.62$,

$p<0.05$) in in the hippocampus (Figure 3A), as recently reported (Hu et al., 2013), but did not alter serine-phosphorylation of GSK3α ($t=0.07$, $p>0.05$) or GSK3β ($t=0.80$, $p>0.05$) in female hippocampi (Fig 3B). The total levels of GSK3α and GSK3β were unaltered by EE, indicating an effect on the regulation, rather than expression, of GSK3 in EE-exposed male wild-type mice. We next tested if altered GSK3 regulation in male and female mice was due to a basal difference in the inhibitory serine-phosphorylation of GSK3. Immunoblots of hippocampal extracts from male and female wild-type mice revealed that the inhibitory serine-phosphorylation of GSK3β is 60% ($t=3.29$, $p<0.05$) higher in female than male mice (Figure 3C), indicating that female mice may be less responsive to environmental manipulation due to increased basal inhibition of GSK3.

We tested if EE, which is well-known to promote neurogenesis (Komitova et al., 2005; Leal-Galicia et al., 2007; Zhao et al., 2008; Li et al., 2008; Hu et al., 2010; Chakrabarti et al., 2011; Mustroph et al., 2012), affected the impaired neurogenesis in GSK3 knockin mice. Housing mice in EE for 25 days significantly increased hippocampal NPC proliferation by 150% ($t=4.17$, $p<0.01$) in male wild-type mice (Figure 4A) as previously reported (Komitova et al., 2005; Leal-Galicia et al., 2007; Zhao et al., 2008; Hu et al., 2010; Chakrabarti et al., 2011; Mustroph et al., 2012). Hippocampal NPC proliferation also was increased by EE in male GSK3 knockin mice by 170% ($t=2.61$, $p<0.05$). This increase did not compensate for the basal deficit in GSK3 knockin mice, so NPC proliferation after EE remained 30% below wild-type mice. Thus, although constitutively active GSK3 impairs basal adult hippocampal neurogenesis, it does not block enhanced proliferation induced by EE in male mice, indicating that the enhancement by EE is independent of GSK3 inhibition by serine phosphorylation.

Female mice differed from male mice in that EE did not significantly increase hippocampal NPC proliferation in wild-type mice ($t=0.67$, $p>0.05$) (Figure 4B), as reported previously (Kempermann et al., 1997; van Praag et al., 1999; Li et al., 2008). EE also did not increase hippocampal NPC proliferation in female GSK3 knockin mice ($t=0.91$, $p>0.05$).

Wild-type male mice housed in EE exhibited a significant increase in the survival of NPCs ($t=2.54$, $p<0.05$) and their differentiation to neurons ($t=2.45$, $p<0.05$) by 240% and 320% respectively (Figure 5A and B), as reported previously (Komitova et al 2011; Hu et al 2010; Chakrabarti et al 2011). However, EE did not increase NPC survival ($t=0.02$, $p>0.05$) or differentiation ($t=0.20$, $p>0.05$) in male GSK3 knockin mice. One-way ANOVA revealed a significant effect of EE in the survival of NPCs ($F(3,24)=5.12$, $p<0.01$) and the differentiation to neurons ($F(3,24)=4.66$, $p<0.05$) in male mice. EE increased the survival ($t=2.68$, $p<0.05$) and differentiation ($t=3.20$, $p<0.05$) of NPCs in female wild-type mice (Figure 5C and D) as previously reported (Kempermann et al., 1997; van Praag et al., 1999; Li et al., 2008). In GSK3 knockin female mice, EE also significantly increased NPC survival ($t=3.06$, $p<0.05$) and neuronal differentiation ($t=3.66$, $p<0.05$) by 400%. One-way ANOVA also revealed a significant effect of EE in the survival of NPCs ($F(3,18)=16.01$, $p<0.01$) and the differentiation to neurons ($F(3,18)=16.47$, $p<0.01$) in female mice.

Thus, EE elevated NPC proliferation in male wild-type and GSK3 knockin mice, but only increased survival and differentiation of NPCs in male wild-type, but not GSK3 knockin mice. Although EE did not increase the proliferation of NPCs in female wild-

type or GSK3 knockin mice, NPC survival and differentiation were increased in both female wild-type and GSK3 knockin mice.

Modulation of NPC proliferation by CRS

We tested if the inhibitory serine-phosphorylation of GSK3 was affected by CRS in male and female mouse hippocampus. In male wild-type mice, stress decreased the hippocampal inhibitory serine-phosphorylation of GSK3α by 25% ($t=6.16$ $p<0.05$) and GSK3β by 55% ($t=4.07$, $p<0.05$) (Figure 6A). However, CRS did not alter serine-phosphorylation of GSKα ($t=1.17$, $p>0.05$), or GSK3β ($t=1.70$, $p>0.05$) in female hippocampi (Figure 6B). The total levels of GSK3α and GSK3β were not changed by chronic stress, indicating that stress alters the regulation, but not expression, of both GSK3 isoforms in male wild-type mice.

Two weeks of CRS reduced NPC proliferation by 30% ($t=2.19$, $p<0.05$) in male wild-type mice (Figure 4A), similar to previous reports of impaired neurogenesis after chronic stress (Cameron and Gould, 1994; Jacobs et al., 2000; Duman et al., 2001; Pham et al., 2003). In contrast, in male GSK3 knockin mice CRS did not significantly impair the already low proliferation of NPCs ($t=0.73$, $p>0.05$). This may indicate shared mechanisms by which hyperactive GSK3 and chronic stress impair NPC proliferation. CRS did not alter NPC proliferation in female wild-type mice ($t=1.09$, $p>0.05$) (Figure 4B), as has been reported previously (Westenbroek et al., 2004; Shors et al., 2007), or in female GSK3 knockin mice ($t=0.62$, $p>0.05$). Thus, NPC proliferation in the hippocampus of female mice is more resistant to environmental influences than in male mice, including both EE and CRS. NPC survival and differentiation were not measured

after CRS because of the incompatible treatment time for stress (2 weeks) and neurogenesis measurements after BrdU administration (4 weeks).

Discussion

Interactions between genetics and the environment can have tremendous influences on susceptibilities to many diseases, including mood disorders. However, much still remains to be learned about the targets of these two factors that mediate differences in disease susceptibility. Neurogenesis may be impaired in mood disorders, and GSK3 has been linked to the regulation of neurogenesis that may be involved in mood regulation (De Sarno et al.; 2002; Li et al., 2004; Eom and Jope, 2009; Kim et al., 2009; Mao et al., 2009). Therefore, in this study, we examined how the combinatorial effects of environmental changes (EE, CRS) and genetics (sex, hyperactive GSK3) affect neurogenesis in mice (Table 1).

Housing male wild-type mice in EE led to increased neurogenesis, which is generally considered to be a healthy response. Similar effects of EE on neurogenesis have been reported previously in male wild-type mice (Komitova et al., 2005; Brenes Saenz et al., 2006; Huang et al., 2006; Leal-Galicia et al., 2007; Zhao et al., 2008; Duman et al., 2008; Greenwood and Fleshner, 2008; Green et al., 2010; Hu et al., 2010; Chakrabarti et al., 2011; Mustroph et al., 2012; Jha et al., 2011 Bechara and Kelly, 2013). We examined whether this healthy response was influenced by sex, constitutively active GSK3, and both together.

Female wild-type mice differed from male wild-type mice in that EE failed to increase the proliferation of NPCs, but nonetheless increased NPC survival and

differentiation. Examination of the effects of EE on the inhibitory serine-phosphorylation of GSK3 demonstrated an increase in male hippocampus that correlated with the increased neurogenesis. However, inhibition of GSK3 clearly was not a requisite for these effects of EE because hippocampal GSK3 was not affected by EE in female wild-type mice that demonstrated enhanced neurogenesis.

Constitutively active GSK3 also influenced some outcomes of EE. EE increased NPC proliferation in GSK3 knockin male mice by the same percentage as in wild-type mice, indicating that inhibitory serine-phosphorylation of GSK3 is not required for EE to enhance NPC proliferation. This differs from the requirement for serine-phosphorylation of GSK3 for treatment with lithium and fluoxetine to increase NPC proliferation (Eom and Jope 2009). However, EE was ineffective in enhancing NPC survival and differentiation in GSK3 knockin mice, indicating a requirement for inhibition of GSK3 for these outcomes in male mice. Modulation by EE in female GSK3 knockin mice is different from male GSK3 knockin mice, demonstrating that there are gender specific differences in neurogenesis in mice with hyperactive GSK3. Female GSK3 knockin mice, like female wild-type mice, exhibited increased survival of NPCs and differentiation to neurons.

Neurogenesis is reduced by CRS (Cameron and Gould, 1994; Jacobs et al., 2000; Duman et al., 2001; Pham et al., 2003), which also increases susceptibility to depression, which has been suggested may be associated with impaired neurogenesis. CRS impaired NPC proliferation in male wild-type mice but not in male GSK3 knockin mice, which may indicate a commonality in mechanisms by which hyperactive GSK3 and chronic stress impair neurogenesis. Chronic stress affects neurogenesis in wild-type male and

female mice in different ways (Westenbroek et al., 2004; Shors et al., 2007), therefore we tested if our CRS paradigm would reveal an effect in female wild-type mice, and whether the same pattern would be exhibited by female GSK3 knockin mice. CRS did not alter NPC proliferation in female wild-type mice or GSK3 knockin mice. Examination of the effects of CRS on the inhibitory serine-phosphorylation of GSK3 demonstrated that CRS reduced GSK3 serine-phosphorylation in the hippocampus of male wild-type mice, which correlated with decreased neurogenesis. Notably, the regulation of GSK3 was much more stable in female than male hippocampus as the serine-phosphorylation of GSK3 in female wild-type mouse hippocampus was unaltered by CRS. Consistent with previous data, this suggests that cells in the female wild-type hippocampus are less responsive to stress, and that NPC proliferation in the female GSK3 knockin hippocampus is similarly less responsive to stress. Furthermore, the divergent response to CRS in male and female wild-type mice supports the possibility that there are gender specific differences in response to injury and recovery following neurological stress (Walker and Mason, 2011) and that estrogen is neuroprotective in many types of brain injury (Lang and McCullough, 2008).

In summary, EE did not alter NPC proliferation in female wild-type mice or GSK3 knockin mice. In addition to the chronic stress results, this suggests that cells in the female mouse hippocampus are less responsive to stress-induced decreased neurogenesis and EE-induced increased neurogenesis. Female GSK3 knockin mice, but not male GSK3 knockin mice, displayed increased NPC survival and differentiation to neurons following EE, suggesting that female mice with hyperactive GSK3 may have increased neuroprotection in response to environmental manipulation. The mechanisms

accounting for the differences observed among wild-type and GSK3 knockin male and female mice remain to be further examined. These differences may be due to increased levels of the hormone estrogen in female mice. By eliminating estrogen production in female mice with ovariecotomization, future studies can determine if estrogen mediates the difference in neurogenesis plasticity in male and female wild-type and GSK3 knockin mice. The differential mechanisms involved in the regulation of neurogenesis and the effect of hyperactive GSK3 in male and female mice may play a role in the development and treatment of mood disorders. The differential mechanisms involved in the regulation of neurogenesis and the effect of hyperactive GSK3 in male and female mice may play a role in the development and treatment of mood disorders.

Acknowledgements

We would like to thank Dr. Tae-Yeon Eom, Dr. Ling Song, Dr. Iria Gonzalez-Dopeso Reyes, Matthew Rice, Dr. Buffie Clodfelder-Miller, Robert McHugh, and Dr. Terry Lewis at the University of Alabama at Birmingham for their generous instruction on various techniques of immunohistochemical preparation, histology, and sterology. We would like to thank Dr. George McNamara and Dr. Melissa Carballosa-Gonzalez at the University of Miami for their help with fluorescent microscopy.

References

Bechara, R. G., & Kelly, Á. M. (2013). Exercise improves object recognition memory and induces BDNF expression and cell proliferation in cognitively enriched rats. *Behav Brain Res 245,* 96-100.

Brenes Sáenz, J. C., Villagra, O. R., & Fornaguera Trías, J. (2006). Factor analysis of Forced Swimming test, Sucrose Preference test and Open Field test on enriched, social and isolated reared rats. *Behav Brain Res 169*, 57-65.

Brown, J., Cooper-Kuhn, C. M, Kempermann, G., Van Praag, H., Winkler, J., Gage F. H., & Kuhn, H. G. (2003). Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. *Eur J Neurosci 17*, 2042-2046.

Cameron, H, A. & Gould, E. (1994). Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. *Neuroscience 61,* 203-209.

Chakrabarti, L., Scafidi, J., Gallo, V., & Haydar, T. F. (2011). Environmental enrichment rescues postnatal neurogenesis defect in the male and female Ts65Dn mouse model of Down syndrome. *Dev Neurosc 33,* 428-441.

Chen, G., Rajkowska, G., Du, F., Seraji-Bozorgzad, N., & Manji, H. K. (2000). Enhancement of hippocampal neurogenesis by lithium. *J Neurochem 75*, 1729-1734.

David, D. J., Samuels, B. A., Rainer, Q., Wang, J. W., Marsteller, D., Mendez, I., et al. (2009). Neurogenesis-dependent and -independent effects of fluoxetine in an animal model of anxiety/depression. *Neuron 62*, 479-493.

De Sarno, P., Li, X., & Jope, R. S. (2002). Regulation of Akt and glycogen synthase kinase-3β phosphorylation by sodium valproate and lithium. *Neuropharmacol 43*, 1158-1164.

Dranovsky, A. & Hen, R. (2006). Hippocampal neurogenesis: regulation by stress and antidepressants. *Biol Psychiatry* 59, 1136-1143.

Duman, C. H., Schlesinger, L., Russell, D. S., & Duman, R. S. (2008). Voluntary exercise produces antidepressant and anxiolytic behavioral effects in mice. *Brain Research 1199*, 148-158.

Duman, R. S., Malberg, J., & Nakagawa, S. (2001). Regulation of adult neurogenesis by psychotropic drugs and stress. *J Pharmacol Exp Ther 299*, 401-407.

Eom, T. Y., & Jope, R. S. (2009). Blocked inhibitory serine-phosphorylation of glycogen synthase kinase-3α/β impairs in vivo neural precursor cell proliferation. *Biological Psychiatry 66*, 494-502.

Falconer, E. M. & Galea, L. A. (2003). Sex differences in cell proliferation, cell death and defensive behavior following acute predator odor stress in adult rats. *Brain Res 975*, 22-36.

Gould, E., Daniels, D. C., Cameron, H. A., & McEwen, B. S. (1992). Expression of adrenal steroid receptors by newly born cells and pyknotic cells in the dentate gyrus of the postnatal rat. *Mol Cell Neurosci 3*, 44-48.

Green, T. A., Alibhai, I. N., Roybal, C. N., Winstanley, C. A., Theobald, D. E., Birnbaum, S. G., et al. (2010). Environmental enrichment produces a behavioral phenotype mediated by low cyclic adenosine monophosphate response element binding (CREB) activity in the nucleus accumbens. *Biological Psychiatry 67*, 28-35.

Greenwood, B. N. & Fleshner, M. (2008). Exercise, learned helplessness, and the stress-resistant brain. *Neuromolecular Medicine 10*, 81-98.

Hanson, N. D., Owens, M. J., & Nemeroff, C. B. (2011). Depression, antidepressants, and neurogenesis: a critical reappraisal. *Neuropsychopharmacology 36*, 2589-2602.

Hashimoto, R., Senatorov, V., Kanai, H., Leeds, P., & Chuang, D. M. (2003). Lithium stimulates progenitor proliferation in cultured brain neurons. *Neuroscience 117*, 55-61.

Hillerer, K. M., Neumann, I. D., Couillard-Despres, S., Aigner, L., & Slattery, D. A. (2013). Sex-dependent regulation of hippocampal neurogenesis under basal and chronic stress conditions in rats. *Hippocampus 23*, 476-487.

Hu, Y. S., Xu, P., Pigino, G., Brady, S. T., Larson, J., & Lazarov, O. (2010). Complex environment experience rescues impaired neurogenesis, enhances synaptic plasticity, and attenuates neuropathology in familial Alzheimer's disease-linked APPswe/PS1DeltaE9 mice. *FASEB J 24*, 1667-1681.

Hu, Y. S., Long, N., Pigino, G., Brady, S. T., & Lazarov, O. (2013). Molecular mechanisms of environmental enrichment: impairments in Akt/GSK3β, neurotrophin-3 and CREB signaling. *PLoS One 8*, e64460.

Huang, F. L., Huang, K. P., Wu, J., & Boucheron, C. (2006). Environmental enrichment enhances neurogranin expression and hippocampal learning and memory but fails to rescue the impairments of neurogranin null mutant mice. *The Journal of Neuroscience 26*, 6230-6237.

Jacobs, B. L., Praag, H., & Gage, F. H. (2000). Adult brain neurogenesis and psychiatry: a novel theory of depression. *Mol Psychiatry 5*, 262-269.

Jha, S., Dong, B., & Sakata, K. (2011). Enriched environment treatment reverses depression-like behavior and restores reduced hippocampal neurogenesis and protein levels of brain-derived neurotrophic factor in mice lacking its expression through promoter IV. *Transl Psychiatry, 1*, e40.

Jope, R. S. & Johnson, G. V. W. (2004). The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci 29*, 95-102.

Jope, R. S. (2011). Glycogen synthase kinase-3 in the etiology and treatment of mood disorders. *Frontiers in Molecular Neuroscience 4*, 16.

Kempermann, G., Kuhn, H. G., & Gage, F. H. (1997). More hippocampal neurons in adult mice living in an enriched environment. *Nature 386*, 493−495.

Kim, W. Y., Wang, X., Wu, Y., Doble, B. W., Patel, S., Woodgett, J. R., et al., (2009). GSK-3 is a master regulator of neural progenitor homeostasis. *Nat Neurosci 12*, 1390-1397.

Kobilo, T., Liu, Q. R., Gandhi, K., Mughal, M., Shaham, Y., & van Praag, H. (2011). Running is the neurogenic and neurotrophic stimulus in environmental enrichment. *Learn Mem* 18, 605-609.

Komitova, M., Mattsson, B., Johansson, B. B., & Eriksson, P. S. (2005). Enriched environment increases neural stem/progenitor cell proliferation and neurogenesis in the subventricular zone of stroke-lesioned adult rats. *Stroke 36*, 1278-1282.

Koo, J. W., Russo, S. J., Ferguson, D., Nestler, E. J., & Duman, R. S. (2010). Nuclear factor-kappaB is a critical mediator of stress-impaired neurogenesis and depressive behavior. *Proc Natl Acad Sci U S A 107*, 2669-2674.

Lang, J. T. & McCullough, L. D. (2008). Pathways to ischemic neuronal cell death: are sex differences relevant? *Journal of Translational Medicine 6*:33.

Leal-Galicia, P., Saldívar-González, A., Morimoto, S., & Arias, C. (2007). Exposure to environmental enrichment elicits differential hippocampal cell proliferation: role of individual responsiveness to anxiety. *Dev Neurobiol 67*, 395-405.

Li, X., Zhu, W., Roh, M. S., Friedman, A. B., Rosborough, K., & Jope R. S. (2004). In vivo regulation of glycogen synthase kinase-3β (GSK3β) by serotonergic activity in mouse brain. *Neuropsychopharmacology 29*, 1426-1431.

Li, Y., Luikart, B. W., Birnbaum, S., Chen, J., Kwon, C. H., Kernie, S. G. et al. (2008). TrkB regulates hippocampal neurogenesis and governs sensitivity to antidepressive treatment. *Neuron 14*, 399-412.

Lie, D. C., Song, H., Colamarino, S. A., Ming, G. L., & Gage, F. H. (2004). Neurogenesis in the adult brain: new strategies for central nervous system diseases. *Annu Rev Pharmacol Toxicol 44*, 399-421.

Mao, Y., Ge, X., Frank, C. L., Madison, J. M., Koehler, A. N., Doud, M. K. et al. (2009). Disrupted in schizophrenia 1 regulates neuronal progenitor proliferation via modulation of GSK3β/β-catenin signaling. *Cell 136*, 1017-1031.

Malberg, J. E., Eisch, A. J., Nestler, E. J., & Duman, R. S. (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci 20*, 9104-9110.

Malberg, J. E. & Duman, R. S. (2003). Cell proliferation in adult hippocampus is decreased by inescapable stress: reversal by fluoxetine treatment. *Neuropsychopharmacology 28*, 1562-1571.

Manev, H., Uz, T., Smalheiser, N. R., & Manev, R. (2001). Antidepressants alter cell proliferation in the adult brain in vivo and in neural cultures in vitro. *Eur J Pharmacol 411*, 67-70.

Manji, H. K., Moore, G. J., Rajkowska, G., & Chen, G. (2000). Neuroplasticity and cellular resilience in mood disorders. *Mol Psychiat 5*, 578-593.

McEwen, B. S. (2008). Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators. *Eur J Pharmacol 583*, 174-185.

McManus, E. J., Sakamoto, K., Armit, L. J., Ronaldson, L., Shpiro, N., Marquez, R., Alessi, D. R. (2005). Role that phosphorylation of GSK3 plays in insulin and Wnt signalling defined by knockin analysis. *EMBO J 24*, 1571-1583.

Mineur, Y. S., Belzung, C., & Crusio, W. E. (2007). Functional implications of decreases in neurogenesis following chronic mild stress in mice. *Neuroscience 150*, 251-259.

Mustroph, M. L., Chen, S., Desai, S. C., Cay, E. B., DeYoung, E. K., & Rhodes, J. S. (2012). Aerobic exercise is the critical variable in an enriched environment that increases hippocampal neurogenesis and water maze learning in male C57BL/6J mice. *Neuroscience 219*, 62-71.

Pham, K., Nacher, J., Hof, P. R., & McEwen, B. S. (2003). Repeated restraint stress suppresses neurogenesis and induces biphasic PSA-NCAM expression in the adult rat dentate gyrus. *Eur J Neurosci 17*, 879-886.

Phiel, C. J. & Klein, P. S. (2001). Molecular targets of lithium action. *Annu Rev Pharmacol Toxicol 41*, 789-813.

Polter, A., Beurel, E., Garner, R., Song, L., Miller, C., Sweatt, J. D., et al. (2010). Deficiency in the inhibitory serine-phosphorylation of glycogen synthase kinase-3 increases sensitivity to mood disturbances. *Neuropsychopharmacology 35*: 1761-1774.

Samuels, B. A. & Hen, R. (2011). Neurogenesis and affective disorders. *Eur J Neurosci 33*, 1152-1159.

Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., et al. (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science 301*, 805-809.

Shors, T. J., Mathew, J., Sisti, H. M., Edgecomb, C., Beckoff, S., & Dalla, C. (2007). Neurogenesis and helplessness are mediated by controllability in males but not in females. *Biol Psychiatry 62*, 487-495.

Silva, R., Mesquita, A. R., Bessa, J., Sousa, J. C., Sotiropoulos, I., Leão, P., et al. (2008). Lithium blocks stress-induced changes in depressive-like behavior and hippocampal cell fate: the role of glycogen-synthase-kinase-3β. *Neuroscience 152*, 656-669.

van Praag, H., Kempermann, G., & Gage, F. H. (1999). Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat Neurosci 2*, 266-270.

Walker, M. D. & Mason, G. (2011). Female C57BL/6 mice show consistent individual differences in spontaneous interaction with environmental enrichment that are predicted by neophobia. *Behav Brain Res 224*, 207-212.

Warner-Schmidt, J. L. & Duman, R. S. (2007). VEGF is an essential mediator of the neurogenic and behavioral actions of antidepressants. *Proc Natl Acad Sci USA 104*, 4647-4652.

Westenbroek, C., Den Boer, J. A., Veenhuis, M., & Ter Horst, G. J. (2004). Chronic stress and social housing differentially affect neurogenesis in male and female rats. *Brain Res Bull 64*, 303-308.

Wexler, E. M., Geschwind, D. H., & Palmer, T. D. (2008). Lithium regulates adult hippocampal progenitor development through canonical Wnt pathway activation. *Mol Psychiatry 13*, 285-292.

Zhao, C., Deng, W., & Gage, F. H. (2008). Mechanisms and functional implications of adult neurogenesis. *Cell 132*, 645-660.

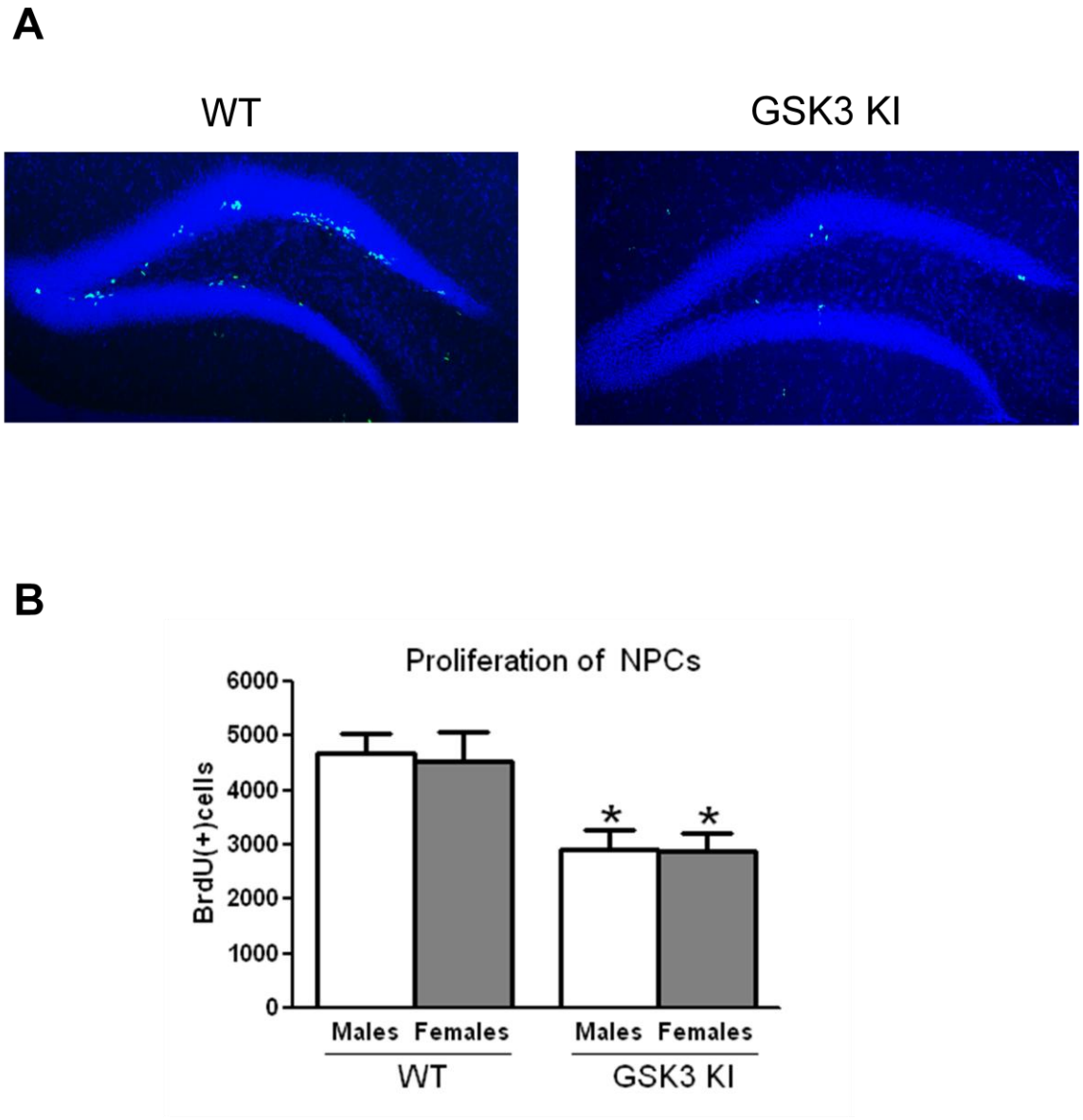


Figure 1. NPC proliferation is impaired in the hippocampus of GSK3 knockin mice.
(A) Immunohistochemical detection of BrdU-positive cells (green) in the hippocampus of male wild-type and GSK3 knockin mice. Nuclei are labeled with bisbenzimide (blue). (B) Unbiased stereological quantitation of BrdU-positive cells in the hippocampal dentate gyrus of male and female wild-type (WT) and GSK3 knockin (KI) mice. NPC proliferation significantly differed between WT males (4685 ± 353) and GSK3 KI males (2906 ± 382) ($t=3.42$, $p<0.05$) and between WT females (4543 ± 519) and GSK3 KI females (2884 ± 337) ($t=2.28$, $p<0.05$) (one-way ANOVA (genotype x sex) followed by Bonferroni's multiple comparison test, $F(3,34)=5.50$, $p<0.05$). Values are means ± S.E.M.; n=11 males/group, n=6-10 females/group *$p<0.05$ compared to WT control mice of the same sex.

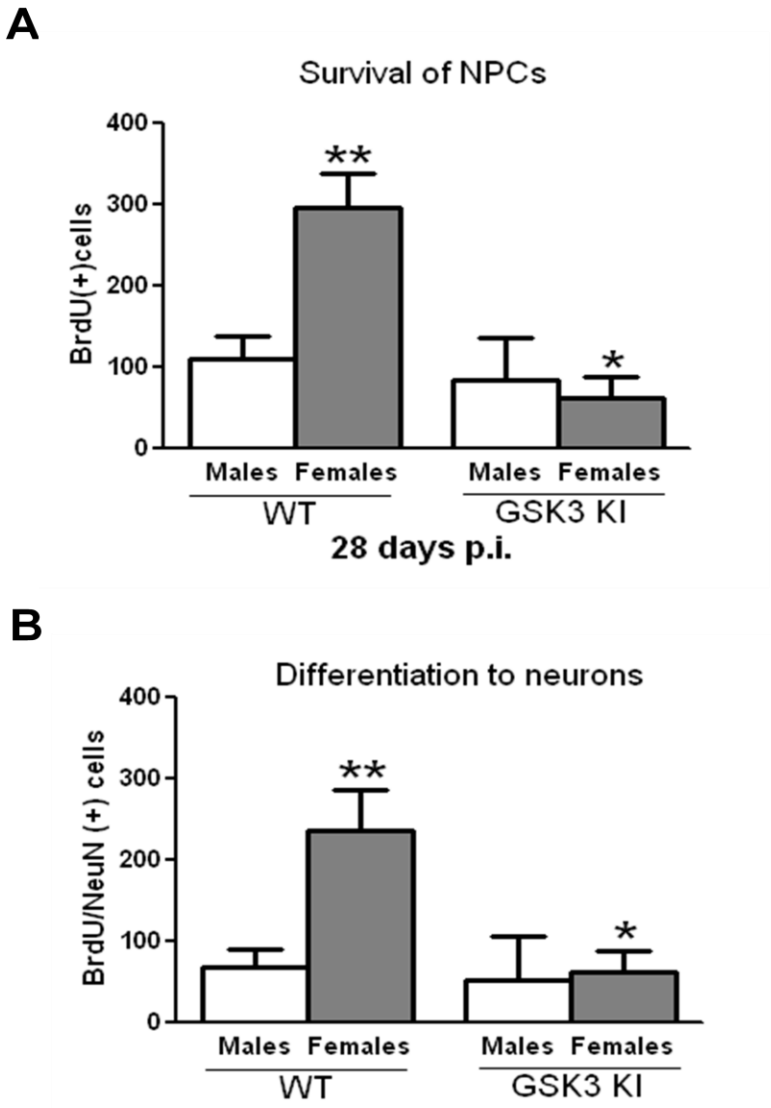


Figure 2. NPC survival and differentiation to neurons is impaired in the hippocampus of female, but not male, GSK3 knockin mice.

Unbiased stereological quantitation of BrdU-positive cells in the hippocampus of male and female wild-type (WT) and GSK3 knockin (KI) mice. (A) Survival of NPCs significantly differed between WT males (110 ± 28) and WT females (300 ± 40) ($t=3.81$, $p<0.05$) and between WT females and GSK3 KI females (62 ± 28) ($t=5.01$, $p<0.05$) (one-way ANOVA (genotype x sex) followed by Bonferroni's multiple comparison test, $F(3,18)=7.56$, $p<0.01$). (B) Differentiation to neurons significantly differed between WT males (69 ± 21) and WT females (236 ± 51) ($t=3.69$, $p<0.05$) and between WT females and GSK3 KI females (62 ± 28) ($t=3.30$, $p<0.05$) (one-way ANOVA (genotype x sex) followed by Bonferroni's multiple comparison test, $F(3,18)=5.36$, $p<0.01$). Values are means ± S.E.M.; n=4-8 males/group, n=4-6 females/group, **$p<0.05$ compared to WT male mice, *$p<0.05$ compared to WT female mice.

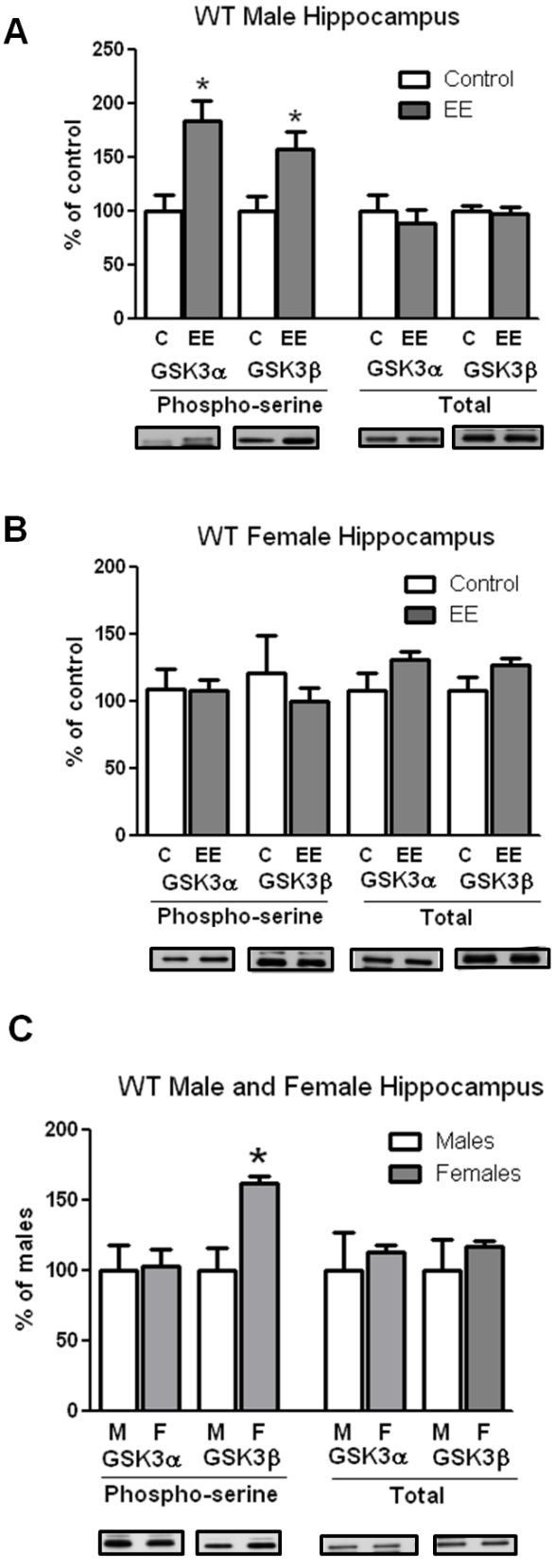

A
WT Male Hippocampus
% of control
250
200
150
100
50
0
Control
EE
*
*
C EE C EE C EE C EE
GSK3α GSK3β GSK3α GSK3β
Phospho-serine
Total
B
WT Female Hippocampus
% of control
200
150
100
50
0
Control
EE
C EE C EE C EE C EE
GSK3α GSK3β GSK3α GSK3β
Phospho-serine
Total
C
WT Male and Female Hippocampus
% of males
200
150
100
50
0
Males
Females
*
M F M F M F M F
GSK3α GSK3β GSK3α GSK3β
Phospho-serine
Total

Figure 3. Environmental enrichment (EE) increases serine-phosphorylation of GSK3 in wild-type male, but not wild-type female, hippocampus.
(A) In the hippocampus of male wild-type (WT) mice, EE significantly increased phospho-GSK3α ($t=3.246$, $p<0.05$) and phospho-GSK3β ($t=2.62$, $p<0.05$). The total levels of GSK3α ($t=0.47$, $p>0.05$) and GSK3β ($t=0.34$, $p>0.05$) were not altered by EE. (B) In the hippocampus of female WT mice, EE did not alter inhibitory phosphorylation of GSKα ($t=0.07$, $p>0.05$), or GSK3β ($t=0.80$, $p>0.05$), or total levels of GSK3α ($t=1.72$, $p>0.05$) or GSK3β ($t=1.76$, $p>0.05$). (C) WT female mice displayed increased phospho-GSK3β ($t=3.29$, $p<0.05$) compared to male WT mice, but there were no differences in phospho-GSK3α ($t=1.26$, $p>0.05$) or total levels of GSK3α ($t=0.87$, $p>0.05$) and GSK3β ($t=0.98$, $p>0.05$). Phospho-GSK3 values were calculated as the percent of ratios to total GSK3 levels and compared to results from control mice not housed in EE. Values are means ± S.E.M; n= 6-10 males/group, n=4-5 females/group; *$p<0.05$ compared to control WT mice.

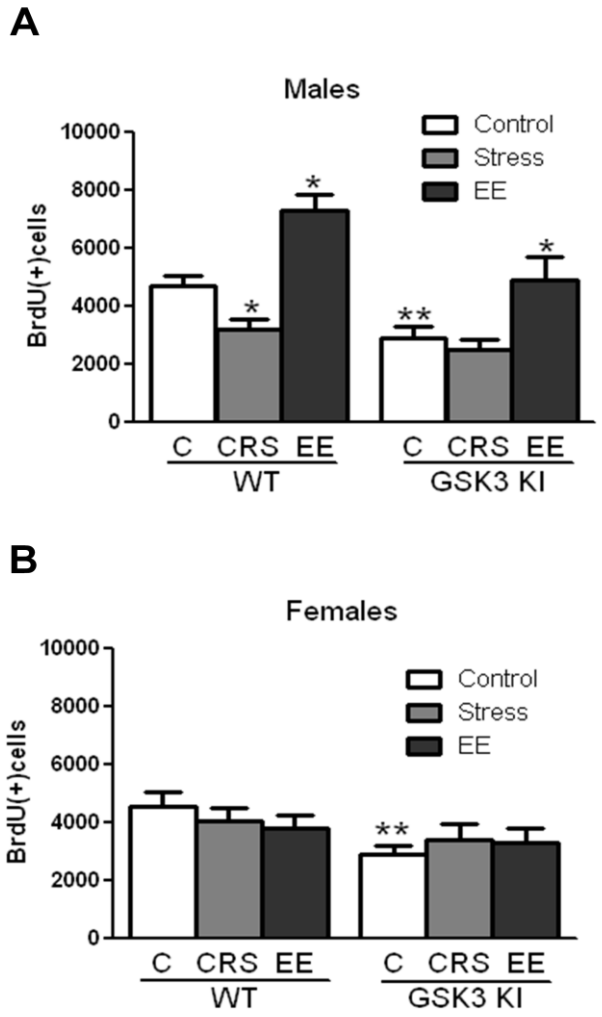


Figure 4. Effects of environmental enrichment (EE) and chronic restraint stress (CRS) on NPC proliferation in the hippocampus.

Unbiased stereological quantitation of BrdU-positive cells in the hippocampus of male and female wild-type (WT) and GSK3 knockin (KI) mice with and without 25 days of EE or two weeks of CRS. (A) NPC proliferation was significantly increased by EE in male WT mice ($t=4.17$, $p<0.01$) and male GSK3 KI mice ($t=2.61$, $p<0.05$), whereas CRS significantly decreased proliferation in male WT mice ($t=2.19$, $p<0.05$) but not in male GSK3 KI mice ($t=0.73$, $p>0.05$) (two-way ANOVA (genotype x treatment) followed by post hoc Bonferroni's multiple comparison test; $F(2,49)=24.93$, $p<0.05$). (B) NPC proliferation was unchanged by EE in female WT mice ($t=0.67$, $p>0.05$) and female GSK3 KI mice ($t=0.91$, $p>0.05$) or CRS in female WT mice ($t=1.09$, $p>0.05$) and female GSK3 KI mice ($t=0.62$, $p>0.05$) (two-way ANOVA (genotype x treatment) followed by post hoc Bonferroni's multiple comparison test; $F(2,40)=0.83$, $p>0.05$). Values are means ± S.E.M.; n=5-11 males/group; n=4-10 females/group; **$p<0.05$ compared to control WT mice; *$p<0.05$ compared to same genotype without treatment.

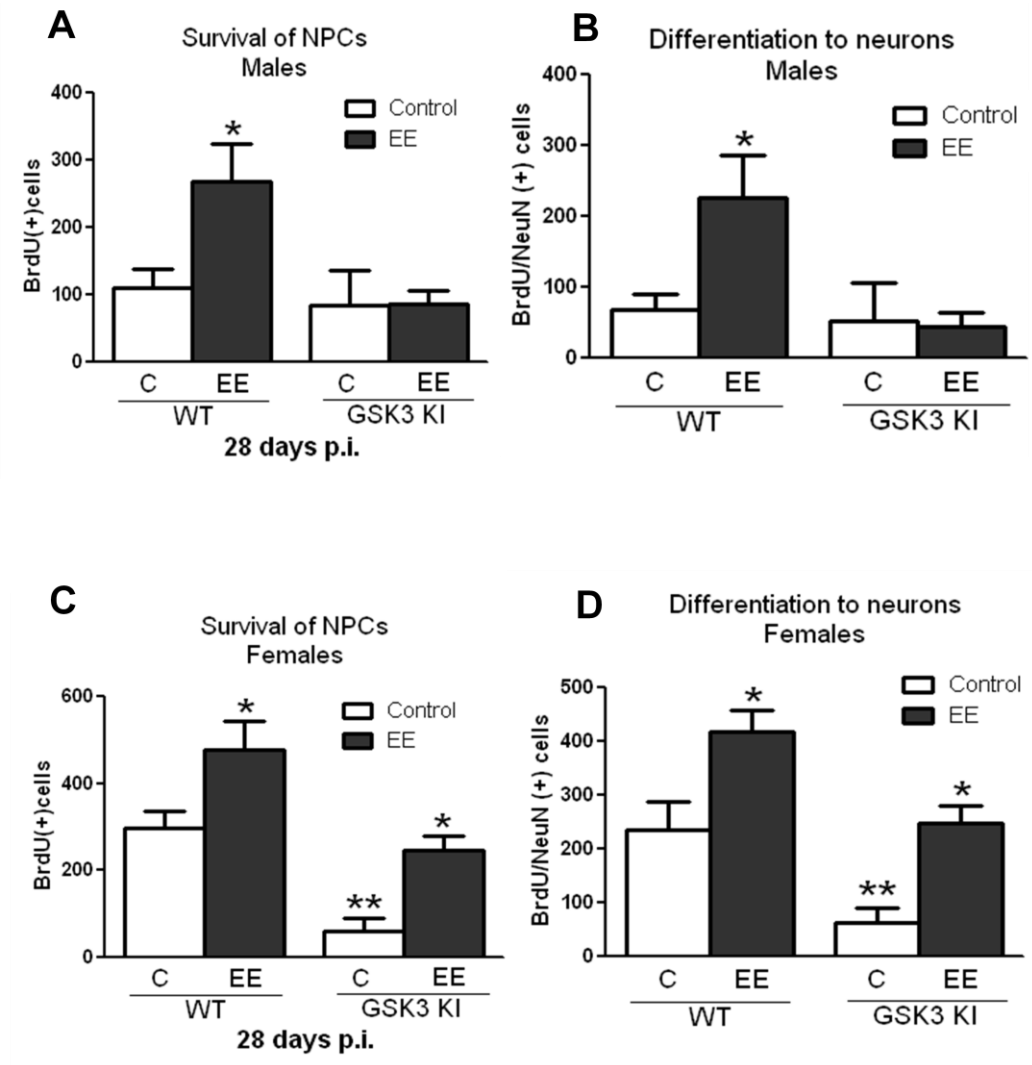

A
Survival of NPCs
Males
BrdU(+)cells
400
300
200
100
0
Control
EE
*
C
EE
C
EE
WT
GSK3 KI
28 days p.i.
B
Differentiation to neurons
Males
BrdU/NeuN (+) cells
400
300
200
100
0
Control
EE
*
C
EE
C
EE
WT
GSK3 KI
C
Survival of NPCs
Females
BrdU(+)cells
600
400
200
0
Control
EE
*
**
*
C
EE
C
EE
WT
GSK3 KI
28 days p.i.
D
Differentiation to neurons
Females
BrdU/NeuN (+) cells
500
400
300
200
100
0
Control
EE
*
**
*
C
EE
C
EE
WT
GSK3 KI

Figure 5. Environmental enrichment (EE) increases survival of NPCs and differentiation to neurons in female, but not male, GSK3 knockin mice.
(A) NPC survival was significantly increased by EE in male wild-type (WT) mice ($t=2.54$, $p<0.05$), but not in male GSK3 knockin (KI) mice ($t=0.02$, $p>0.05$) (one-way ANOVA (genotype x treatment) followed by post hoc Bonferroni's multiple comparison test, $F(3,24)=5.12$, $p<0.01$). (B) Differentiation to neurons was also significantly increased by EE in male WT mice ($t=2.45$, $p<0.05$), but not in male GSK3 KI mice ($t=0.20$, $p>0.05$) (one-way ANOVA (genotype x treatment) followed by post hoc Bonferroni's multiple comparison test, $F(3,24)=4.66$, $p<0.05$). (C) NPC survival was significantly increased by EE in female WT ($t=2.68$, $p<0.05$) and GSK3 KI mice ($t=3.06$, $p<0.05$) (one-way ANOVA (genotype x treatment) followed by post hoc Bonferroni's multiple comparison test, $F(3,18)=16.01$, $p<0.01$). (D) Differentiation to neurons was also significantly increased by EE in female WT ($t=3.20$, $p<0.05$) and GSK3 KI mice ($t=3.66$, $p<0.05$) (one-way ANOVA (genotype x treatment) followed by post hoc Bonferroni's multiple comparison test, $F(3,18)=16.47$, $p<0.01$). Values are means ± S.E.M.; n=4-8 males/group; n=4-6 females/group; **$p<0.05$ compared to WT male mice, *$p<0.05$ compared to same genotype without treatment.

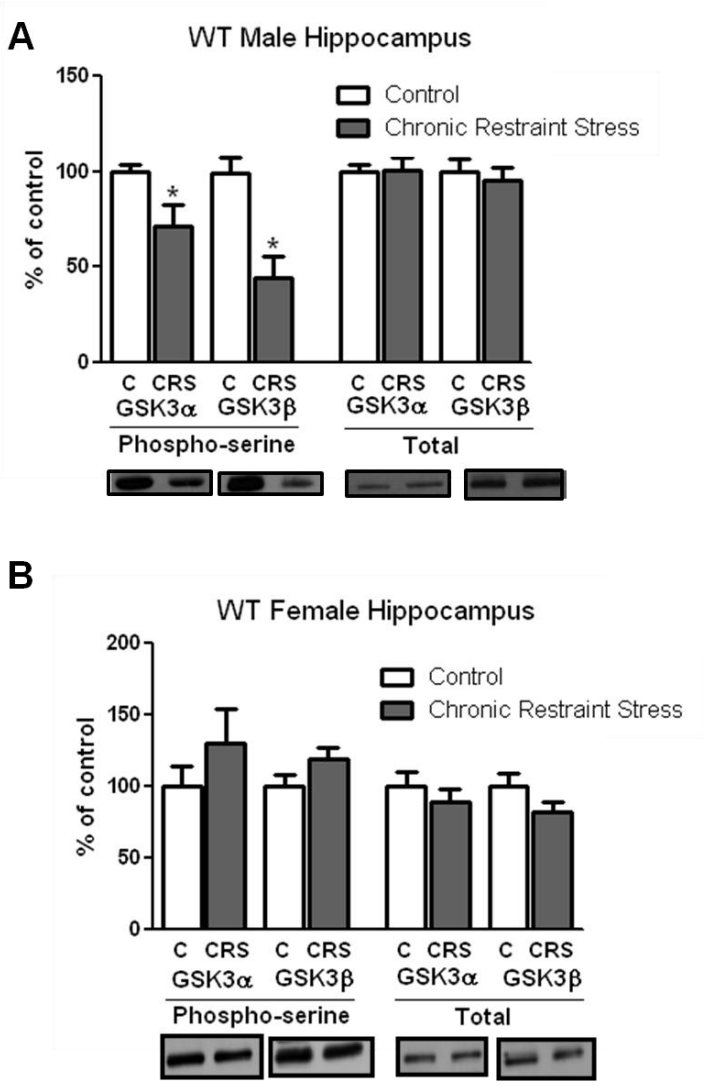


Figure 6. Serine phosphorylation of GSK3α and GSK3β is decreased in the hippocampus of wild-type mice following chronic restraint stress (CRS).
(A) In the hippocampus of male wild-type (WT) mice, CRS decreased phospho-GSK3α ($t=6.16$ $p<0.05$) and phospho-GSK3β ($t=4.07$, $p<0.05$). There was no change in the total level of GSK3α ($t=1.41$, $p>0.05$) or GSK3β ($t=0.51$, $p>0.05$). (B) In the hippocampus of female WT mice, CRS did not affect inhibitory phosphorylation of GSKα ($t=1.17$, $p>0.05$), or GSK3β ($t=1.70$, $p>0.05$), or total levels of GSK3α ($t=0.78$, $p>0.05$) or GSK3β ($t=1.42$, $p>0.05$). Phospho-GSK3 values were calculated as ratios to total GSK3 levels and compared to results from control mice not subjected to CRS. Values are means ± S.E.M; n=7-8 males/group, n=4-5 females/group; *$p<0.05$ compared to control mice.

	Inhibition of GSK3		NPC proliferation		NPC survival and differentiation
	EE	CRS	EE	CRS	EE
WT males	↑	↓	↑	↓	↑
GSK3 KI males	N/A	N/A	↑	↔	↔
WT females	↔	↔	↔	↔	↑
GSK3 KI females	N/A	N/A	↔	↔	↑

Table 1. Summary of results.
Environmental enrichment (EE) and chronic restraint stress (CRS) affect inhibition of GSK3, NPC proliferation, survival and differentiation in various ways in wild-type (WT) and GSK3 knockin (KI) male and female mice. N/A= Not applicable.

GLYCOGEN SYNTHASE KINASE-3 INHIBITORS: RESCUERS OF COGNITIVE IMPAIRMENTS

by

MARGARET K. KING, MARTA PARDO, YUYAN CHENG, KIMBERLEE DOWNEY, RICHARD S. JOPE, AND ELÉONORE BEUREL

Pharmacology and Therapeutics

Format adapted for dissertation

Abstract

Impairment of cognitive processes is a devastating outcome of many diseases, injuries, and drugs affecting the central nervous system (CNS). Most often, very little can be done by available therapeutic interventions to improve cognitive functions. Here we review evidence that inhibition of glycogen synthase kinase-3 (GSK3) ameliorates cognitive deficits in a wide variety of animal models of CNS diseases, including Alzheimer's disease, Fragile X syndrome, Down syndrome, Parkinson's disease, spinocerebellar ataxia type 1, traumatic brain injury, and others. GSK3 inhibitors also improve cognition following impairments caused by therapeutic interventions, such as cranial irradiation for brain tumors. These findings demonstrate that GSK3 inhibitors are able to ameliorate cognitive impairments caused by a diverse array of diseases, injury, and treatments. The improvements in impaired cognition instilled by administration of GSK3 inhibitors appear to involve a variety of different mechanisms, such as supporting long-term potentiation and diminishing long-term depression, promotion of neurogenesis, reduction of inflammation, and increasing a number of neuroprotective mechanisms. The potential for GSK3 inhibitors to repair cognitive deficits associated with many conditions warrants further investigation of their potential for therapeutic interventions, particularly considering the current dearth of treatments available to reduce loss of cognitive functions.

1. Introduction

Cognitive abilities define our species and our individual identities. Yet these functions are often threatened, as it seems that nearly every neurological and psychiatric disease includes a component of cognitive disability. This is particularly true of ageing-associated diseases, which afflict an ever-increasing portion of the population. With the recognition of the importance of disease-associated cognitive disabilities, much effort, although perhaps insufficient, is being directed towards finding ways to protect and restore cognitive functions. Here we review the rapidly accumulating evidence that inhibitors of glycogen synthase kinase-3 (GSK3) represent one of the strongest candidate classes of agents for this purpose. Although most widely studied for their potential therapeutic actions in Alzheimer's disease (AD), in fact more than a dozen distinct conditions in rodent models involving cognitive impairments have been shown to be ameliorated by the administration of GSK3 inhibitors (Figure 1). Thus, this substantial evidence suggests that GSK3 inhibitors should be more widely considered as interventions for protecting and restoring cognitive abilities that are jeopardized in many individuals with neurological and psychiatric diseases.

2. GSK3 and inhibitors

GSK3 refers to two paralogs, GSK3α and GSK3β, that are commonly referred to as isoforms because of their similar sequences and functions although they are derived from different genes and differential actions have been identified (Kaidanovich-Beilin and Woodgett, 2011). They are ubiquitously expressed, serine/threonine kinases that are involved in a large number of cellular functions (Jope and Johnson, 2004). The activity of

GSK3 is most commonly regulated by phosphorylation on a regulatory serine, serine-21 in GSK3α and serine-9 in GSK3β. Phosphorylation of these regulatory serines inhibits the activity of GSK3. GSK3 can be phosphorylated on these serines by several kinases, such as Akt, protein kinase C, protein kinase A, and others. This provides a mechanism for many intracellular signaling pathways to control the activity of GSK3. However, it appears that this also provides a mechanism for disease-associated impairments in signal transduction pathways to result in failure to adequately inhibit GSK3. This failure can permit GSK3 to remain abnormally active, which appears to allow GSK3 to contribute to disease pathologies, including cognitive impairments, as discussed in later sections of this review.

The increasing evidence that GSK3 contributes to the pathology of several prevalent diseases, perhaps most notably AD and mood disorders, has generated much interest in applying GSK3 inhibitors therapeutically. Lithium was the first GSK3 inhibitor to be identified (Klein and Melton, 1996; Stambolic et al., 1996), and lithium remains the most widely used experimentally and clinically. Lithium directly binds and inhibits GSK3 (Klein and Melton, 1996; Stambolic et al., 1996), and lithium administration also increases the inhibitory serine-phosphorylation of GSK3 (Jope, 2003). Lithium is widely used therapeutically as a mood stabilizer in patients with mood disorders, and much evidence indicates that inhibition of GSK3 makes an important contribution to its mood stabilizing therapeutic effect (Jope, 2011). In human patients, therapeutic levels of lithium are in the range of 0.5-1.2 mM lithium in the serum, and this serum concentration of lithium is often achieved in rodents by administration of food pellets containing 0.2-0.4% lithium (Jope, 2011). Many actions of lithium have been

shown to be due to inhibition of GSK3, but lithium also has other actions, such as inhibition of inositol monophosphatase, that should not be discounted unless the effects of lithium have been verified to be due to inhibition of GSK3 using other selective inhibitors of GSK3 or molecular manipulations of GSK3 (Phiel and Klein, 2001). The utility of lithium and therapeutic promise of GSK3 inhibitors led to the development of many selective inhibitors of GSK3 during the last decade that are beginning to be more widely used (Eldar-Finkelman and Martinez, 2011). Many of these are ATP-competitive inhibitors of GSK3, but particularly promising are GSK3 inhibitors that are not ATP-competitive, since ATP-competitive inhibitors tend to also inhibit other kinases and may prove to be more toxic. Among the frequently used ATP-competitive GSK3 inhibitors are indirubin derivatives (Leclerc et al., 2001), paullone derivatives (Leost et al., 2000), SB415286 and SB216763, although care must be taken concerning their solubilities as originally described (Coghlan et al., 2000), and AR-A014418 (Bhat et al., 2003), although the reports of behavioral effects of AR-A014418 are mitigated by other studies indicating that it does not significantly enter the CNS (Vasdev et al., 2005; Selenica et al., 2007; Hicks et al., 2010). Reports of the kinase specificities of several GSK3 inhibitors are particularly valuable (Davies et al., 2000; Murray et al., 2004; Bain et al., 2007), enabling investigators to choose multiple GSK3 inhibitors with different off-target actions. Other GSK3 inhibitors that are not competitive with the ATP binding site in GSK3 are particularly promising (Eldar-Finkelman and Martinez, 2011). L803-mts is a cell-permeable, 11 residue peptide that is a substrate-competitive specific inhibitor of GSK3 (Plotkin et al., 2003; Kaidanovich-Beilin et al., 2004; Licht-Murava et al., 2011). TDZD-8 is a highly selective ATP non-competitive inhibitor of GSK3 (Martinez et al.,

2002). VP0.7 is an allosteric (not competitive with ATP or substrate) selective GSK3 inhibitor that binds to the C-terminal lobe of the enzyme (Palomo et al., 2011). Here we review the evidence for cognitive effects involving GSK3. Studies of potential actions of GSK3 in cognition have primarily utilized lithium, in part because it is the GSK3 inhibitor that has been available the longest, so reports of lithium's effects predominate in this review, but newer GSK3 inhibitors and molecular modifications of GSK3 have also been studied.

3. Effects of GSK3 inhibitors on cognition in healthy rodents and humans

3.1. Cognition in lithium-treated rodents

In contrast to the many conditions where impaired cognitive behaviors in rodents are improved by lithium treatment, which are discussed below, lithium often has been reported to have little effect on cognitive tasks in healthy rodents. For example, recent reports concluded that chronic dietary lithium treatment for several weeks did not alter performance in the Morris water maze in Wistar rats (Vasconcellos et al., 2003; de Vasconcellos et al., 2005), or in mice ranging in initial treatment age from one week to 12 months (Yazlovitskaya et al., 2006; Watase et al., 2007; Thotala et al., 2008; Sy et al., 2011). Chronic dietary lithium treatment also did not alter contextual fear conditioning in mice (Watase et al., 2007), performance in the object location test in mice (Dai et al., 2012), or contextual fear conditioning, spatial memory, novel object recognition, and T maze spontaneous alternation task in mice (Contestabile et al., 2013). Chronic dietary lithium treatment that began in adolescence (4 weeks old) and was continued for 8 weeks, or 4 weeks of dietary lithium treatment in adult mice, did not alter performance in the

visual object novelty detection task, temporal ordering for visual objects task, or spatial learning in the coordinate and categorical processing tasks (King and Jope, 2013). Chronic lithium treatment (2 mmol/kg; i.p.) did not alter acquisition of a non-matching to place rule in rats (Tsaltas et al., 2007b), nor did lithium treatment (47.5 mg/kg, i.p.) alter behavior in the olfactory discrimination test, the social recognition task, or short-term and long-term memory evaluated in the step-down inhibitory avoidance task in rats (Castro et al., 2012). These findings support the older literature that cognitive tasks in rodents are often unaffected by therapeutically relevant levels of lithium. However, enhanced performance following chronic lithium treatment has been found in several studies. Chronic lithium treatment (2 mmol/kg; i.p.) of mice for several weeks improved spatial working memory in a delayed alternation T-maze task and facilitated long-term retention of passive avoidance learning (Tsaltas et al., 2007a). Chronic dietary lithium treatment increased freezing behavior in a cued fear conditioning task (Watase et al., 2007), and enhanced learning in the passive avoidance task (Yuskaitis et al., 2010). Adult male rats treated with dietary lithium for 4 weeks displayed improved spatial discrimination learning in the hole-board task, increased working memory and long-term memory in the T-maze delayed alternation task and enhanced place conditioned learning in the social place-preference conditioning task (Nocjar et al., 2007). Thus, certain cognitive tasks may be improved by lithium treatment of healthy rodents, but most often lithium has been found to not significantly affect performance.

3.2. Cognition in rodents treated with other inhibitors of GSK3

Few studies have investigated the effects of GSK3 inhibitors other than lithium on cognitive behaviors in healthy rodents, and most found no effects, which is in agreement with most studies of the effects of lithium. Treatment with the GSK3 inhibitors SB216763 (0.6 mg/kg; i.p) or SB415286 (1.0 mg/kg; i.p.) for 3 days in two week old wild-type mice did not alter performance in the Morris water maze measured 2-3 months later (Thotala et al., 2008). Chronic treatment of mice with SB216763 (2 mg/kg; i.p.) every other day for 2 weeks had no effect on performance in the contextual and tone trace conditioning tests or spatial learning in the delayed non-matching-to-place radial arm maze (Guo et al., 2012). Inhibition of GSK3 with intracerebroventricular (icv) infusion of SB216763 (20 ng/µl) in rats did not affect performance in the Morris water maze (Tian et al., 2012). Acute treatment with the GSK3 inhibitors TDZD-8 (5 mg/kg; i.p.) or VP0.7 (5 mg/kg; i.p.) did not alter performance of mice in the visual object novelty detection task, temporal ordering for visual objects task, spatial learning in the coordinate and categorical processing tasks (Franklin et al., 2013).These findings match well with those of lithium treatments, in that cognitive behaviors of healthy rodents often are unaffected by administration of GSK3 inhibitors. In contrast, a report that 4 weeks of icv infusion of SB216763 (78 pmol/day) in rats impaired performance in the Morris water maze task (Hu et al., 2009) may be indicative of toxic effects of long-term central administration of an ATP-competitive inhibitor of GSK3. On the other hand, acute treatment with the dual phosphodiesterase-7 and GSK3 inhibitor VP1.15 (3 mg/kg; i.p.) improved performance in the spatial object recognition test, the Y-maze task, and cued fear memory in mice

(Lipina et al., 2013), raising the possibility that GSK3 inhibitors may play a role in future developments of cocktails of drugs that may be tested as cognitive enhancers.

3.3. Cognition in rodents after molecular modification of GSK3

Whereas administration of GSK3 inhibitors to healthy rodents generally has little effect on cognitive tasks, studies of mice expressing modified GSK3 have begun to provide more information about the influences of GSK3 on cognitive processes. Molecular reduction of GSK3 appears to be particularly detrimental for memory rather than learning. Heterozygote GSK3β knockout (GSK3β+/-) mice learned to find a fixed but hidden platform submerged in a pool in the Morris water maze equivalently to wild-type mice, but in later re-testing GSK3β+/- mice failed to locate the hidden platform, whereas it was easily located by wild-type mice (Kimura et al., 2008). In a contextual fear-conditioning test, GSK3β+/- mice were not impaired in the ability to form and consolidate memory, but subsequent testing revealed impaired reconsolidation. This was further confirmed by the finding that GSK3 inhibition with AR-A014418 (30 mg/kg; i.p.) applied before the reconsolidation step significantly impaired memory reconsolidation, which was interpreted as a display of retrograde amnesia in mice deficient in GSK3β (Kimura et al., 2008). Mice lacking GSK3α demonstrated learning in the passive avoidance task equivalently to wild-type mice, but had an impaired ability to form and consolidate memory in a fear conditioning test (Kaidanovich-Beilin et al., 2009). These findings suggest that molecularly reducing GSK3 may have detrimental effects on memory, in contrast to the administration of GSK3 inhibitors. This detrimental effect may be due to the reduction of GSK3 during development in transgenic mice causing

aberrations in the development of memory processes, or due to differences between molecular and pharmacological modifications of GSK3 in the extent or duration of reduction of each GSK3 isoform's activity. Thus, molecular reduction of GSK3 may not model well therapeutically applied drugs that inhibit GSK3.

The effects of excessive GSK3 activity also have been examined on cognition. Several studies investigated the effects of GSK3 overexpression, predominantly for modeling AD, and as discussed in section 4 these often found impaired cognition that was associated with AD-linked pathology and neuronal loss. Thus, this approach does not directly address potential regulatory actions of increased GSK3 activity that is not associated with neurodegeneration. Dewachter et al. (2009) took the less severe approach of studying GSK3β knockin mice, in which the inhibitory serine-9 of GSK3β was mutated to alanine to render GSK3β constitutively active and unable to be inhibited through the serine-9 phosphorylation mechanism. GSK3β knockin mice displayed impaired inhibitory avoidance learning and object recognition memory, but no deficits in cued and contextual fear conditioning tasks and conditioned taste aversion, and the mice were free of neurodegeneration (Dewachter et al., 2009). These findings suggest that loss of inhibitory control of GSK3 can result in impaired cognition, which may play a role in diseases and conditions exhibiting both activated GSK3 and cognition impairments.

3.4. Cognition in lithium-treated humans

It is unclear whether lithium alters cognition in healthy human subjects. Early reports of enhanced neurocognitive test performance after lithium treatment have been attributed to methodological limitations, such as practice-induced improvements (Dias et

al., 2012). In contrast, several investigators reported that lithium causes transient and/or mild detrimental effects on several cognitive domains in healthy subjects, such as verbal learning and memory, without altering others, such as visual memory or attention (Weingartner et al., 1985; Linnoila et al., 1986; Stip et al., 2000; Wingo et al., 2009). On the other hand, a meta-analysis concluded that lithium seems not to impair cognition in healthy human subjects after administration for 2.5 weeks or longer (Wingo et al., 2009). Thus, therapeutic lithium levels appear not to improve cognition in healthy human subjects and may cause variable mild impairments in some individuals, whereas the effects of other GSK3 inhibitors have not been tested in healthy human subjects.

Altogether, the majority of studies indicate that administration of a moderate dose of lithium or another GSK3 inhibitor has no effect, or causes relatively minor impairments, on cognition in healthy rodents or humans. This stands in stark contrast to the significantly enhanced cognitive abilities provided by administration of GSK3 inhibitors in conditions associated with cognitive disabilities that are discussed in the following sections.

4. Effects of GSK3 inhibitors on cognition in Alzheimer's disease (AD) models

AD is a neurodegenerative disorder that culminates in neurodegeneration and severe impairments in cognition. AD neuropathology is characterized by extracellular plaques of aggregated amyloid-β peptide (Aβ) and intracellular neurofibrillary tangles containing hyperphosphorylated tau, a microtubule-binding protein (Hardy et al., 1998). The Aβ hypothesis of the pathophysiology of AD posits that Aβ induces the formation of tau-containing neurofibrillary tangles and neuronal death, which contribute to

progressively worsening cognitive abilities (Hardy and Selkoe, 2002). GSK3 is intimately linked to AD neuropathology, as Aβ increases GSK3β activity (Takashima et al., 1996), GSK3 promotes the production of Aβ (Phiel et al., 2003), and GSK3 promotes apoptotic signaling induced by Aβ (reviewed in Mines et al., 2011). Furthermore, GSK3 phosphorylates tau and likely contributes to the hyperphosphorylation of tau in neurofibrillary tangles (Mandelkow et al., 1992; Hong et al., 1997; Muñoz-Montaño et al., 1997; Xie et al., 1998; Lovestone et al., 1999; Bhat et al., 2003). These findings suggest that GSK3 plays a central role in the pathophysiology of AD and have led many investigators to study the effects of GSK3 on cognition in rodent models of AD and in patients with AD (Martinez et al., 2011).

Genetically and pharmacologically increasing GSK3 activity have been used to model events that may occur in AD, manipulations that exacerbate cognitive impairments and neuropathology in rodent models of AD. Conditional overexpression of GSK3β in mouse cortical and hippocampal neurons resulted in impaired performance in the Morris water maze, hyperphosphorylation of tau, reactive astrocytosis and microgliosis, and neuronal death (Lucas et al., 2001; Hernández et al., 2002). Suppression of overexpressed GSK3β reversed the spatial memory deficit in the novel object recognition task, reduced tau hyperphosphorylation, and decreased reactive gliosis and neuronal death (Engel et al., 2006). Deletion of tau expression in GSK3β-overexpressing mice significantly reduced impairment in the Morris water maze, indicating that GSK3-mediated tau phosphorylation contributed to this cognitive impairment (Gómez de Barreda et al., 2010). The GSK3-tau interaction was further implicated by cognitive impairments in GSK3β x Tau-P301L mice with increased GSK3β expression and expression of

tauopathy-associated mutated tau. These mice displayed impaired novel object recognition memory and passive avoidance learning, which occurred prior to the deposition of tau aggregates, suggesting that early tau pathology due to increased GSK3β activity may cause synaptic deficits that underlie the cognitive impairments in the GSK3β x Tau-P301L mice (Terwel et al., 2008).

Activation of GSK3 also has been reported to be detrimental for cognitive tasks in studies of potential pathological mechanisms in AD. Intracerebroventricular infusion of the phosphatidylinositol 3-kinase inhibitor wortmannin and the protein kinase C inhibitor GF-109203X in adult rats caused spatial memory deficits in the Morris water maze and hyperphosphorylation of tau (Liu et al., 2003). Induction of diabetes by streptozotocin treatment in human amyloid precursor protein (APP) transgenic mice increased GSK3β activity and impaired performance in the Barnes circular maze task (Jolivalt et al., 2010). Increased GSK3 activity in rats following expression of the C-terminal fragment of protein phosphatase 2A, which can dephosphorylate the inhibitory serine in GSK3, impaired performance in the Morris water maze, increased Aβ levels, and caused hyperphosphorylation of tau (Wang et al., 2010). Although all of these interventions can be expected to have multiple effects, they add to the correlative evidence between activation of GSK3 and worsened pathology and cognitive abilities. Altogether, a variety of molecular and pharmacological approaches suggest that increased GSK3 activity likely contributes to pathology and cognitive impairments in rodent models of AD.

Further evidence implicating actions of GSK3 in cognitive impairments in rodent models of AD has come from studies showing that administration of the GSK3 inhibitor lithium reduces neuropathology and cognitive deficits. Lithium (2 mEq/kg; i.p.)

administered to rats daily for 2 weeks prior, and 2 weeks following, intrahippocampal infusion of Aβ fibrils blocked spatial memory deficits in the Morris water maze (De Ferrari et al., 2003). Treatment with lithium (200 mM; icv) in adult male rats reversed the spatial memory impairment in the Morris water maze and abolished hyperphosphorylation of tau caused by activation of GSK3 (Liu et al., 2003). Lithium treatment (20 mg/kg; i.p.) daily for 3 months improved impaired learning in the Morris water maze in human APP transgenic mice, which was associated with decreased Aβ levels and reduced tau phosphorylation (Rockenstein et al., 2007). Dietary chronic lithium treatment for 4 weeks ameliorated a working memory deficit in the Y-maze paradigm in aged APP-intracellular domain-overexpressing transgenic mice (Ghosal et al., 2009). Lithium treatment (3 mEq/kg; i.p.) daily for 12 weeks in aged transgenic mice expressing mutated APP and presenilin-1 ameliorated deficits in the Morris water maze and reduced Aβ plaques and inflammation (Toledo and Inestrosa, 2010). Chronic lithium treatment in the food for 5 weeks in hemizygous TgCRND8 mice that develop amyloid deposition at 3 months of age attenuated cognitive deficits in the step down inhibitory avoidance test and the Morris water maze (Fiorentini et al., 2010). Chronic dietary lithium treatment for 6 weeks protected aged 3xTg-AD mice from lipopolysaccharide-induced impairment in the Morris water maze (Sy et al., 2011). These reports demonstrate that treatment with lithium can rescue a variety of cognitive impairments in rodent models of AD if treatment is initiated early in the pathological process, but not all studies found improvements after lithium treatment. Dietary chronic lithium treatment begun at 6 months of age in TgCRND8 mice (after amyloid deposition) did not improve cognition in the step down inhibitory avoidance test or the Morris water maze, although

the number and size of Aβ plaques was significantly reduced (Fiorentini et al., 2010). In aged 3xTg-AD mice, daily lithium treatment (300 µl of 0.6mol/L; i.p.) for 4 weeks did not rescue deficits in working memory in the T-maze paradigm (Caccamo et al., 2007). In APPwDI/NOS2-/- mice, chronic dietary lithium treatment for 8 months did not rescue memory in the radial-arm maze (Sudduth et al., 2012). Thus, lithium treatment appears most effective in improving cognitive abilities in rodent models of AD when treatment began before major pathologies were established.

In addition to lithium, newer small molecule inhibitors of GSK3 have been found to rescue cognitive deficits in several rodent models of AD. Treatment with the GSK3 inhibitor NP12 (200 mg/kg; oral gavage) daily for 3 months in aged double APP-tau transgenic mice diminished deficits in the Morris water maze, and decreased tau phosphorylation and amyloid deposition (Serenó et al., 2009). Aβ-induced deficits in the Morris water maze were ameliorated by chronic administration of the GSK3 inhibitor SB216763 (78 pmol/day; icv), which also reduced tau phosphorylation and neurodegeneration (Hu et al., 2009). APP/Presenilin-1 double transgenic mice treated with the GSK3 inhibitor indirubin-3'-monoxime (20 mg/kg; i.p.) 3 times per week for 8 weeks exhibited reduced impairments in the Morris water maze, which correlated with attenuation of Aβ production and tau hyperphosphorylation (Ding et al., 2010). In 3XTg-AD mice, treatment with the GSK3 inhibitor MMBO (1 or 3 mg/kg; orally) for 25 days attenuated impairments in the Y-maze task and the novel object recognition test and decreased tau phosphorylation (Onishi et al., 2011). Treatment with the GSK3 inhibitor AR-A014418 (5 mg/kg; i.p.) daily for 4 weeks alleviated deficits in the Morris water maze in APP23/PS45 double transgenic mice, which was accompanied by reduced Aβ

deposition and neuritic plaques (Ly et al., 2013). Transgenic mice that co-express AD mutations in APP and presenilin-1 (5XFAD mice) treated with the selective, substrate-competitive GSK3 inhibitor L803-mts (80 µg; intranasally) every other day for 120 days exhibited enhanced cognition in the contextual fear conditioning test, which was associated with decreased Aβ (Avrahami et al., 2013). Thus, administration of GSK3 inhibitors can ameliorate cognitive impairments in a number of mouse models of AD.

Inhibiting GSK3 using genetic approaches to specifically knockdown either GSK3α or GSK3β can also ameliorate cognitive impairments in AD mouse models. Genetically inactivating GSK3β by crossing human APP transgenic AD mice with dominant-negative GSK3β transgenic mice improved learning in the Morris water maze, reduced Aβ load and decreased tau phosphorylation in the double transgenic mice compared to human APP mice (Rockenstein et al., 2007). Reduced expression of GSK3α in PDAPP (+/-) transgenic mice attenuated deficits in the Barnes maze (Hurtado et al., 2012). Thus pharmacological or genetic approaches to selectively inhibit GSK3 appear to rescue cognitive impairments and attenuate neuropathology occurring in AD in rodent models.

The capacity of GSK3 inhibitors to alleviate symptoms of AD in mouse models has led to several studies in human patients. These studies indicate that lithium treatment protects patients with bipolar disorder from developing AD and increases measures of cognition in patients with AD and dementia or mild cognitive impairment. For example, elderly patients with bipolar disorder (mean age 68.2 years) that had been treated continuously with lithium had reduced prevalence of AD compared to the general elderly population (Nunes et al., 2007). Additionally, bipolar patients continuously treated with

lithium exhibited a reduced rate of dementia compared to bipolar patients treated with anticonvulsants, antidepressants or antipsychotics (Kessing et al., 2010). In a randomized, single-blind, placebo-controlled, parallel-group multicenter 10 week study, patients with early AD treated with lithium had a significant decrease in Alzheimer's Disease Assessment Scale-Cognitive subscale scores, indicating improved cognitive function, and as the lithium serum concentration increased, cognitive impairment decreased in individuals with early AD (Leyhe et al., 2009). Patients with amnestic mild cognitive impairment who received lithium for 12 months also performed better on the Alzheimer's Disease Assessment Scale-Cognitive subscale and in attention tasks compared to amnestic patients that did not receive lithium (Forlenza et al., 2011). In a placebo-controlled trial, long-term lithium treatment slowed the progression of cognitive and functional deficits in patients with amnestic mild cognitive impairment (Forlenza et al., 2012). Patients with dementia that were currently taking, or had taken, lithium for 48.4 ± 51.8 months performed better on the Mini-mental State Examination than patients who had never taken lithium (Terao et al., 2006). AD patients treated with lithium for 15 months performed better than untreated AD patients on the Mini-mental State Examination, and significant differences between lithium-treated and untreated AD patients began 3 months after the start of treatment and increased progressively (Nunes et al., 2013). However, significant cognitive improvement on the Mini-mental State Examination was not found in elderly individuals with mild to moderate AD treated with lithium for up to one year (Macdonald et al., 2008), and lithium treatment for 6 weeks did not improve cognition in a pilot study of AD patients (Pomara, 2009). Promising results with lithium contributed to the development of a phase II pilot study of the GSK3

inhibitor Tideglusib in patients with mild to moderate AD. This study established the safety and tolerability of this GSK3 inhibitor, and although there were trends towards improvement, further trials will be required to determine if cognitive impairments in AD are significantly improved (del Ser et al., 2013).

Altogether, although cognitive enhancement in animal models of AD is clearly evident following treatment with lithium or other GSK3 inhibitors, further studies will be needed, particularly including treatment that begins early in the disease, to determine if cognitive decline in patients with AD can be slowed by GSK3 inhibitors.

5. Effects of GSK3 inhibitors on cognition in Fragile X syndrome

Fragile X syndrome is the most common inherited cause of intellectual disability. Fragile X syndrome is caused by a trinucleotide repeat expansion in the X chromosome that silences the Fragile X Mental Retardation 1 gene, suppressing expression of Fragile X Mental Retardation Protein (Pieretti et al., 1991; Verkerk et al., 1991; Mines and Jope, 2011). The Fragile X (FX) mouse model lacks expression of Fragile X Mental Retardation Protein and displays many characteristics of Fragile X syndrome, including cognitive impairments, locomotor hyperactivity, social interaction deficits, increased audiogenic seizure susceptibility, and autistic-like behaviors, among others (Bakker et al., 1994; Kooy, 2003).

There have been several reports demonstrating that inhibition of GSK3 improves impaired cognition in FX mice. The first of these demonstrated that chronic dietary lithium treatment for three weeks reversed a learning deficit in adult male FX mice in the passive avoidance task (Yuskaitis et al., 2010), as did chronic dietary lithium treatment

that began at weaning (3 weeks old) and was continued to the ages of 8-11 weeks (Liu et al., 2011). Chronic dietary lithium treatment that began in adolescence (4 weeks old) and was continued for 8 weeks, or 4 weeks of dietary lithium treatment in adult FX mice, reversed deficits in FX mice in the visual object novelty detection task and temporal ordering for visual objects task, and improved spatial learning in the coordinate and categorical processing tasks (King and Jope, 2013). In a small open-label trial of lithium treatment in children and young adults with Fragile X syndrome, lithium treatment significantly improved cognition in the Repeatable Battery for the Assessment of Neuropsychological Status List Learning measure (Berry-Kravis et al., 2008). Thus, lithium administration to FX mice at a variety of ages improves cognitive abilities in several tasks, and preliminary evidence indicates that lithium also may be effective in patients.

Similar cognitive enhancing effects of other specific inhibitors of GSK3 indicate that the beneficial effects of lithium in FX mice likely result from its inhibition of GSK3. Chronic treatment of adult FX mice with the GSK3 inhibitor SB216763 (2 mg/kg; i.p.) every other day for 2 weeks improved performance in the contextual and tone trace conditioning tests and enhanced spatial learning in the delayed non-matching-to-place radial arm maze (Guo et al., 2012). Acute treatment of FX mice with the specific GSK3 inhibitors TDZD-8 (5 mg/kg; i.p.) or VP0.7 (5 mg/kg; i.p.) reversed impairments in visual object novelty detection task, temporal ordering for visual objects task, and spatial learning in the coordinate and categorical processing tasks (Franklin et al., 2013). Altogether, GSK3 inhibitors have provided more cognitive benefits in FX mice, as well as in humans with Fragile X syndrome, than any other therapeutic intervention.

6. Effects of GSK3 inhibitors on cognition in other diseases and disease models

6.1 Down syndrome

Down syndrome, caused by an extra copy of chromosome 21, is associated with intellectual disabilities that include language, verbal and spatial learning, and memory. The trisomic Ts65Dn mouse model re-capitulates some of the impaired cognitive abilities characteristic of Down syndrome (Reeves et al., 1995). Four weeks of lithium treatment in the food normalized impaired behavior of Ts65Dn mice in contextual fear conditioning, spatial memory, and novel object recognition tasks, changes that were associated with lithium-induced recovery of impairments in dentate gyrus LTP and neurogenesis (Contestabile et al., 2013).

6.2. Parkinson's disease

Parkinson's disease is a neurodegenerative disorder characterized by progressive degeneration of nigrostriatal dopaminergic neurons that causes memory impairments, sleep abnormalities, anxiety, depression, bradykinesia, tremor, and muscular rigidity (Dawson and Dawson, 2003; Chaudhuri et al., 2006). Administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MTPT) is widely used to model Parkinson's disease in rodents (Schober, 2004). Lithium treatment (47.5 mg/kg, i.p.) for seven days prior to a single bilateral intranasal administration of MPTP attenuated deficits in olfactory discrimination, social recognition, and short-term inhibitory avoidance memory impairment evaluated in the step-down inhibitory avoidance task induced by MPTP in Wistar rats, improvements that were associated with less striatal dopamine depletion in lithium-treated rats (Castro et al., 2012).

6.3. Spinocerebellar ataxia type 1 (SCA1)

SCA1 is a dominantly inherited neurodegenerative disorder caused by excess CAG repeats in the ataxin 1 gene, and is characterized by progressive loss of motor control and cognitive impairments (Watase et al., 2007). Sca1(1544Q/2Q) mice, a knockin mouse model created by targeting 154 CAG repeats into the endogenous mouse locus, display many features of human SCA1, including cognitive deficits, loss of motor coordination, premature death, Purkinje cell loss, and age-related hippocampal synaptic dysfunction (Watase et al., 2002). Chronic dietary lithium treatment ameliorated cognitive deficits in adult Sca1(1544Q/2Q) mice in the Morris water maze test and in contextual fear conditioning, whereas lithium treatment did not improve impaired cued fear conditioning, indicating that lithium treatment protects hippocampus-dependent cognitive functions in Sca1(1544Q/2Q) mice (Watase et al., 2007).

6.4. Traumatic brain injury (TBI)

TBI results from direct damage to the brain that causes many pathological effects that can continue to evolve over time, including changes in neuronal architecture and death, which contribute to learning and memory deficits (Dixon et al., 1991; Thompson et al., 2005; Blennow et al., 2012). Lithium pretreatment (1 mmol/kg; i.p.) for 2 weeks protected adult mice against TBI-induced impairments in the Morris water maze task, which was accompanied by attenuated neuronal degeneration (Zhu et al., 2010). Post-injury treatment with lithium (1 mEq/kg; s.c.) also improved performance in the Morris water maze that was accompanied by reduced hippocampal CA3 neuron loss (Dash et al., 2011). Following experimental TBI, rats treated with lithium (1 mEq/kg; s.c.) for two

weeks displayed improved hippocampal-dependent short term and long term spatial memory for platform location and quadrant preference in the Morris water maze, and daily treatment with the GSK3 inhibitor SB216763 (5 mg/kg; i.p.) for 5 days improved short term, but not long term, spatial memory (Dash et al., 2011). Post-TBI treatment with lithium (1.5 mEq/kg; i.p.) for up to 3 weeks also attenuated TBI-induced deficits in the Morris water maze and increased hippocampal-dependent learning and memory in the Y-maze test measured 10 days post-injury, which was associated with attenuated pathological markers (Yu et al., 2012).

6.5. Ischemic stroke

Ischemic stroke survivors exhibit impairments in cognition, sensation, perception and movement (Carmichael, 2003), and lithium reduces ischemia-induced neuronal damage in rodents (Nonaka and Chuang, 1998). Treatment of adult male rats with lithium (1 mmol/kg; i.p.) for 2 weeks prior to, and 9 days after, transient brain ischemia attenuated deficits in performance in the Morris water maze, which was associated with decreased ischemia-induced neuronal death (Yan et al., 2007).

6.6. HIV encephalitis

HIV patients with excessive neuroinflammation often exhibit severe cognitive deficits (Cherner et al., 2002). There is much interest in the potential therapeutic use of GSK3 inhibitors in HIV patients because of their neuroprotective and anti-inflammatory actions (Dewhurst et al., 2007; Crews et al., 2009). For example, 14 days of lithium treatment (2 mg/kg; i.p.) in 4 month old HIV-gp-120 transgenic mice provided protection

from gp120-mediated hippocampal toxicity and reduced dendritic damage (Everall et al., 2002). The first pilot study in fifteen cognitively impaired HIV patients found that 10 weeks of lithium treatment did not improve cognitive performance (The Dana Consortium, 1996). However, a subsequent study in eight patients with HIV-associated neurocognitive impairment found that lithium treatment for 12 weeks improved cognitive performance in all patients and cognitive impairment was eliminated in six patients (Letendre et al., 2006). Another study in eleven patients demonstrated that lithium treatment for 10 weeks did not improve cognition, but imaging indicated that lithium reduced CNS injury (Schifitto et al., 2009). Thus, although lithium clearly is neuroprotective, further studies appear warranted to clarify if GSK3 inhibitors reduce cognitive impairments in patients with HIV encephalitis.

6.7. Cerebral malaria

Cerebral malaria results from infection with *Plasmodium falciparum* and causes long-term cognitive impairments even in survivors with successful eradication of the parasite (Falchook et al., 2003; Boivin et al., 2007). Dai et al (2012) found that experimental cerebral malaria induced in mice caused significant hemorrhage in brain regions, cognitive impairment, and activation of GSK3 after eight days. Lithium treatment (20 mg/kg; i.p.) for 10 days in conjunction with chloroquine administration normalized cognitive deficits in infected mice in the object location test, suggesting that lithium may ameliorate some of the long-term neurological deficits associated with cerebral malaria (Dai et al., 2012).

6.8. Diabetes

People with diabetes have a higher rate of impaired learning, memory, and mental flexibility, and are at a higher risk for developing Alzheimer's disease than the general population, and learning deficits also occur in insulin-deficient mice. Insulin-deficient diabetes induced in rats by streptozotocin caused long-term memory deficits in the autoshaping learning task that were reversed by treatment with lithium given after the training task (Ponce-Lopez et al., 2011). Insulin-deficient diabetes induced in mice by treatment with streptozotocin impaired performances in the Barnes maze and the object recognition task that were attenuated by treatment with the GSK3 inhibitor AR-A014418 (30 μmol/kg; i.p.) (King et al., 2013). These results suggest that GSK3 inhibition may be useful for attenuating diabetes-associated cognitive deficits.

6.9. Postoperative cognition dysfunction

Postoperative cognition dysfunction, characterized by impairment of recent memory, concentration, language comprehension, and social integration, occurs in over 60% of older patients following surgery and anesthesia and can persist for weeks or months after surgery (Hovens et al., 2012). Treatment of 18 month old male rats with lithium (2 mmole/kg; i.p.) for seven days prior to exploratory laparotomy attenuated surgery-induced impaired performance in the Morris water maze (Zhao et al., 2011).

7. GSK3 inhibitors can improve treatment-induced cognitive impairments

GSK3 inhibition has been found to reduce cognitive impairments that were induced in rodents by several different treatments. Cranial irradiation therapy is a

common treatment for brain tumors, and although cancer cure rates are improved, learning disorders and memory deficits commonly occur following treatment in children and adults (Roman and Sperduto, 1995). Pretreatment of mouse pups with lithium (40 mg/kg; i.p.) for one week prior to cranial irradiation improved performance in the Morris water maze task tested six weeks after irradiation (Yazlovitskaya et al., 2006). Similarly, pretreatment with the GSK3 inhibitors SB216763 (0.6 mg/kg; i.p.) or SB415286 (1 mg/kg; i.p.) for 3 days before cranial irradiation improved Morris water maze performance in irradiated mice (Thotala et al., 2008). In addition, Khasraw et al (2012) noted that lithium treatment reduces radiation-induced gliosis that can contribute to decreased neurogenesis and cognitive deficits. A phase I clinical trial in which five cancer patients were treated with lithium one week before cranial irradiation showed no decline in short term memory of these patients in global and spatial memory test (Yang et al., 2007).

In addition to cranial radiation, GSK3 inhibitors also provided protection from cognitive impairments induced by a variety of other treatments. Chronic lithium treatment (5.0 to 7.5 mEq/kg; orally; 3 times/day) of 8 rhesus monkeys between the ages of 13 and 30 years restored working memory on the delayed response task after impairment induced by cirazoline treatment, an adrenergic receptor agonist (Birnbaum et al., 2004). Chronic stress impaired spatial memory in the Morris water maze task in rats, and this was prevented by four weeks of lithium treatment in the food (Vasconcellos et al., 2003; de Vasconcellos et al., 2005). Infusion of the protein kinase A inhibitor H-89 into the hippocampal CA1 region of rats impaired spatial memory retention in the Morris water maze task, which was prevented by four weeks of pretreatment with lithium (600

mg/L in the drinking water) (Sharifzadeh et al., 2007). Administration of the anesthetic sevoflurane to rats activated GSK3 and impaired memory consolidation, both of which were reversed by acute lithium treatment (100 mg/kg; i.p.) (Liu et al., 2010). Deficits in an autoshaping learning task induced in male rats by intracerebroventricular infusion of streptozotocin for 2 weeks were reversed by acute treatment with lithium (100 mg/kg; i.p.) (Ponce-Lopez et al., 2011). Intracerebroventricular infusion of angiotensin II to rats increased GSK3β activity and induced deficits in the Morris water maze, which was reversed by treatment with the GSK3 inhibitor SB216763 (20 ng/µl; icv) (Tian et al., 2012). Memory impairment in the contextual fear conditioning task induced by administration to mice of MK-801, an N-methyl-D-aspartate receptor antagonist, was reversed by treatment twice daily for 3 days with the GSK3 inhibitor AZD1080 (4 or 15 µmol/kg; oral gavage) (Georgievska et al., 2013). Lithium treatment (1 or 4 ml/kg of 0.15 M lithium) also attenuated ouabain-induced impairments in the Morris water maze (Wang et al., 2013). Adult offspring of poly(I:C)-exposed mothers, an infection-based mouse model of neuropsychiatric disease, displayed deficits in the spontaneous alternation in the Y-maze cognitive task that were alleviated by acute administration of the GSK3 inhibitor TDZD-8 (1 or 10 mg/kg; i.p.) (Willi et al., 2013). The diversity of chemicals and treatments used to induce cognitive deficits that were ameliorated by GSK3 inhibitors indicates that protection was unlikely due to blocking the action of the insult, but more likely due to protection of a fundamental component of the cognitive process.

8. Effects of lithium treatment on cognition in patients with bipolar disorder

Lithium was the first mood stabilizer used to successfully treat bipolar disorder, previously known as manic-depression, and much evidence indicates that inhibition of GSK3 by lithium is an important component of its therapeutic action (Jope, 2011). Although previously often overlooked, it is now recognized that significant cognitive deficits are associated with bipolar disorder, such as impairments in verbal memory, processing speed, and attention, and that these can persist even during clinical remission (Clark et al., 2002; Martinez-Aran et al., 2004). Furthermore, several studies have concluded that bipolar disorder patients exhibit cognitive impairments in all phases of the disorder and that these are a fundamental characteristic of bipolar disorder, not a consequence of medications (Smigan and Perris, 1983; Engelsmann et al., 1988; Robinson and Ferrier, 2006; Mur et al., 2008; López-Jaramillo et al., 2010).

Since lithium is the classical mood stabilizer, many investigators have examined its effects on cognition in bipolar disorder patients, but results have been contradictory and the issue remains unsettled (Balanzá-Martínez et al., 2010; Dias et al., 2012). Many studies concluded that cognitive abilities of lithium-treated bipolar disorder patients did not differ from those free of medication (Marusarz et al., 1981; Lund et al., 1982; Smigan and Perris, 1983; Engelsmann et al., 1988; Joffe et al.,1988; Sharma and Singh, 1988; Jauhar et al., 1993; Van Gorp et al., 1998; El-Badri et al., 2001; Clark et al., 2002; Altshuler et al., 2004; Mur et al., 2008; López-Jaramillo et al., 2010; Arts et al., 2011). On the other hand, several studies found that lithium has a mild negative effect on cognition in bipolar disorder patients. Cognitive domains that have been reported to be worsened after lithium treatment of bipolar disorder patients include verbal learning and

memory, attention, and short-term memory (Christodoulou et al., 1981; Elsass et al., 1981; Loo et al., 1981; Shaw et al., 1987; Hatcher et al., 1990; Honig et al., 1999; Pachet and Wisniewski, 2003; Wingo et al., 2009). However, there are also reports of no lithium-induced impairments of visuospatial skills, visual memory, delayed verbal memory, attention, and executive performance (Honig et al., 1999; Pachet and Wisniewski, 2003, Wingo et al., 2009). A review of the effects of lithium in bipolar disorder patients concluded that there were five consistent findings: "impairment on tasks of psychomotor speed, impaired functioning in the majority of studies examining verbal memory, no impairment on tasks of visuospatial constructional ability or attention/ concentration, and no negative cumulative effect" (Pachet and Wisniewski, 2003). A meta-analysis concluded that in bipolar disorder patients "lithium treatment appears to have only few and minor negative effects on cognition" (Wingo et al., 2009). These investigators also pointed out that in many studies there have been methodological and statistical limitations, non-homogeneous patient populations, diverse research designs, varied diagnostic methods, and a number of uncontrolled variables (Pachet and Wisniewski, 2003; Wingo et al., 2009; Balanzá-Martínez et al., 2010; Dias et al., 2012). Altogether, the effect of lithium on cognitive dysfunction in bipolar disorder patients remains unresolved, but it is evidently not beneficial for the cognitive facet of bipolar disorder and may be moderately detrimental for specific functions, but a conclusive resolution will require further well-designed studies. Thus, despite the many conditions in which lithium improves cognitive performance reviewed here, it is rather ironical that lithium does not do so in bipolar disorder, the preeminent condition that lithium has proven therapeutic efficacy on the defining characteristic of the disorder.

9. Mechanisms underlying improved cognition following GSK3 inhibition

Multiple mechanisms appear to contribute to the cognition-protecting actions of GSK3 inhibitors, several of which include the following.

9.1. Long-term potentiation (LTP) and long-term depression (LTD)

The most direct mechanisms known by which GSK3 may regulate cognitive functions are by influencing the processes of LTP and LTD (Bradley et al., 2012). LTP and LTD are components of synaptic plasticity that are thought to be critical regulators of learning and memory, and GSK3 is intimately involved in both processes. LTP increases the inhibitory serine-phosphorylation of GSK3 and overexpression or activation of GSK3 impairs LTP (Hooper et al., 2007; Zhu et al., 2007). These findings indicate that GSK3 has to be inactivated for optimal establishment of LTP, raising the possibility that pathologically active GSK3 may impair LTP in conditions associated with impaired cognition. In contrast, LTD increases GSK3 activity and inhibition of GSK3 prevents the induction of LTD, demonstrating that active GSK3 supports the induction of LTD (Peineau et al., 2007). Thus, inhibition of GSK3 facilitates LTP, but GSK3 activity is required for LTD, indicating that lithium and other GSK3 inhibitors may prevent impairments in LTP and reduce the induction of LTD. Support for this relationship comes from studies demonstrating that the rescue of abnormal LTP and/or LTD following treatment with GSK3 inhibitors is accompanied by increased cognition in mouse models of Fragile X syndrome (Choi et al., 2011; Franklin et al., 2013), AD (Ma et al., 2010; Li et al., 2012), and Down syndrome (Contestabile et al., 2013). These studies indicate that

GSK3 inhibition may improve learning and memory impairments in some conditions by regulating hippocampal synaptic plasticity.

9.2. Neurogenesis

Part of the improvement in cognition provided by administration of GSK3 inhibitors in rodent models of diseases with impaired cognition may be due to increased neurogenesis. The discovery that new neurons are generated in the adult hippocampus led to an explosion of studies to identify the functional consequences of neurogenesis, and to determine if impaired neurogenesis contributes to CNS diseases and is bolstered by therapeutic drugs (Lie et al., 2004). Neurogenesis appears to support certain forms of learning and memory and may be defective in some conditions associated with impairments in cognition (van Praag et al., 2005; Leuner et al., 2006; Deng et al., 2010; Massa et al., 2011). Evidence indicates that dysregulated GSK3 may contribute to deficient neurogenesis in some conditions because neurogenesis is impaired by constitutively active GSK3 in mice (Eom and Jope, 2009), and molecular deletion of GSK3 in mouse neural progenitors increased neurogenesis (Kim et al., 2009). Thus, in diseases such as depression, Fragile X syndrome, and Alzheimer's disease in which GSK3 in the CNS is abnormally active, an outcome may be diminished neurogenesis and consequently cognitive impairments. Conversely, neurogenesis is increased by treatment with lithium or other drugs that inhibit GSK3 (Chen et al., 2000: Hashimoto et al., 2003; Silva et al., 2008; Wexler et al., 2008; Kim et al., 2009; Morales-Garcia et al., 2012), and treatment with the GSK3 inhibitor SB216763 (2 mg/kg; i.p.) every other day for 2 weeks increased neurogenesis that is impaired in mice expressing DISC1 mutations (Mao et al., 2009).

Thus, administration of GSK3 inhibitors may improve cognition in part by restoring impairments in neurogenesis. This relationship has been supported by studies reporting increased neurogenesis following administration of GSK3 inhibitors that was correlated with improved cognition in mouse models of Fragile X syndrome (Guo et al., 2012), Down syndrome (Contestabile et al., 2013), and AD (Serenó et al., 2009; Fiorentini et al., 2010; Jo et al., 2011).

9.3. Inflammation

GSK3 inhibitors may improve deficient cognitive functions in part by their anti-inflammatory effects. Many CNS diseases are accompanied by neuroinflammation, which often exacerbates impaired neuronal function and survival. One critical outcome of neuroinflammation is the impairment of a broad range of cognitive functions (Streck et al., 2008). Since GSK3 was first identified as an important promoter of inflammatory responses in peripheral cells (Martin et al., 2005), an equivalent role for GSK3 has been established in the CNS (Beurel, 2011). Thus, GSK3 inhibitors diminish many inflammatory responses by both astrocytes and microglia in the CNS, as well as by peripheral immune cells, reducing inflammation in the CNS (Beurel and Jope, 2009; Cheng et al., 2009; Yuskaitis and Jope, 2009). Transcription factors represent a key group of targets by which GSK3 inhibitors reduce inflammation, including suppressing promoters of inflammation such as transcription factors in the STAT (signal transducer and activator of transcription) family and NF-κB, and amplifying anti-inflammatory actions of the transcription factor CREB (cyclic AMP response element binding protein) (Martin et al., 2005; Beurel and Jope, 2008). Anti-inflammatory actions of GSK3

inhibitors are sufficient to profoundly diminish diseases in which inflammation has a major impact, such as sepsis and the mouse model of multiple sclerosis (Martin et al., 2005; De Sarno et al., 2008; Beurel et al., 2011; Beurel et al., 2013). Inflammation is well-known to accompany and exacerbate neurodegenerative diseases, such as AD, and neuroinflammation is also now recognized as an important component of the disease process in psychiatric diseases, such as depression (Raison and Miller, 2013) and schizophrenia (Mansur et al., 2012). Thus, by reducing inflammation, GSK3 inhibitors may improve cognitive functions otherwise damaged by inflammatory molecules in many neurological and psychiatric diseases involving neuroinflammation, such as ischemia, traumatic brain injury and AD.

9.4. Neuroprotection

One of the most notorious actions of dysregulated GSK3 is its promotion of apoptosis, an outcome thought to frequently contribute to neurological diseases (Beurel and Jope, 2006). It appears that GSK3 promotes the intrinsic apoptotic signaling pathway following exposure to apparently any insult capable of inducing this response. Reciprocally, the ability of GSK3 inhibitors to reduce apoptosis is an important part of their well-recognized characteristic as neuroprotective agents. This neuroprotective capacity may underlie several of the reported cognition-protective effects of GSK3 inhibitors discussed above, particularly in neurodegenerative diseases. For example, GSK3 can promote apoptosis in conditions modeling AD (Mines et al., 2011) and Parkinson's disease (King et al., 2001; Chen et al., 2004). Thus, administration of GSK3

inhibitors may diminish cognition impairments in some conditions by reducing apoptotic loss of neurons rather than by a direct effect on cognitive mechanisms.

In addition to apoptosis, GSK3 inhibitors also provide neuroprotective resilience by a number of mechanisms, such as reducing neuronal dysfunction resulting from endoplasmic reticulum stress (Song et al., 2002; Meares et al., 2011) and oxidative stress (Schäfer et al., 2004; King and Jope, 2005), and by promoting the production of intracellular chaperone proteins (Chu et al., 1996; Bijur and Jope, 2000). Besides being implicated in neurodegenerative diseases, these conditions associated with impaired neuronal function have been linked to many psychiatric and neurological diseases (Bown et al., 2000; Lin and Beal, 2006; Andreazza et al., 2010; Roussel et al., 2013). Thus, bolstering neuronal resilience, as well as reducing apoptosis, may contribute to the capacity of GSK3 inhibitors to reduce disease-associated impairments in cognition.

10. Conclusions

Impairment of cognition is a devastating outcome of many conditions affecting the CNS. In a remarkable number and broad spectrum of conditions that cause cognitive impairments in rodents, inhibition of GSK3 protects cognitive processes or promotes their repair (Figure 1). One limitation of the reported pharmacological intervention studies is that the majority have relied on lithium, and have not compared its effects with other GSK3 inhibitors. Thus there is a great need for cognition studies using other GSK3 inhibitors, as well as for further studies of rodents with molecular modifications of GSK3, to verify that GSK3 is the therapeutic target protecting cognitive processes, and to provide alternatives to lithium for therapeutic interventions. The current state of research

is also limited by the relatively small number of different GSK3 inhibitors that have been tested in cognition studies, which is surprising because many GSK3 inhibitors have been developed during the last decade (Eldar-Finkelman and Martinez, 2011). Additionally, little information is available from comparative studies using several GSK3 inhibitors in cognition studies, or in studies of the duration of their effectiveness. Thus, the reviewed studies have provided strong initial evidence that GSK3 inhibitors have the potential to ameliorate cognitive impairments, but further studies are needed to identify the most efficacious drugs and those that are effective during prolonged administration. Furthermore, we should emphasize that there is likely significant overlap in behaviors that are commonly labeled as being related to cognition, depression, mania, anxiety, and others, so that drug effects on one may certainly affect others. Additionally, clarification of the mechanisms underlying the cognitive enhancing actions of GSK3 inhibitors in each condition would provide a better understanding of the causes of cognitive decline and of the mechanisms that may be exploited in the development of improved interventions. It is unlikely that a single mechanism accounts for all of the reported cognition-protecting effects of GSK3 inhibitors. We have discussed four mechanisms that are likely to play a role in different conditions, but this short list does not exclude additional mechanisms of action. Most importantly, considering the prevalence and devastating consequences of loss of cognitive functions, and the dearth of efficacious interventions, the findings summarized here emphasize the importance of greater development and utilization of GSK3 inhibitors to treat conditions causing cognitive impairments.

Acknowledgments

Research in the authors' laboratories was supported by grants from the NIMH (MH038752, MH090236, MH095380)

References

Andreazza, A. C., Shao, L., Wang, J. F., & Young, L. T. (2010). Mitochondrial complex I activity and oxidative damage to mitochondrial proteins in the prefrontal cortex of patients with bipolar disorder. *Arch Gen Psychiatry 67*, 360-368.

Altshuler, L. L., Ventura, J., van Gorp, W. G., Green, M. F., Theberge, D. C., & Mintz, J. (2004). Neurocognitive function in clinically stable men with bipolar I disorder or schizophrenia and normal control subjects. *Biol Psychiatry 56*, 560-569.

Arts, B., Jabben, N., Krabbendam, L., & van Os, J. (2011). A 2-year naturalistic study on cognitive functioning in bipolar disorder. *Acta Psychiatr Scand* 123, 190-205.

Avrahami, L., Farfara, D., Shaham-Kol, M., Vassar, R., Frenkel, D., & Eldar-Finkelman, H. (2013). Inhibition of glycogen synthase kinase-3 ameliorates β-amyloid pathology and restores lysosomal acidification and mammalian target of rapamycin activity in the Alzheimer disease mouse model: in vivo and in vitro studies. *J Biol Chem 288*, 1295-1306.

Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C. J., McLauchlan, H., et al. (2007). The selectivity of protein kinase inhibitors: a further update. *Biochem J 408*, 297-315.

Bakker, C. E., et al., & Dutch-Belgian Fragile X Consortium. (1994). Fmr1 knockout mice: a model to study fragile X mental retardation. *Cell 78*, 23-33.

Balanzá-Martínez, V., Selva, G., Martínez-Arán, A., Prickaerts, J., Salazar, J., González-Pinto, A., et al. (2010). Neurocognition in bipolar disorders--a closer look at comorbidities and medications. *Eur J Pharmacol 626*, 87-96.

Berry-Kravis, E., Sumis, A., Hervey, C., Nelson, M., Porges, S. W., Weng, N., et al. (2008). Open-label treatment trial of lithium to target the underlying defect in fragile X syndrome. *J Dev Behav Pediatr 29*, 293-302.

Beurel, E. (2011). Regulation by glycogen synthase kinase-3 of inflammation and T cells in CNS diseases. *Front Mol Neurosci 4*, 18.

Beurel, E., & Jope, R. S. (2006). The paradoxical pro- and anti-apoptotic actions of GSK3 in the intrinsic and extrinsic apoptosis signaling pathways. *Prog Neurobiol 79*, 173-189.

Beurel, E., & Jope, R. S. (2008). Differential regulation of STAT family members by glycogen synthase kinase-3. *J Biol Chem 283*, 21934-21944.

Beurel, E., & Jope, R. S. (2009). Lipopolysaccharide-induced interleukin-6 production is controlled by glycogen synthase kinase-3 and STAT3 in the brain. *J Neuroinflammation 11*, 6-9.

Beurel, E., Yeh, W. I., Michalek, S. M., Harrington, L. E., & Jope, R. S. (2011). Glycogen synthase kinase-3 is an early determinant in the differentiation of pathogenic Th17 cells. *J Immunol 186*, 1391-1398.

Beurel, E., Kaidanovich-Beilin, O., Yeh, W. I., Song, L., Palomo, V., Michalek, S. M., et al. (2013). Regulation of Th1 cells and experimental autoimmune encephalomyelitis (EAE) by glycogen synthase kinase-3. *J Immunol 190*, 5000-5011.

Bhat, R., Xue, Y., Berg, S., Hellberg, S., Ormö, M., Nilsson, Y., et al. (2003). Structural insights and biological effects of glycogen synthase kinase 3-specific inhibitor AR-A014418. *J Biol Chem 278*, 45937-45945.

Bijur, G. N., & Jope, R. S. (2000). Opposing actions of phosphatidylinositol 3-kinase and glycogen synthase kinase-3β in the regulation of HSF-1 activity. *J Neurochem 75*, 2401-2408.

Birnbaum, S. G., Yuan, P. X., Wang, M., Vijayraghavan, S., Bloom, A. K., Davis, D. J., et al. (2004). Protein kinase C overactivity impairs prefrontal cortical regulation of working memory. *Science 306*, 882-884.

Blennow, K., Hardy, J., & Zetterberg, H. (2012). The neuropathology and neurobiology of traumatic brain injury. *Neuron 76*, 886-899.

Boivin, M. J., Bangirana, P., Byarugaba, J., Opoka, R. O., Idro, R., Jurek, A. M. et al. (2007). Cognitive impairment after cerebral malaria in children: A prospective study. *Pediatrics 119*, e360–e366.

Bown, C., Wang, J. F., MacQueen, G., & Young, L. T. (2000). Increased temporal cortex ER stress proteins in depressed subjects who died by suicide. *Neuropsychopharmacol 22*, 327-332.

Bradley, C. A., Peineau, S., Taghibiglou, C., Nicolas, C. S., Whitcomb, D. J., Bortolotto, Z. A., et al. (2012). A pivotal role of GSK-3 in synaptic plasticity. *Front Mol Neurosci 5*, 13.

Caccamo, A., Oddo, S., Tran, L. X., & LaFerla, F. M. (2007). Lithium reduces tau phosphorylation but not Aβ or working memory deficits in a transgenic model with both plaques and tangles. *Am J Pathol 170*, 1669-1675.

Carmichael, S. T. (2003). Plasticity of cortical projections after stroke. *Neuroscientist 9*, 64-75.

Castro, A. A., Ghisoni, K., Latini, A., Quevedo, J., Tasca, C. I., & Prediger, R. D. (2012). Lithium and valproate prevent olfactory discrimination and short-term memory

impairments in the intranasal 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) rat model of Parkinson's disease. *Behav Brain Res 229*, 208-215.

Chaudhuri, K. R., Healy, D. G., Schapira, A. H., & National Institute for Clinical Excellence. (2006). Non-motor symptoms of Parkinson's disease: diagnosis and management. *Lancet Neurol 11*, 1116-1125.

Chen, G., Rajkowska, G., Du, F., Seraji-Bozorgzad, N., & Manji, H. K. (2000). Enhancement of hippocampal neurogenesis by lithium. *J Neurochem 75*, 1729-1734.

Chen, G., Bower, K. A., Ma, C., Fang, S., Thiele, C. J., & Luo, J. (2004). Glycogen synthase kinase 3β (GSK3β) mediates 6-hydroxydopamine-induced neuronal death. *FASEB J 18*, 1162-1164.

Cheng, Y. L., Wang, C. Y., Huang, W. C., Tsai, C. C., Chen, C. L., Shen, C. F., et al. (2009). Staphylococcus aureus induces microglial inflammation via a glycogen synthase kinase 3β-regulated pathway. *Infect Immun 77*, 4002-4008.

Cherner, M., Masliah, E., Ellis, R. J., Marcotte, T. D., Moore, D. J., Grant, I., et al. (2002). Neurocognitive dysfunction predicts postmortem findings of HIV encephalitis. *Neurology 59*, 1563-1567.

Choi, C. H., Schoenfeld, B. P., Bell, A. J., Hinchey, P., Kollaros, M., Gertner, M. J., et al. (2011). Pharmacological reversal of synaptic plasticity deficits in the mouse model of fragile X syndrome by group II mGluR antagonist or lithium treatment. *Brain Res 1380*, 106-119.

Christodoulou, G. N., Kokkevi, A., Lykouras, E. P., Stefanis, C. N., & Papadimitriou, G. N. (1981). Effects of lithium on memory. *Am J Psychiatry 138*, 847–848.

Chu, B., Soncin, F., Price, B. D., Stevenson, M. A., & Calderwood, S. K. (1996). Sequential phosphorylation by mitogen-activated protein kinase and glycogen synthase kinase 3 represses transcriptional activation by heat shock factor-1. *J Biol Chem 271*, 30847-30857.

Clark, L., Iversen, S. D., & Goodwin, G. M. (2002). Sustained attention deficit in bipolar disorder. *Br J Psychiatry 180*, 313–319.

Coghlan, M. P., Culbert, A. A., Cross, D. A., Corcoran, S. L., Yates, J. W., Pearce, N. J., et al. (2000) Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem Biol 7*, 793-803.

Contestabile, A., Greco, B., Ghezzi, D., Tucci, V., Benfenati, F., & Gasparini, L. (2013). Lithium rescues synaptic plasticity and memory in Down syndrome mice. *J Clin Invest 123*, 348-361.

Crews, L., Patrick, C., Achim, C. L., Everall, I. P., & Masliah, E. (2009). Molecular pathology of neuro-AIDS (CNS-HIV). *Int J Mol Sci 10*, 1045-1063.

Dai, M., Freeman, B., Shikani, H. J., Bruno, F. P., Collado, J. E., Macias, R., et al. (2012). Altered regulation of Akt signaling with murine cerebral malaria, effects on long-term neuro-cognitive function, restoration with lithium treatment. *PLoS One 7*, e44117.

Dash, P. K., Johnson, D., Clark, J., Orsi, S. A., Zhang, M., Zhao, J., et al. (2011). Involvement of the glycogen synthase kinase-3 signaling pathway in TBI pathology and neurocognitive outcome. *PLoS One 6*, e24648

Davies, S. P., Reddy, H., Caivano, M., & Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J 351*, 95-105.

Dawson, T. M., & Dawson, V. L. (2003). Molecular pathways of neurodegeneration in Parkinson's disease. *Science 302*, 819-822.

De Ferrari, G. V., Chacón, M. A., Barría, M. I., Garrido, J. L., Godoy, J. A., Olivares, G., et al. (2003). Activation of Wnt signaling rescues neurodegeneration and behavioral impairments induced by β-amyloid fibrils. *Mol Psychiatry 8*, 195-208.

De Sarno, P., Axtell, R. C., Raman, C., Roth, K. A., Alessi, D. R., & Jope, R. S. (2008). Lithium prevents and ameliorates experimental autoimmune encephalomyelitis. *J Immunol. 181*, 338-345.

de Vasconcellos, A. P., Zugno, A. I., Dos Santos, A. H., Nietto, F. B., Crema, L. M., Gonçalves, M., et al. (2005). Na+,K(+)-ATPase activity is reduced in hippocampus of rats submitted to an experimental model of depression: effect of chronic lithium treatment and possible involvement in learning deficits. *Neurobiol Learn Mem 84*, 102-110.

del Ser, T., Steinwachs, K. C., Gertz, H. J., Andrés, M. V., Gómez-Carrillo, B., Medina, M., et al. (2013). Treatment of Alzheimer's disease with the GSK-3 inhibitor tideglusib: a pilot study. *J Alzheimers Dis 33*, 205-215.

Deng, W., Aimone, J. B., & Gage, F. H. (2010). New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? *Nat Rev Neurosci 11*, 339-350.

Dewachter, I., Ris, L., Jaworski, T., Seymour, C. M., Kremer, A., Borghgraef, P., et al. (2009). GSK3β, a centre-staged kinase in neuropsychiatric disorders, modulates long term memory by inhibitory phosphorylation at serine-9. *Neurobiol Dis 35*, 193-200.

Dewhurst, S., Maggirwar, S. B., Schifitto, G., Gendelman, H. E., & Gelbard, H. A. (2007). Glycogen synthase kinase 3 β (GSK-3β) as a therapeutic target in neuroAIDS. *J Neuroimmune Pharmacol 2*, 93–96

Dias, V. V., Balanzá-Martinez, V., Soeiro-de-Souza, M. G., Moreno, R. A., Figueira, M. L., Machado-Vieira, R., et al. (2012). Pharmacological approaches in bipolar disorders and the impact on cognition: a critical overview. *Acta Psychiatr Scand 126*, 315-331.

Ding, Y., Qiao, A., & Fan, G. H. (2010). Indirubin-3'-monoxime rescues spatial memory deficits and attenuates β-amyloid-associated neuropathology in a mouse model of Alzheimer's disease. *Neurobiol Dis 39*, 156-168.

Dixon, C. E., Clifton, G. L., Lighthall, J. W., Yaghmai, A. A., & Hayes, R. L. (1991). A controlled cortical impact model of traumatic brain injury in the rat. *J Neurosci Methods 39*, 253-262.

El-Badri, S. M., Ashton, C. H., Moore, P. B., Marsh, V. R., & Ferrier, I. N. (2001). Electrophysiological and cognitive function in young euthymic patients with bipolar affective disorder. *Bipolar Disord 3*, 79 -87.

Eldar-Finkelman, H., & Martinez, A. (2011). GSK-3 inhibitors: preclinical and clinical focus on CNS. *Front Mol Neurosci 4*, 32.

Elsass, P., Mellerup, E. T., Rafaelsen, O. J., & Theilgaard, A. (1981). Effect of lithium on reaction time. A study of diurnal variations. *Psychopharmacology 72*, 279-282.

Engel, T., Hernández, F., Avila, J., & Lucas, J. J. (2006). Full reversal of Alzheimer's disease-like phenotype in a mouse model with conditional overexpression of glycogen synthase kinase-3. *J Neurosci 26*, 5083-5090.

Engelsmann, F., Katz, J., Ghadirian, A. M., & Schachter, D. (1988). Lithium and memory: a long-term following study. *J Clin Psychopharmacol 8*, 207–212.

Eom, T. Y., & Jope, R. S. (2009). Blocked inhibitory serine-phosphorylation of glycogen synthase kinase-3α/β impairs in vivo neural precursor cell proliferation. *Biol Psychiatry 66*, 494-502.

Everall, I. P., Bell, C., Mallory, M., Langford, D., Adame, A., Rockestein, E., et al. (2002). Lithium ameliorates HIV-gp120-mediated neurotoxicity. Mol Cell Neurosci. 21, 493-501.

Falchook, G. S., Malone, C. M., Upton, S., & Shandera, W. X. (2003). Postmalaria neurological syndrome after treatment of Plasmodium falciparum malaria in the United States. *Clin Infect Dis 37*, e22-24

Fiorentini, A., Rosi, M. C., Grossi, C., Luccarini, I., & Casamenti, F. (2010). Lithium improves hippocampal neurogenesis, neuropathology and cognitive functions in APP mutant mice. *PLoS One 5*, e14382.

Forlenza, O. V., Diniz, B. S., Radanovic, M., Santos, F. S., Talib, L. L., & Gattaz, W. F. (2011). Disease-modifying properties of long-term lithium treatment for amnestic mild cognitive impairment: randomised controlled trial. *Br J Psychiatry 198*, 351-356.

Forlenza, O. V., de Paula, V. J., Machado-Vieira, R., Diniz, B. S., & Gattaz, W.F. (2012). Does lithium prevent Alzheimer's disease? *Drugs Aging 29*, 335-342.

Franklin, A. V., King, M. K., Palomo, V., Martinez, A., McMahon, L., & Jope, R. S. (2013). Glycogen synthase kinase-3 inhibitors reverse deficits in long-term potentiation and cognition in Fragile X mice. *Biol Psychiatry*, in press .

Georgievska, B., Sandin, J., Doherty, J., Mörtberg, A., Neelissen, J., Andersson, A., et al. (2013). AZD1080, a novel GSK3 inhibitor, rescues synaptic plasticity deficits in rodent brain and exhibits peripheral target engagement in humans. *J Neurochem 125*, 446-456.

Ghosal, K., Vogt, D. L., Liang, M., Shen, Y., Lamb, B. T., & Pimplikar, S.W. (2009). Alzheimer's disease-like pathological features in transgenic mice expressing the APP intracellular domain. *Proc Natl Acad Sci U S A 106*, 18367-18372.

Gómez de Barreda, E., Pérez, M., Gómez Ramos, P., de Cristobal, J., Martín-Maestro, P., Morán, A., et al. (2010). Tau-knockout mice show reduced GSK3-induced hippocampal degeneration and learning deficits. *Neurobiol Dis 37*, 622-629.

Guo, W., Murthy, A. C., Zhang, L., Johnson, E. B., Schaller, E. G., Allan, A. M., et al. (2012). Inhibition of GSK3β improves hippocampus-dependent learning and rescues neurogenesis in a mouse model of fragile X syndrome. *Hum Mol Genet 21*, 681-691.

Hardy, J., Duff, K., Hardy, K. G., Perez-Tur, J., & Hutton, M. (1998). Genetic dissection of Alzheimer's disease and related dementias: amyloid and its relationship to tau. *Nat Neurosci 1*, 355-358.

Hardy, J., & Selkoe, D. J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science 297*, 353-536.

Hashimoto, R., Senatorov, V., Kanai, H., Leeds, P., & Chuang, D. M. (2003). Lithium stimulates progenitor proliferation in cultured brain neurons. *Neuroscience 117*, 55-61.

Hatcher, S., Sims, R., & Thompson, D. (1990). The effects of chronic lithium treatment on psychomotor performance related to driving. *Br J Psychiatry 157*, 275–278.

Hernández, F., Borrell, J., Guaza, C., Avila, J., & Lucas, J. J. (2002). Spatial learning deficit in transgenic mice that conditionally over-express GSK-3β in the brain but do not form tau filaments. *J Neurochem 83*, 1529-1533.

Hicks, J. W., VanBrocklin, H. F., Wilson, A. A., Houle, S., & Vasdev, N. (2010). Radiolabeled small molecule protein kinase inhibitors for imaging with PET or SPECT. *Molecules 15*, 8260-8278.

Hong, M., Chen, D. C., Klein, P. S., & Lee, V. M. (1997). Lithium reduces tau phosphorylation by inhibition of glycogen synthase kinase-3. *J Biol Chem 272*, 25326-25332.

Honig, A., Arts, B. M., Ponds, R. W., & Riedel, W. J. (1999). Lithium induced cognitive side effects in bipolar disorder: a qualitative analysis and implications for daily practice. *Int Clin Psychopharmacol 14*, 167-171.

Hooper, C., Markevich, V., Plattner, F., Killick, R., Schofield, E., Engel, T., et al. (2007). Glycogen synthase kinase-3 inhibition is integral to long-term potentiation. *Eur J Neurosci 25*, 81-86

Hovens, I. B., Schoemaker, R. G., van der Zee, E. A., Heineman, E., Izaks, G. J., & van Leeuwen, B. L. (2012). Thinking through postoperative cognitive dysfunction: How to bridge the gap between clinical and pre-clinical perspectives. *Brain Behav Immun 26*, 1169-1179.

Hu, S., Begum, A. N., Jones, M. R., Oh, M. S., Beech, W. K., Beech, B. H., et al. (2009). GSK3 inhibitors show benefits in an Alzheimer's disease (AD) model of neurodegeneration but adverse effects in control animals. *Neurobiol Dis* 33, 193-206.

Hurtado, D. E., Molina-Porcel, L., Carroll, J. C., Macdonald, C., Aboagye, A. K., Trojanowski, J. Q., et al. (2012). Selectively silencing GSK-3 isoforms reduces plaques and tangles in mouse models of Alzheimer's disease. *J Neurosci 32*, 7392-7402.

Jauhar, P., Mcclure, I., Hillary, C., & Watson, A. (1993). Psychomotor performance of patients on maintenance lithium therapy. *Hum Psychopharmacol 8*, 141-144.

Jo, J., Whitcomb, D. J., Olsen, K. M., Kerrigan, T. L., Lo, S. C., Bru-Mercier, G., et al. (2011). Aβ(1-42) inhibition of LTP is mediated by a signaling pathway involving caspase-3, Akt1 and GSK-3β. *Nat Neurosci 14*, 545-547.

Joffe, R. T., MacDonald, C., & Kutcher, S. P. (1988). Lack of differential cognitive effects of lithium and carbamazepine in bipolar affective disorder. *J Clin Psychopharmacol 8*, 425-428.

Jolivalt, C. G., Hurford, R., Lee, C. A., Dumaop, W., Rockenstein, E., & Masliah, E. (2010). Type 1 diabetes exaggerates features of Alzheimer's disease in APP transgenic mice. *Exp Neurol 223*, 422-431.

Jope, R. S. (2003). Lithium and GSK3: one inhibitor, two inhibitory actions, multiple outcomes. *Trends Pharmacol Sci 24*, 441-443.

Jope, R. S. (2011). Glycogen synthase kinase-3 in the etiology and treatment of mood disorders. *Frontiers in Molecular Neuroscience 4*, 16-26.

Jope, R. S., & Johnson, G. V. W. (2004). The glamour and gloom of glycogen synthase kinase-3 (GSK3). *Trends Biochem Sci 29*, 95-102.

Kaidanovich-Beilin, O., A. Milman, A. Weizman, C. G. Pick, & H. Eldar-Finkelman. (2004). Rapid antidepressive-like activity of specific glycogen synthase kinase-3 inhibitor and its effect on β-catenin in mouse hippocampus. *Biol Psychiatry 55*, 781-784.

Kaidanovich-Beilin, O., Lipina, T. V., Takao, K., van Eede, M., Hattori, S., Laliberté, C., et al. (2009). Abnormalities in brain structure and behavior in GSK-3α mutant mice. *Mol Brain 2*, 35.

Kaidanovich-Beilin, O., & Woodgett, J. R. (2011). GSK-3: Functional insights from cell biology and animal models. *Front Mol Neurosci 4*, 40.

Kessing, L. V., Forman, J. L., & Andersen, P. K. (2010). Does lithium protect against dementia? *Bipolar Disord 12*, 87-94.

Khasraw, M., Ashley, D., Wheeler, G., & Berk, M. (2012). Using lithium as a neuroprotective agent in patients with cancer. *BMC Med 10*, 131.

Kim, W. Y., Wang, X., Wu, Y., Doble, B. W., Patel, S., Woodgett, J. R., et al. (2009). GSK-3 is a master regulator of neural progenitor homeostasis. *Nat Neurosci 12*, 1390-1397.

Kimura, T., Yamashita, S., Nakao, S., Park, J. M., Murayama, M., Mizoroki, T., et al. (2008). GSK-3β is required for memory reconsolidation in adult brain. *PLoS One 3*, e3540.

King, M. R., Anderson, N. J., Guernsey, L. S., & Jolivalt, C. G. (2013). Glycogen synthase kinase-3 inhibition prevents learning deficits in diabetic mice. *J Neurosci Res 91*, 506-514.

King, T. D., Bijur, G. N., & Jope, R. S. (2001). Caspase-3 activation induced by inhibition of mitochondrial complex I is facilitated by glycogen synthase kinase-3β and attenuated by lithium. *Brain Res 919*, 106-114.

King, T. D., & Jope, R. S. (2005). Inhibition of glycogen synthase kinase-3 protects cells from intrinsic but not extrinsic oxidative stress. *Neuroreport 16*, 597-601.

King, M. K., & Jope, R. S. (2013) Lithium treatment alleviates impaired cognition in a mouse model of Fragile X syndrome. *Genes, Brain and Behavior* (in press)

Klein, P. S., & Melton, D. A. (1996). A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci USA 93*, 8455-8459.

Kooy, R. F. (2003). Of mice and the fragile X syndrome. *Trends Genet 19*, 148-154.

Leclerc, S., Garnier, M., Hoessel, R., Marko, D., Bibb, J. A., Snyder, G. L., et al. (2001). Indirubins inhibit glycogen synthase kinase-3β and CDK5/p25, two protein kinases involved in abnormal tau phosphorylation in Alzheimer's disease. A property common to most cyclin-dependent kinase inhibitors? *J Biol Chem 276*, 251-260.

Leost, M., Schultz, C., Link, A., Wu, Y. Z., Biernat, J., Mandelkow, E. M., et al. (2000). Paullones are potent inhibitors of glycogen synthase kinase-3β and cyclin-dependent kinase 5/p25. *Eur J Biochem 267*, 5983-5994.

Letendre, S. L., Woods, S. P., Ellis, R. J., Atkinson, J. H., Masliah, E., van den Brande, G., et al. (2006). Lithium improves HIV-associated neurocognitive impairment. *AIDS 20*, 1885-1888.

Leuner, B., Gould, E., & Shors, T. J. (2006). Is there a link between adult neurogenesis and learning? *Hippocampus 16*, 216-224.

Leyhe T, Eschweiler GW, Stransky E, Gasser T, Annas P, Basun H, et al. (2009). Increase of BDNF serum concentration in lithium treated patients with early Alzheimer's disease. *J Alzheimers Dis 16*, 649-656.

Li, X. H., Lv, B. L., Xie, J. Z., Liu, J., Zhou, X. W., & Wang, J. Z. (2012). AGEs induce Alzheimer-like tau pathology and memory deficit via RAGE-mediated GSK-3 activation. *Neurobiol Aging 33*, 1400-1410.

Licht-Murava, A., Plotkin, B., Eisenstein, M., & Eldar-Finkelman, H. (2011). Elucidating substrate and inhibitor binding sites on the surface of GSK-3β and the refinement of a competitive inhibitor. *J Mol Biol 408*, 366-378.

Lie, D. C., Song, H., Colamarino, S. A., Ming, G. L., & Gage, F. H. (2004). Neurogenesis in the adult brain: new strategies for central nervous system diseases. *Annu Rev Pharmacol Toxicol 44*, 399-421.

Lin, M. T., & Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature 443*, 787-795.

Linnoila, M., Rudorfer, M. V., Dubyoski, K. V., Rawlings, R. R., & Eckardt, M. J. (1986). Effects of one-week lithium treatment on skilled performance, information processing, and mood in healthy volunteers. *J Clin Psychopharmacol 6*, 356-359.

Lipina, T. V., Palomo, V., Gil, C., Martinez, A., & Roder, J. C. (2013). Dual inhibitor of PDE7 and GSK-3-VP1.15 acts as antipsychotic and cognitive enhancer in C57BL/6J mice. *Neuropharmacology 64*, 205-214.

Liu, S. J., Zhang, A. H., Li, H. L., Wang, Q., Deng, H. M., Netzer, W. J., et al. (2003). Overactivation of glycogen synthase kinase-3 by inhibition of phosphoinositol-3 kinase and protein kinase C leads to hyperphosphorylation of tau and impairment of spatial memory. *J Neurochem 87*, 1333-1344.

Liu, X. S., Xue, Q. S., Zeng, Q. W., Li, Q., Liu, J., Feng, X. M., et al. (2010). Sevoflurane impairs memory consolidation in rats, possibly through inhibiting phosphorylation of glycogen synthase kinase-3β in the hippocampus. *Neurobiol Learn Mem 94*, 461-467.

Liu, Z. H., Chuang, D. M., & Smith, C. B. (2011). Lithium ameliorates phenotypic deficits in a mouse model of fragile X syndrome. *Int J Neuropsychopharmacol 14*, 618-630.

Loo, H., Bonnel, J., Etevenon, P., Benyacoub, J., & Slowen P. (1981). Intellectual efficiency in manic-depressive patients treated with lithium. A control study. *Acta Psychiatr Scand 64*, 423-430.

López-Jaramillo, C., Lopera-Vásquez, J., Ospina-Duque, J, García J, Gallo A, Cortez V, et al. (2010). Lithium treatment effects on the neuropsychological functioning of patients with bipolar I disorder. *J Clin Psychiatry 71*, 1055-1060.

Lovestone, S., Davis, D. R., Webster, M. T., Kaech, S., Brion, J. P., Matus, A., et al. (1999). Lithium reduces tau phosphorylation: effects in living cells and in neurons at therapeutic concentrations. *Biol Psychiatry 45*, 995-1003.

Lucas, J. J., Hernández, F., Gómez-Ramos, P., Morán, M. A., Hen, R., & Avila J. (2001). Decreased nuclear β-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3β conditional transgenic mice. *EMBO J 20*, 27-39.

Lund, Y., Nissen, M., & Rafaelsen, O. J. (1982). Long-term lithium treatment and psychological functions. *Acta Psychiatr Scand 65*, 233–244.

Ly, P. T., Wu, Y., Zou, H., Wang, R., Zhou, W., Kinoshita, A., et al. (2013). Inhibition of GSK3β-mediated BACE1 expression reduces Alzheimer-associated phenotypes. *J Clin Invest 123*, 224-235.

Ma, T., Hoeffer, C. A., Capetillo-Zarate, E., Yu, F., Wong, H., Lin, M. T., et al. (2010). Dysregulation of the mTOR pathway mediates impairment of synaptic plasticity in a mouse model of Alzheimer's disease. *PLoS One 5*: 9.

Macdonald, A., Briggs, K., Poppe, M., Higgins, A., Velayudhan, L., & Lovestone, S. (2008). A feasibility and tolerability study of lithium in Alzheimer's disease. *Int J Geriatr Psychiatry 23*, 704-711.

Mandelkow, E. M., Drewes, G., Biernat, J., Gustke, N., Van Lint, J., Vandenheede, J. R., et al. (1992). Glycogen synthase kinase-3 and the Alzheimer-like state of microtubule-associated protein tau. *FEBS Lett 314*, 315-321.

Mansur, R. B., Zugman, A., Asevedo, E. M., da Cunha G. R., Bressan, R. A., & Brietzke, E. (2012). Cytokines in schizophrenia: possible role of anti-inflammatory medications in clinical and preclinical stages. *Psychiatry Clin Neurosci 66*, 247-260.

Mao, Y., Ge, X., Frank, C. L., Madison, J. M., Koehler, A. N., Doud, M. K., et al. (2009). Disrupted in schizophrenia 1 regulates neuronal progenitor proliferation via modulation of GSK3β/β-catenin signaling. *Cell 136*, 1017-1031.

Martin, M., Rehani, K., Jope, R. S., & Michalek, S. M. (2005). Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat Immunol 6*, 777-784.

Martinez, A., Alonso, M., Castro, A., Pérez, C., & Moreno, F. J. (2002). First non-ATP competitive glycogen synthase kinase 3β (GSK-3β) inhibitors: thiadiazolidinones (TDZD) as potential drugs for the treatment of Alzheimer's disease. *J Med Chem 45*, 1292-1299.

Martinez, A., Gil, C., & Perez, D. I. (2011). Glycogen synthase kinase 3 inhibitors in the next horizon for Alzheimer's disease treatment. *Int J Alzheimers Dis 2011*, 280502.

Martinez-Aran, A., Vieta, E., Reinares, M., Colom, F., Torrent, C., Sanchez-Moreno, J., et al. (2004). Cognitive function across manic or hypomanic, depressed and euthymic states in bipolar disorder. *Am J Psychiatry 161*, 262–270.

Marusarz, T. Z., Wolpert, E. A., & Koh, S. D. (1981) Memory processing with lithium carbonate. *J Clin Psychiatry 42*, 190-192.

Massa, F., Koehl, M., Wiesner, T., Grosjean, N., Revest, J. M., Piazza, P. V., et al. (2011). Conditional reduction of adult neurogenesis impairs bidirectional hippocampal synaptic plasticity. *Proc Natl Acad Sci U S A 108*, 6644-6649.

Meares, G. P., Mines, M. A., Beurel, E., Eom, T. Y., Song, L., Zmijewska, A. A., et al. (2011). Glycogen synthase kinase-3 regulates endoplasmic reticulum (ER) stress-induced CHOP expression in neuronal cells. *Exp Cell Res 317*, 1621-1628.

Mines, M. A., & Jope, R. S. (2011). Glycogen synthase kinase-3: a promising therapeutic target for fragile x syndrome. *Front Mol Neurosci 4*, 35.

Mines, M. A., Beurel, E., & Jope, R. S. (2011). Regulation of cell survival mechanisms in Alzheimer's disease by glycogen synthase kinase-3. *Int J Alzheimers Dis 2011*, 861072.

Morales-Garcia, J. A., Luna-Medina, R., Alonso-Gil, S., Sanz-Sancristobal, M., Palomo, V., Gil, C., et al. (2012). Glycogen synthase kinase 3 inhibition promotes adult hippocampal neurogenesis in vitro and in vivo. *ACS Chem Neurosci 3*, 963-971.

Muñoz-Montaño, J. R., Moreno, F. J., Avila, J., & Diaz-Nido, J. (1997). Lithium inhibits Alzheimer's disease-like tau protein phosphorylation in neurons. *FEBS Lett 411*, 183-188.

Mur, M., Portella, M. J., Martínez-Arán, A., Pifarré, J., & Vieta, E. (2008). Neuropsychological profile in bipolar disorder: a preliminary study of monotherapy lithium-treated euthymic bipolar patients evaluated at a 2-year interval. *Acta Psychiatr Scand 118*, 373–381.

Murray, J. T., Campbell, D. G., Morrice, N., Auld, G. C., Shpiro, N., Marquez, R., et al. (2004). Exploitation of KESTREL to identify NDRG family members as physiological substrates for SGK1 and GSK3. *Biochem J 384*, 477-488.

Nocjar, C., Hammonds, M. D., & Shim, S. S. (2007). Chronic lithium treatment magnifies learning in rats. *Neuroscience 150*, 774-788.

Nonaka, S., & Chuang, D. M. (1998). Neuroprotective effects of chronic lithium on focal cerebral ischemia in rats. *Neuroreport 9*, 2081-2084.

Nunes, P. V., Forlenza, O. V., & Gattaz, W. F. (2007). Lithium and risk for Alzheimer's disease in elderly patients with bipolar disorder. *Br J Psychiatry 190*, 359-360.

Nunes, M. A., Viel, T. A., & Buck, H. S. (2013). Microdose lithium treatment stabilized cognitive impairment in patients with Alzheimer's disease. *Curr Alzheimer Res 10*, 104-107.

Onishi, T., Iwashita, H., Uno, Y., Kunitomo, J., Saitoh, M., Kimura, E., et al. (2011). A novel glycogen synthase kinase-3 inhibitor 2-methyl-5-(3-{4-[(S)-methylsulfinyl]phenyl}-1-benzofuran-5-yl)-1,3,4-oxadiazole decreases tau phosphorylation and ameliorates cognitive deficits in a transgenic model of Alzheimer's disease. *J Neurochem 119*, 1330-1340.

Pachet, A. K., & Wisniewski, A. M. (2003). The effects of lithium on cognition: an updated review. *Psychopharmacology 170*, 225-234.

Palomo, V., Soteras, I., Perez, D. I., Perez, C., Gil, C., Campillo, N. E., et al. (2011). Exploring the binding sites of glycogen synthase kinase 3. Identification and characterization of allosteric modulation cavities. *J Med Chem. 54*, 8461-8470.

Peineau, S., Taghibiglou, C., Bradley, C., Wong, T. P., Liu, L., Lu, J., et al. (2007). LTP inhibits LTD in the hippocampus via regulation of GSK3β. *Neuron 53*: 703-717.

Phiel, C. J. & Klein, P. S. (2001). Molecular targets of lithium action. *Annu Rev Pharmacol Toxicol 41*, 789-813.

Phiel, C. J., Wilson, C. A., Lee, V. M. Y., & Klein, P. S. (2003). GSK-3α regulates production of Alzheimer's disease amyloid-β peptides. *Nature 423*, 435-439.

Pieretti, M., Zhang, F. P., Fu, Y. H., Warren, S. T., Oostra, B. A., Caskey, C. T., et al. (1991). Absence of expression of the FMR-1 gene in fragile X syndrome. *Cell 66*, 817-822.

Plotkin, B., Kaidanovich, O., Talior, I., & Eldar-Finkelman, H. (2003). Insulin mimetic action of synthetic phosphorylated peptide inhibitors of glycogen synthase kinase-3. *J Pharmacol Exp Ther 305*, 974-980.

Pomara, N. (2009). Lithium treatment in Alzheimer's disease does not promote cognitive enhancement, but may exert long-term neuroprotective effects. *Psychopharmacology (Berl) 205*,169-170.

Ponce-Lopez, T., Liy-Salmeron, G., Hong, E., & Meneses, A. (2011). Lithium, phenserine, memantine and pioglitazone reverse memory deficit and restore phospho-GSK3β decreased in hippocampus in intracerebroventricular streptozotocin induced memory deficit model. *Brain Res 1426*, 73-85.

Raison, C. L., & Miller, A. H. (2013). The evolutionary significance of depression in Pathogen Host Defense (PATHOS-D). *Mol Psychiatry 18*, 15-37.

Reeves, R. H., Irving, N. G., Moran, T. H., Wohn, A., Kitt, C., Sisodia, S. S., et al. (1995). A mouse model for Down syndrome exhibits learning and behaviour deficits. *Nat Genet 11*, 177-184.

Robinson, L. J., & Ferrier, I. N. (2006). Evolution of cognitive impairment in bipolar disorder: a systematic review of cross-sectional evidence. *Bipolar Disord 8*, 103-116.

Rockenstein, E., Torrance, M., Adame, A., Mante, M., Bar-on, P., Rose, J. B., et al. (2007). Neuroprotective effects of regulators of the glycogen synthase kinase-3β signaling pathway in a transgenic model of Alzheimer's disease are associated with reduced amyloid precursor protein phosphorylation. *J Neurosci 27*, 1981-1991.

Roman, D. D., & Sperduto, P. W. (1995). Neuropsychological effects of cranial radiation: current knowledge and future directions. *Int J Radiat Oncol Biol Phys 31*, 983–998.

Roussel, B. D., Kruppa, A. J., Miranda, E., Crowther, D. C., Lomas, D. A., & Marciniak, S. J. (2013). Endoplasmic reticulum dysfunction in neurological disease. *Lancet Neurol 12*,105-118.

Schäfer, M., Goodenough, S., Moosmann, B., & Behl, C. (2004). Inhibition of glycogen synthase kinase 3β is involved in the resistance to oxidative stress in neuronal HT22 cells. *Brain Res 1005*, 848-849.

Schifitto, G., Zhong, J., Gill, D., Peterson, D. R., Gaugh, M. D., Zhu, T., et al. (2009). Lithium therapy for human immunodeficiency virus type 1-associated neurocognitive impairment. *J Neurovirol 15*, 176-186.

Selenica, M. L., Jensen, H. S., Larsen, A. K., Pedersen, M. L., Helboe, L., Leist, M., et al. (2007). Efficacy of small-molecule glycogen synthase kinase-3 inhibitors in the postnatal rat model of tau hyperphosphorylation. *Br J Pharmacol 152*, 959-979.

Serenó, L., Coma, M., Rodríguez, M., Sánchez-Ferrer, P., Sánchez, M. B., Gich, I., et al. (2009). A novel GSK-3β inhibitor reduces Alzheimer's pathology and rescues neuronal loss in vivo. *Neurobiol Dis 35*, 359-367.

Sharifzadeh, M., Aghsami, M., Gholizadeh, S., Tabrizian, K., Soodi, M., Khalaj, S., et al. (2007). Protective effects of chronic lithium treatment against spatial memory retention deficits induced by the protein kinase AII inhibitor H-89 in rats. *Pharmacology 80*, 158-165.

Sharma, I., & Singh, P. (1988). Cognitive functions in patients of primary affective disorder on prophylactic lithium treatment. *Indian J Med Res 88*, 246-252.

Shaw, E. D., Stokes, P. E., Mann, J. J., & Manevitz, A. Z. (1987). Effects of lithium carbonate on the memory and motor speed of bipolar outpatients. *J Abnorm Psychol 96*, 64-69.

Schober, A. (2004). Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP. *Cell Tissue Res 318*, 215-224.

Silva, R., Mesquita, A. R., Bessa, J., Sousa, J. C., Sotiropoulos, I., Leão, P., et al. (2008). Lithium blocks stress-induced changes in depressive-like behavior and hippocampal cell fate: the role of glycogen-synthase-kinase-3β. *Neuroscience 152*, 656-669.

Smigan, L., & Perris, C. (1983). Memory functions and prophylactic treatment with lithium. *Psychol Med 13*, 529–536.

Song, L., De Sarno, P., & Jope, R. S. (2002). Central role of glycogen synthase kinase-3β in endoplasmic reticulum stress-induced caspase-3 activation. *J Biol Chem 277*, 44701-44708.

Stambolic, V., Ruel, L., & Woodgett, J. R. (1996). Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Curr Biol 6*, 1664-1668.

Stip, E., Dufresne, J., Lussier, I., & Yatham, L. (2000). A double-blind, placebo-controlled study of the effects of lithium on cognition in healthy subjects: mild and selective effects on learning. *J Affect Disord 60*, 147-157.

Streck, E. L., Comim, C. M., Barichello, T., & Quevedo, J. (2008). The septic brain. *Neurochem Res 33*, 2171-2177.

Sudduth, T. L., Wilson, J. G., Everhart, A., Colton, C. A., & Wilcock, D. M. (2012). Lithium treatment of APPSwDI/NOS2-/- mice leads to reduced hyperphosphorylated tau, increased amyloid deposition and altered inflammatory phenotype. *PLoS One 7*, e31993.

Sy, M., Kitazawa, M., Medeiros, R., Whitman, L., Cheng, D., Lane, T. E., et al. (2011). Inflammation induced by infection potentiates tau pathological features in transgenic mice. *Am J Pathol 178*, 2811-2822.

Takashima, A., Noguchi, K., Michel, G., Mercken, M., Hoshi, M., Ishiguro, K., et al. (1996). Exposure of rat hippocampal neurons to amyloid beta peptide (25-35) induces the inactivation of phosphatidylinositol-3 kinase and the activation of tau protein kinase I/glycogen synthase kinase-3β. *Neurosci Lett 203*, 33-36.

Terao, T., Nakano, H., Inoue, Y., Okamoto, T., Nakamura, J., & Iwata, N. (2006). Lithium and dementia: a preliminary study. *Prog Neuropsychopharmacol Biol Psychiatry 30*, 1125-1128.

Terwel, D., Muyllaert, D., Dewachter, I., Borghgraef, P., Croes, S., Devijver, H., et al. (2008). Amyloid activates GSK-3β to aggravate neuronal tauopathy in bigenic mice. *Am J Pathol 172*, 786-798.

The Dana Consortium on Therapy for HIV Dementia and Related Cognitive Disorders. (1996). Clinical confirmation of the American Academy of Neurology algorithm for HIV-1-associated cognitive/motor disorder. *Neurology 47*, 1247-1253.

Thompson, H. J., Lifshitz, J., Marklund, N., Grady, M. S., Graham, D. I., Hovda, D. A., et al. (2005). Lateral fluid percussion brain injury: a 15-year review and evaluation. *J Neurotrauma 22*, 42-75.

Thotala, D. K., Hallahan, D. E., & Yazlovitskaya, E. M. (2008). Inhibition of glycogen synthase kinase 3β attenuates neurocognitive dysfunction resulting from cranial irradiation. *Cancer Res 68*, 5859-5868.

Tian, M., Zhu, D., Xie, W., & Shi, J. (2012). Central angiotensin II-induced Alzheimer-like tau phosphorylation in normal rat brains. *FEBS Lett 586*, 3737-3745.

Toledo, E. M., & Inestrosa, N. C. (2010). Activation of Wnt signaling by lithium and rosiglitazone reduced spatial memory impairment and neurodegeneration in brains of an APPswe/PSEN1DeltaE9 mouse model of Alzheimer's disease. *Mol Psychiatry 15*, 272-285.

Tsaltas, E., Kyriazi, T., Poulopoulou, C., Kontis, D., & Maillis, A. (2007a). Enhancing effects of lithium on memory are not by-products of learning or attentional deficits. *Behav Brain Res 180*, 241-245.

Tsaltas, E., Kontis, D., Boulougouris, V., Papakosta, V. M., Giannou, H., Poulopoulou, C., et al. (2007b). Enhancing effects of chronic lithium on memory in the rat. *Behav Brain Res 177*, 51-60.

Van Gorp, W. G., Altshuler, L., Theberge, D. C., Wilkins, J., & Dixon, W. (1998). Cognitive impairment in euthymic bipolar patients with and without prior alcohol dependence. A preliminary study. *Arch Gen Psychiatry 55*, 41-46.

van Praag, H., Shubert, T., Zhao, C., & Gage, F. H. (2005). Exercise enhances learning and hippocampal neurogenesis in aged mice. *J Neurosci 25*, 8680-8685.

Vasconcellos, A. P., Tabajara, A. S., Ferrari, C., Rocha, E., & Dalmaz, C. (2003). Effect of chronic stress on spatial memory in rats is attenuated by lithium treatment. *Physiol Behav 79*, 143-149.

Vasdev, N., Garcia, A., Stableford, W. T., Young, A. B., Meyer, J. H., Houle, S., et al. (2005). Synthesis and ex vivo evaluation of carbon-11 labelled N-(4-methoxybenzyl)-N'-(5-nitro-1,3-thiazol-2-yl)urea ([11C]AR-A014418): a radiolabelled glycogen synthase kinase-3β specific inhibitor for PET studies. *Bioorg Med Chem Lett 15*, 5270-5273.

Verkerk, A. J., Pieretti, M., Sutcliffe, J. S., Fu, Y. H., Kuhl, D. P., Pizzuti, A., et al. (1991). Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell 65*, 905-914.

Wang, X., Blanchard, J., Kohlbrenner, E., Clement, N., Linden, R. M., Radu, A., et al. (2010). The carboxy-terminal fragment of inhibitor-2 of protein phosphatase-2A induces Alzheimer disease pathology and cognitive impairment. *FASEB J 24*, 4420-4432.

Wang, Y. C., Wang, E. N., Wang, C. C., Huang, C. L., & Huang, A. C. (2013) Effects of lithium and carbamazepine on spatial learning and depressive behavior in a rat model of bipolar disorder induced by ouabain. *Pharmacol Biochem Behav 105*, 118-127.

Watase, K., Weeber, E. J., Xu, B., Antalffy, B., Yuva-Paylor, L., Hashimoto, K., et al. (2002). A long CAG repeat in the mouse Sca1 locus replicates SCA1 features and reveals the impact of protein solubility on selective neurodegeneration. *Neuron 34*, 905-919.

Watase, K., Gatchel, J. R., Sun, Y., Emamian, E., Atkinson, R., Richman, R., et al. (2007). Lithium therapy improves neurological function and hippocampal dendritic arborization in a spinocerebellar ataxia type 1 mouse model. *PLoS Med 4*, 836-847.

Weingartner, H., Rudorfer, M. V., & Linnoila, M. (1985). Cognitive effects of lithium treatment in normal volunteers. *Psychopharmacology 86*, 472-474.

Wexler, E. M., Geschwind, D. H., & Palmer, T. D. (2008). Lithium regulates adult hippocampal progenitor development through canonical Wnt pathway activation. *Mol Psychiatry 13*, 285-292.

Willi, R., Harmeier, A., Giovanoli, S., & Meyer, U. (2013). Altered GSK3β signaling in an infection-based mouse model of developmental neuropsychiatric disease. *Neuropharmacology 73C*, 56-65.

Wingo, A. P., Wingo, T. S., Harvey, P. D., & Baldessarini, R. J. (2009). Effects of lithium on cognitive performance: a meta-analysis. *J Clin Psychiatry 70*, 1588-1597.

Xie, H., Litersky, J. M., Hartigan, J. A., Jope, R. S., & Johnson, G. V. (1998). The interrelationship between selective tau phosphorylation and microtubule association. *Brain Res 798*, 173-183.

Yan, X. B., Wang, S. S., Hou, H. L., Ji, R., & Zhou, J. N. (2007). Lithium improves the behavioral disorder in rats subjected to transient global cerebral ischemia. *Behav Brain Res 177*, 282-289.

Yang, E. S., Lu, B., & Hallahan, D. E. (2007). Lithium-mediated neuroprotection during cranial irradiation: A phase 1 trial. In ASTRO (American Society for Therapeutic Radiology and Oncology) - 49th annual Conference. Los Angeles CA.

Yazlovitskaya, E. M., Edwards, E., Thotala, D., Fu, A., Osusky, K. L., Whetsell, W. O. Jr., et al. (2006). Lithium treatment prevents neurocognitive deficit resulting from cranial irradiation. *Cancer Res 66*, 11179-11186.

Yu, F., Zhang, Y., & Chuang, D. M. (2012). Lithium reduces BACE1 overexpression, beta amyloid accumulation, and spatial learning deficits in mice with traumatic brain injury. *J Neurotrauma 29*, 2342-2351.

Yuskaitis, C. J., & Jope, R. S. (2009). Glycogen synthase kinase-3 regulates microglial migration, inflammation, and inflammation-induced neurotoxicity. *Cell Signal 21*, 264-73.

Yuskaitis, C. J., Mines, M. A., King, M. K., Sweatt, J. D., Miller, C. A., & Jope, R. S. (2010). Lithium ameliorates altered glycogen synthase kinase-3 and behavior in a mouse model of fragile X syndrome. *Biochem Pharmacol 79*, 632-646.

Zhao, L., Wang, F., Gui, B., Hua, F., & Qian, Y. (2011). Prophylactic lithium alleviates postoperative cognition impairment by phosphorylating hippocampal glycogen synthase kinase-3β (Ser9) in aged rats. *Exp Gerontol 46*, 1031-1036.

Zhu, L. Q., Wang, S. H., Liu, D., Yin, Y. Y., Tian, Q., Wang, X. C., et al. (2007). Activation of glycogen synthase kinase-3 inhibits long-term potentiation with synapse-associated impairments. *J Neurosci 27*, 12211-12220.

Zhu, Z. F., Wang, Q. G., Han, B. J., & William, C. P. (2010). Neuroprotective effect and cognitive outcome of chronic lithium on traumatic brain injury in mice. *Brain Res Bull 83*, 272-277.

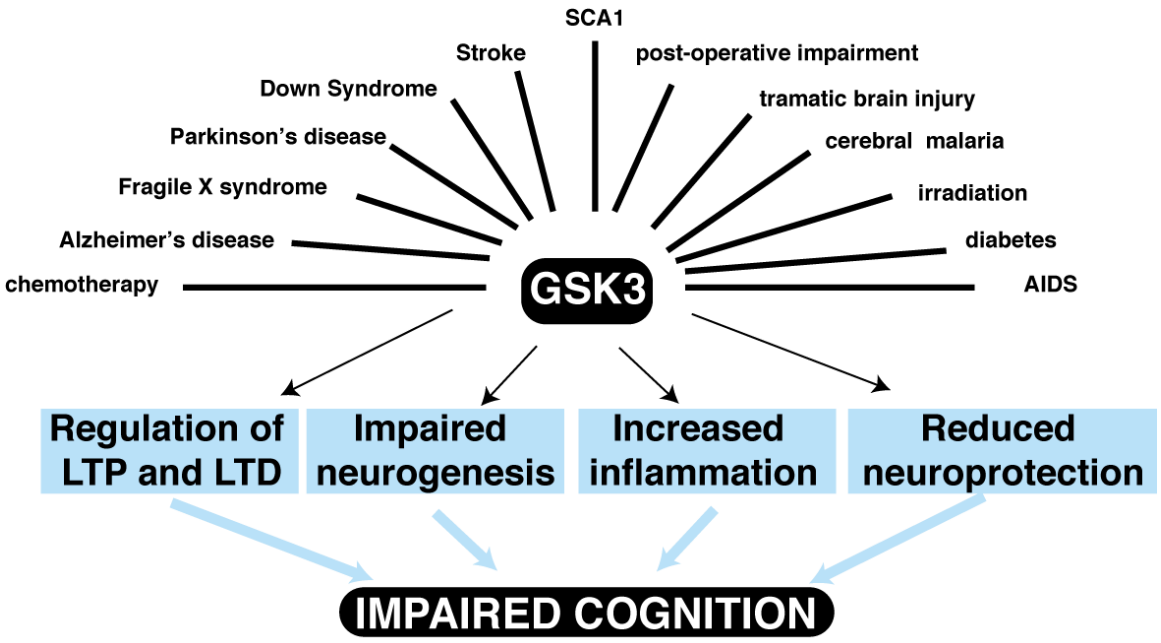


Figure 1. GSK3 inhibitors improve impaired cognition in multiple conditions.
Schematic representation of conditions in which inhibition of GSK3 improves impairments in cognitive processes. The improvements in impaired cognition following administration of GSK3 inhibitors likely involve a variety of different mechanisms, such as supporting long-term potentiation and diminishing long-term depression, promotion of neurogenesis, reduction of inflammation, and increasing a number of neuroprotective mechanisms.

LITHIUM TREATMENT ALLEVIATES IMPAIRED COGNITION IN A MOUSE MODEL OF FRAGILE X SYNDROME

by

MARGARET K. KING AND RICHARD S. JOPE

Genes, Brain, and Behavior

Format adapted for dissertation

Abstract

Fragile X Syndrome (FXS) is caused by suppressed expression of fragile X mental retardation protein (FMRP), which results in intellectual disability accompanied by many variably manifested characteristics, such as hyperactivity, seizures, and autistic-like behaviors. Treatment of mice that lack FMRP, *Fmr1* knockout (KO) mice, with lithium has been reported to ameliorate locomotor hyperactivity, prevent hypersensitivity to audiogenic seizures, improve passive avoidance behavior, and attenuate sociability deficits. To focus on the defining characteristic of FXS, which is cognitive impairment, we tested if lithium treatment ameliorated impairments in four cognitive tasks in *Fmr1* KO mice, tested if the response to lithium differed in adolescent and adult mice, and tested if therapeutic effects persisted after discontinuation of lithium administration. *Fmr1* KO mice displayed impaired cognition in the novel object detection task, temporal ordering for objects task, and coordinate and categorical spatial processing tasks. Chronic lithium treatment of adolescent (from 4-8 weeks of age) and adult (from 8-12 weeks of age) mice abolished cognitive impairments in all four cognitive tasks. Cognitive deficits returned after lithium treatment was discontinued for 4 weeks. These results demonstrate that *Fmr1* KO mice exhibit severe impairments in these cognitive tasks, that lithium is equally effective in normalizing cognition in these tasks whether it is administered to young or adult mice, and that lithium administration must be continued for the cognitive improvements to be sustained. These findings provide further evidence that lithium administration may be beneficial for individuals with FXS.

Introduction

Fragile X syndrome (FXS) is the most common cause of inherited intellectual disability and is the most prevalent monogenetic cause of autism spectrum disorders. FXS is caused by a trinucleotide CGG repeat expansion on the X chromosome that suppresses expression of Fragile X Mental Retardation Protein (FMRP) (Verkerk *et al.* 1991; Pierreti *et al.* 1991), which is thought to cause the intellectual, behavioral, and physical abnormalities characteristic of FXS. In mouse hippocampus, FMRP expression is highest at postnatal day 7 (Lu *et al.* 2004), and FMRP is important for establishing functional neuronal networks (Gatto & Broadie 2009). Since individuals with FXS lack FMRP during postnatal development, crucial questions are whether cognitive deficits can be ameliorated pharmacologically, and if improvements depend on early intervention.

FXS is modeled in *Fmr1* knockout (KO) mice (Bakker *et al.* 1994) that display several characteristics of FXS, including impaired social interactions, locomotor hyperactivity, and decreased passive avoidance learning (Kooy *et al.* 1996; Mineur *et al.* 2002; Yan *et al.* 2004). Remarkably, all of these behavioral phenotypes are normalized in *Fmr1* KO mice by lithium treatment (Min *et al.* 2009; Mines *et al.* 2010; Yuskaitis *et al.* 2010a; Liu *et al.* 2011). Furthermore, lithium is the only agent that has improved performance on a cognitive task in FXS patients in a formal trial setting (Berry-Kravis *et al.* 2008). Since lithium is safely used in patients with bipolar disorder, including children and adolescents (Alessi *et al.* 1994; Ryan *et al.* 1999; Findling *et al.* 2011), these findings suggest that lithium is a promising therapeutic agent for FXS.

Here we tested if lithium treatment can reverse several cognitive deficits in *Fmr1* KO mice. We also tested if the beneficial effects of lithium treatment on cognitive tasks

in *Fmr1* KO mice differ between young and adult mice, since the lack of FMRP during development may have established irreversible morphological and neuronal abnormalities that preclude effective intervention in adults. Administration of lithium to lactating mothers or to pups immediately upon weaning at 3 weeks of age results in retarded growth of the pups (Min *et al*., 2009), therefore lithium administration was initiated when mice reached 4 weeks of age to compare its effects on performance in cognitive tasks in young (from 4-8 weeks of age) mice with adult-treated (from 8-12 weeks of age) mice. Additionally, the effects of lithium withdrawal from mice treated during adolescence were examined to test if lithium-induced improvements in cognitive task performance by *Fmr1* KO mice required continual lithium treatment or if they remained stable once repaired, which may occur if lithium treatment resulted in long-lasting repairs of deficits in neural circuitry or neurogenesis in *Fmr1* KO mice. Significant deficits in *Fmr1* KO mice were found in object novelty detection, temporal order memory, and spatial learning tests, and each of these was improved by chronic lithium treatment of adolescent and adult mice, whereas the cognitive deficits were reinstated after four weeks of lithium withdrawal. These results further support the potential benefits of lithium treatment in FXS.

Materials and methods

Mice

This study used male C57Bl/6J littermates, with or without a disruption of the *Fmr1* gene (originally kindly provided by Dr. W. Greenough, University of Illinois). Mice were weaned 3 weeks after birth, group housed, tested between 1000 and 1400, and 7-20 mice

were used in each experiment as described in the figure legends. The *Fmr1* KO mice were generated by breeding male C57BL/6J hemizygous *Fmr1* KO mice and female C57Bl/6J heterozygous *Fmr1* KO mice to generate male homozygous *Fmr1* KO mice and wild-type (WT) littermates. Genotypes were determined by PCR using the Jackson Laboratory protocol for genotyping *Fmr1* mice. To test chronic lithium treatment, *Fmr1* KO mice and WT mice were given water *ad libitum,* and were fed either normal 18% protein rodent diet or the same diet with 0.2% lithium carbonate (both from Teklad, Madison, WI) with provision of an additional bottle containing saline to prevent hyponatremia. This is a therapeutically relevant treatment regimen that produces serum lithium concentrations of 0.6-0.8 mM (Chen *et al.* 2000; O'Brien *et al.* 2004; Shaltiel *et al.* 2008; Jope 2011; Contestabile *et al.* 2013), within the 0.5-1.2 mM range that is therapeutic in human patients. Adult mice were treated with lithium for 4 weeks and throughout the behavioral tests. For lithium treatment during adolescence, mice were treated with lithium from 4 until 8 weeks of age and throughout the behavioral tests, then lithium was discontinued for 4 weeks, and the mice were retested. Mice were housed in light and temperature controlled rooms and treated in accordance with NIH and University of Miami Institutional Animal Care and Use Committee regulations.

Object novelty detection task

Recognition memory for a novel object compared to a familiar object was assessed by the object novelty detection task (Hoge & Kesner 2007; Hunsaker & Kesner 2008; Hunsaker *et al.* 2012). For this task, a Plexiglas box (26 cm long x 20 cm wide x 16 cm tall) and four objects in duplicate (4-6 cm diameter x 2-6 cm height) were used. During

the first session, two copies of Object 1 were placed at each end of the box, and the mouse was allowed to explore the objects for 5 min. The mouse was then removed to an opaque holding container for 5 min, and the objects were replaced with two copies of Object 2 for the next session. After 5 min exploring during session 2, the mouse was placed in the holding container and the copies of Object 2 were replaced with duplicates of Object 3. Following exploration during session 3, the mouse was removed and the objects were replaced by an unused copy of Object 1 and a novel Object 4 for the mouse to explore during the 5 min test session. More time exploring the novel Object 4 compared to the familiar Object 1 indicates that the mouse remembered previously exploring Object 1, but equal exploration time between the two objects indicates that the mouse has impaired recognition memory. Object exploration was defined as the mouse sniffing or touching the object with its nose, vibrissa, mouth, or forepaws, and time spent near or standing on top of the objects without interacting with the object was not counted as exploration. Exploration time of the novel and familiar object is presented, and changes in object exploration ratio were calculated as: (exploration time of Object 4 – exploration time of Object 1)/ (exploration time of Object 1 + exploration time of Object 4). This calculation constrains the ratios to be between -1 and 1, and a ratio approaching 1 indicates an intact memory of Object 1.

For this and all other behavioral assessments, the sessions were filmed, a white noise generator (55 dB) was used, and each apparatus and object was cleaned with 70% ethanol between each test session.

Temporal ordering for objects task

Temporal order memory was assessed using the temporal ordering for objects task (Mitchell & Laiacono, 1998; Hannesson *et al*.; 2004; Hoge & Kesner 2007; Hunsaker *et al.* 2012). Similar to the object novelty detection task, the same box was used and a mouse received three sessions to explore two copies of a new set of objects (Objects 5, 6, 7). For the 5 min test session, an unused copy of Object 5 and an unused copy of Object 7 were placed in the box and the mouse was allowed to explore. A mouse with normal temporal order memory spends more time exploring the first object (Object 5) presented compared to the most recent object (Object 7). Time exploring Object 5 and Object 7 are presented, and changes in object exploration ratio were calculated as: (exploration time of Object 5 – exploration time of Object 7)/ (exploration time of Object 5 + exploration time of Object 7).

Coordinate spatial processing task

Spatial memory was assessed in mice using the coordinate and categorical spatial processing tasks (Goodrich-Hunsaker *et al.* 2005; Goodrich-Hunsaker *et al.* 2008; Hunsaker *et al.* 2009; Hunsaker *et al.* 2012). The coordinate spatial processing task consisted of a 15 min habituation session, a 5 min holding time, and a 5 min test session. For the habituation session a mouse was placed at the edge of the table facing 2 different objects spaced 45 cm apart, and the mouse was allowed to explore the table and the objects for 15 min. Then the mouse was placed in an opaque holding container for 5 min. For the test session, the objects were moved closer together so that they were 30 cm

apart, and the mouse was allowed to explore the objects for 5 min. Mice that have intact spatial memory display increased exploration of the objects during the test session compared with the last 5 min of the habituation session. For the coordinate spatial processing task, the exploration ratio was calculated as: (exploration time during the 5 min test session)/ (exploration time during the 5 min test session + exploration time during the last 5 min of the habituation session). Increased exploration during the 5 min test session compared to the last 5 min of the habituation session is indicated by a ratio >0.5.

Categorical spatial processing task

Like the coordinate spatial processing task, the categorical spatial processing task (Goodrich-Hunsaker *et al.* 2005; Goodrich-Hunsaker *et al.* 2008; Hunsaker *et al.* 2009; Hunsaker *et al.* 2012), is used to assess spatial memory with 2 novel objects, which are different from the objects used in the coordinate spatial processing task. For the habituation session, a mouse was placed on the edge of the table facing 2 different objects that were spaced 45 cm apart and allowed to explore the table and objects for 15 min. Then the mouse was placed in an opaque container for 5 min, and the position of the objects was interchanged, while the distance was maintained. For the test session, the mouse was allowed to explore the objects for 5 min. Increased exploration of the objects during the test session compared with the last 5 min of the habituation phase indicates that the mice remember the object positions. The same exploration ratio was calculated for the categorical spatial processing task as in the coordinate spatial processing task.

Statistical analysis

Statistical significance was assessed by two factor ANOVA with genotype and treatment as factors followed by Bonferroni's multiple comparison tests (for lithium treatment in WT and *Fmr1* KO adult and adolescent mice), or by one factor ANOVA (for discontinued lithium treatment in WT and *Fmr1* KO mice), or Student's t-test (for time spent with each object in the object novelty detection task and the temporal ordering for objects task).

Results

Chronic lithium treatment significantly improves object novelty detection in Fmr1 KO mice

We tested if cognition was impaired in *Fmr1* KO mice in four hippocampus-dependent learning tasks, if chronic lithium treatment repaired cognitive deficits in *Fmr1* KO mice and if there were different outcomes after lithium was administered to adolescent mice (from 4-8 weeks of age) or adult mice (from 8-12 weeks of age). The object novelty detection task is a dentate gyrus-dependent task that assesses the ability to discriminate between familiar and novel objects, indicated by more time spent exploring a novel object than a familiar object (Otto & Eichenbaum 1992; Knight 1996; Dolan & Fletcher 1997; Lisman 1999; Hunsaker & Kesner 2008). Previous reports show that novel object recognition is impaired in *Fmr1* KO mice (Ventura *et al.* 2004; Pacey *et al.* 2011; Bhattacharya & Klann 2012). WT mice spent significantly more time exploring the novel object than the familiar object, whereas *Fmr1* KO mice spent equivalent amounts of time

exploring each object (Figure 1A). Thus, there was a significant interaction between genotype and treatment and the object exploration ratio differed between *Fmr1* KO and WT mice (Figure 1B). The impairment in object novelty detection in *Fmr1* KO mice was corrected by lithium treatment, and lithium was equally effective after administration to adult or adolescent mice (Figure 1A). Lithium treatment of adolescent or adult WT mice did not alter performance in the object novelty detection task. In either adolescent or adult *Fmr1* KO mice that were treated with lithium, the exploration ratio was significantly increased to a level equivalent to that of WT mice (Figure 1B). These results demonstrate that object novelty detection is impaired in *Fmr1* KO mice, and that lithium treatment of adolescent or adult *Fmr1* KO mice corrects this impairment.

Lithium treatment normalizes temporal order memory in Fmr1 KO mice

The temporal ordering for objects is a hippocampal CA1-dependent task that is exhibited by mice spending less time with an object most recently presented in the previous habituation session (Honey *et al.* 1998; Wallenstein *et al.* 1998; Lisman 1999; Rolls & Kesner 2006; Hoge & Kesner 2007; Hunsaker *et al.* 2008; Hunsaker *et al.* 2012). WT mice, but not *Fmr1* KO mice, spent more time exploring the first object presented than the most recent object presented (Figure 2A). There was a significant interaction between genotype and treatment in the temporal ordering task, and the object exploration ratio differed significantly between *Fmr1* KO and WT mice (Figure 2B). The impairment in temporal order memory in *Fmr1* KO mice was corrected by lithium treatment, and lithium was similarly effective after administration to adult mice or adolescent mice (Figure 2A). Lithium treatment of adolescents or adults significantly increased the

exploration ratio in *Fmr1* KO mice, whereas lithium treatment did not affect the performance of WT mice in this task (Figure 2B). Thus, temporal order memory is impaired in *Fmr1* KO mice, and is repaired by lithium treatment of adolescent or adult *Fmr1* KO mice.

Treatment with lithium repairs spatial memory impairment in Fmr1 KO mice

The coordinate and categorical spatial learning tasks assess metrical and topological spatial pattern separation, respectively, in similar spaces (Save *et al.* 1992; Tsien *et al.* 1996; Long & Kesner 1996; Lisman 1999; Goodrich-Hunsaker *et al.* 2005; Hunsaker *et al.* 2009; Goodrich-Hunsaker *et al.* 2008; Hunsaker *et al.* 2009; Hunsaker *et al.* 2012). The coordinate spatial learning task involves measuring the time spent exploring two objects after the objects have been moved closer together compared to the last 5 min of the habituation period. There was a significant interaction between genotype and treatment in the coordinate spatial learning task indicating that *Fmr1* KO mice exhibited an impaired object exploration ratio compared to WT mice, demonstrating a deficit in coordinate spatial memory in *Fmr1* KO mice (Figure 3A). Lithium treatment of adult or adolescent *Fmr1* KO mice normalized coordinate spatial memory to that of WT mice, but did not affect the performance of WT mice.

The categorical spatial learning task assesses the time spent exploring two objects after the position of the objects is transposed, with the distance unchanged, following the habituation phase. *Fmr1* KO mice spent significantly less time than WT mice exploring the objects after the objects had been transposed, and there was a significant interaction between genotype and treatment (Figure 3B), revealing impaired categorical spatial

memory in *Fmr1* KO mice. Administration of lithium did not alter the amount of time that WT mice spent exploring the objects that were transposed, but lithium treatment of adult or adolescent *Fmr1* KO mice significantly increased the exploration ratio. Thus, the results of the coordinate and categorical spatial learning tests reveal impaired spatial pattern learning in *Fmr1* KO mice, and that this is significantly improved by lithium treatment of either adolescents or adults.

Learning deficits in Fmr1 KO mice are reinstated following lithium withdrawal

To determine if lithium's enhancing effects on cognition in *Fmr1* KO mice are sustained following lithium withdrawal, chronic lithium treatment was discontinued after the behavior tests in mice treated from 4 weeks until 8 weeks of age. Four weeks later the *Fmr1* KO and WT mice were retested in all cognitive tasks. Prior testing in the same paradigms had no effect on re-test performance in the WT mice or *Fmr1* KO mice that were not treated with lithium (Figures 4 and 5). Following lithium withdrawal, WT mice exhibited normal object novelty detection, indicating that there was no effect of lithium withdrawal in WT mice (Figure 4A). However, *Fmr1* KO mice that were withdrawn from lithium treatment spent significantly less time exploring the novel object, revealing that impaired object novelty detection returned following lithium withdrawal in *Fmr1* KO mice. *Fmr1* KO mice that had been withdrawn from lithium demonstrated a significantly reduced object exploration ratio compared to WT mice that had been withdrawn from lithium (Figure 4B). The results show that the impairment in object novelty detection in *Fmr1* KO mice returned following lithium withdrawal.

Discontinuation of lithium treatment also reinstated the temporal order memory deficit in *Fmr1* KO mice without affecting WT mice. *Fmr1* KO mice that were discontinued from lithium treatment demonstrated deficient temporal order memory, whereas temporal order memory was unaltered by lithium withdrawal in WT mice (Figure 4C). The object exploration ratio was significantly reduced in *Fmr1* KO, compared with WT, mice withdrawn from lithium (Figure 4D). Thus the effect of lithium in the temporal order task was not sustained in *Fmr1* KO mice following four weeks of lithium withdrawal.

Coordinate and categorical spatial memory impairments also returned in *Fmr1* KO mice after lithium was withdrawn. Although WT mice maintained intact spatial memory, untreated and previously treated *Fmr1* KO mice displayed significantly reduced object exploration ratios compared to WT mice in the coordinate spatial task (Figure 5A) and in the categorical spatial task (Figure 5B). Thus, the improvements in coordinate and categorical spatial processing in *Fmr1* KO mice induced by lithium treatment were reversed when lithium treatment was discontinued.

Discussion

Here we report impaired cognition in adult *Fmr1* KO mice in four hippocampus-dependent learning and memory tasks, and that each of these was significantly improved by chronic lithium treatment. Furthermore, lithium treatment was equally effective in ameliorating cognitive deficits when administered to adult or adolescent *Fmr1* KO mice. Importantly, lithium administration did not affect the performance of adult or adolescent WT mice in these cognitive tasks, demonstrating an *Fmr1* KO-specific improvement in

learning and memory. Discontinuation of lithium treatment caused cognitive impairments to return in *Fmr1* KO mice, but lithium withdrawal did not alter the performance of WT mice.

Although the predominant characteristic of FXS is intellectual disability, severe cognitive deficits were initially difficult to identify in the *Fmr1* KO mouse model. *Fmr1* KO mice display modest cognitive deficits in several hippocampus-dependent tasks, such as the Morris water maze, radial arm maze, and operant conditioning paradigms (Bakker 1994; Kooy *et al.* 1996; D'Hooge *et al.* 1997; Fisch *et al.* 1999; Paradee *et al.* 1999; Peier *et al.* 2000; Mineur *et al.* 2002). *Fmr1* KO mice also exhibit deficits in fear motivated learning tasks, including passive and active avoidance behaviors, and contextual, conditioned and trace fear memory (Yan *et al.* 2004; Qin *et al.* 2005; Zhao *et al.* 2005; Brennan *et al.* 2006; Hayashi *et al.* 2007; Baker *et al.* 2010; Guo *et al.* 2011). Recently, severe deficits in non-aversive learning and memory tasks, including novel object recognition and context discrimination, have been identified in *Fmr1* KO mice (Pacey *et al.* 2011; Eadie *et al.* 2012; Bhattacharya & Klann 2012). In the present study, *Fmr1* KO mice exhibited significant impairments in recognition memory, working memory, and short-term memory that are assessed in the object novelty detection task and the temporal ordering for objects task, and spatial memory measured in the coordinate and categorical spatial processing tasks (Goodrich-Hunsaker *et al.* 2005; Hoge & Kesner 2007; Hunsaker & Kesner 2008; Goodrich-Hunsaker *et al.* 2008; Hunsaker *et al.* 2009; Hunsaker *et al.* 2012). As previously discussed (Mineur *et al.* 2002; Ventura *et al.* 2004; Spencer *et al.* 2008; Hagerman *et al.* 2009; Baker *et al.* 2010; Bhogal & Jongens 2010; Guo *et al.* 2011; Bagni *et al.* 2012), similar deficits have been identified in patients with FXS, such as

impaired recognition memory, working memory, short-term memory, and spatial memory (Kemper *et al.* 1988; Cornish *et al.* 1999; Orstein *et al.* 2008; Gatto & Broadie 2009). Thus, the cognitive deficits displayed by *Fmr1* KO mice may model some of the impairments in nonverbal measures of cognitive functions in FXS patients.

Chronic lithium treatment proved to be remarkably effective in essentially normalizing severe deficits in *Fmr1* KO mice in novel object detection, temporal ordering for objects, and coordinate and categorical spatial processing tasks. The lithium treatments were designed to test the hypothesis that treatment of younger *Fmr1* KO mice would be more effective than treatment of adult *Fmr1* KO mice. This was based on the finding that FMRP is more highly expressed in young than adult mouse brain (Lu *et al.* 2004), raising the possibility that its absence may produce irreversible deficits in adult *Fmr1* KO mice. However, lithium treatment was equally effective in adolescent and adult *Fmr1* KO mice in reversing cognitive deficits. This is an encouraging finding that suggests some cognitive impairments may be pharmacologically reversible even with post-adolescent administration in FXS, although caution must be exercised in translating results from *Fmr1* KO mice. However, it is encouraging that lithium administration improved performance on a cognitive task in a small trial in FXS patients (Berry-Kravis *et al.* 2008).

The improvements in cognitive tasks reported here add to an extensive number of abnormal phenotypes that are improved by lithium treatment of *Fmr1* KO mice. Phenotypes in *Fmr1* KO mice that have been reported to be improved by lithium treatment include locomotor hyperactivity, audiogenic seizure hypersensitivity, increased spine density, macroorchidism, excess protein synthesis, social behavior deficits,

deficient passive avoidance learning, and synaptic plasticity (Min *et al.* 2009; Yuskaitis *et al.* 2010a, Yuskaitis *et al.* 2010b; Mines *et al.* 2010; Liu *et al.* 2011; Choi *et al.* 2011; Liu *et al.* 2012). Improvement of cognition by lithium treatment correlates well with previous findings in *Fmr1* KO mice of altered synaptic plasticity, measured as long-term potentiation (LTP) and long-term depression (LTD). *Fmr1* KO mice display enhanced metabotropic glutamate receptor (mGluR)-dependent LTD at hippocampal CA1 synapses (Huber *et al.* 2002; Hou *et al.* 2006; Nosyreva & Huber 2006) and deficient LTP at medial perforant path synapses in the dentate gyrus (Eadie *et al.* 2012). Lithium treatment in adolescent *Fmr1* KO mice (from 5-6 weeks of age until 9-11 months of age) or adult *Fmr1* KO mice (from 8 weeks of age to 4-5 months of age) normalized mGluR-dependent LTD in the hippocampus, without affecting WT mice (Choi *et al.* 2011). Lithium inhibits glycogen synthase kinase-3 (GSK3) (Klein & Melton 1996), lithium treatment reduces abnormally hyperactive GSK3 in *Fmr1* KO mice (Min *et al.* 2009; Yuskaitis *et al.* 2010a), and hyperactive GSK3 impairs LTP and promotes LTD (Hooper *et al.* 2007; Zhu *et al.* 2007). Taken together, these findings suggest that inhibition of GSK3 by lithium contributes to the normalization of synaptic plasticity and cognition in *Fmr1* KO mice, although this conjecture will require further examination. The cognitive-enhancing actions of lithium in *Fmr1* KO mice are clearly dependent on the continued presence of lithium, since cognitive deficits were equivalent in *Fmr1* KO mice withdrawn from lithium and *Fmr1* KO mice that had never been given lithium. Thus, lithium treatment must be sustained in *Fmr1* KO mice for cognitive benefits to persist.

In summary, lithium treatment of adolescent or adult *Fmr1* KO mice is safe, and effectively remediates performance in several cognitive tasks, as well as providing many

previously reported beneficial effects in *Fmr1* KO mice. These results extend previous findings that lithium ameliorates synaptic plasticity and/or cognitive deficits in *Fmr1* KO flies (McBride *et al.* 2005), mice (Yuskaitis *et al.* 2010a; Liu *et al.* 2011; Choi *et al.* 2011) and FXS patients (Berry-Kravis *et al.* 2008). Thus, there is increasing evidence that lithium may provide therapeutic benefits in FXS.

Acknowledgements

This research was supported by grants from the NIMH (MH038752) and from the FRAXA Foundation. The authors would like to thank Marjelo A. Mines, Eleonore Beurel, Marta Pardo, and Elyse Barrett for their contributions to this project.

References

Alessi, N., Baylor, M.W., Ghaziuddin, M., & Zubieta, J.K. (1994) Update on lithium carbonate therapy in children and adolescents. *J Am Acad Child Adolesc Psychiatry* **33**, 291-304.

Bagni, C., Tassone, F., Neri, G., & Hagerman, R. (2012) Fragile X syndrome: causes, diagnosis, mechanisms, and therapeutics. *J Clin Invest* **122,** 4314-4322.

Baker, K.B., Wray, S.P., Ritter, R., Mason, S., Lanthorn, T.H., & Savelieva, K.V. (2010) Male and female Fmr1 knockout mice on C57 albino background exhibit spatial learning and memory impairments. *Genes Brain Behav* **9**, 562-574.

Bakker, C.E., et al. & Consortium (1994) Fmr1 knockout mice: a model to study fragile X mental retardation. The Dutch-Belgian Fragile X Consortium. *Cell* **78**, 23-33.

Berry-Kravis, E., Sumis, A., Hervey, C., Nelson, M., Porges, S.W., Weng, N., Weiler, I.J., & Greenough, W.T. (2008) Open-label treatment trial of lithium to target the underlying defect in fragile X syndrome. *J Dev Behav Pediatr* **29**, 293-302.

Bhakar, A.L., Dölen, G., & Bear, M.F. (2012) The pathophysiology of fragile X (and what it teaches us about synapses). *Annu Rev Neurosci* **35**, 417-443.

Bhogal, B. & Jongens, T.A. (2010) Fragile X syndrome and model organisms: identifying potential routes of therapeutic intervention. *Dis Model Mech* **3,** 693-700.

Bhattacharya, A. & Klann E. (2012) Fragile X syndrome therapeutics S(C)TEP through the developmental window. *Neuron* **74**, 1-3.

Brennan, F.X., Albeck, D.S., & Paylor, R. (2006) Fmr1 knockout mice are impaired in a leverpress escape/avoidance task. *Genes Brain Behav* **5**, 467-471.

Chen, G., Rajkowska, G., Du, F., Seraji-Bozorgzad, N., & Manji, H.K. (2000) Enhancement of hippocampal neurogenesis by lithium. *J Neurochem* **75**, 1729–1734.

Choi, C.H., Schoenfeld, B.P., Bell, A.J., Hinchey, P., Kollaros, M., Gertner, M.J., Woo, N.H., Tranfaglia, M.R., Bear, M.F., Zukin, R.S., McDonald, T.V., Jongens, T.A., & McBride, S.M. (2011) Pharmacological reversal of synaptic plasticity deficits in the mouse model of fragile X syndrome by group II mGluR antagonist or lithium treatment. *Brain Res* **1380**, 106-119.

Contestabile, A., Greco, B., Ghezzi, D., Tucci, V., Benfenati, F., & Gasparini, L. (2013) Lithium rescues synaptic plasticity and memory in Down syndrome mice. *J Clin Invest* **123**, 348-361.

Cornish, K.M., Munir, F., & Cross, G. (1999) Spatial cognition in males with Fragile-X syndrome: evidence for a neuropsychological phenotype. *Cortex* **35**, 263-271.

Cornish, K., Munir, F., & Wilding, J. (2001) A neuropsychological and behavioural profile of attention deficits in fragile X syndrome. *Rev Neurol* **33,** Suppl 1:S24-29.

D'Hooge, R., Nagels, G., Franck, F., Bakker, C.E., Reyniers, E., Storm, K., Kooy, R.F., Oostra, B.A., Willems, P.J., & De Deyn, P.P. (1997) Mildly impaired water maze performance in male Fmr1 knockout mice. *Neuroscience* **76**, 367-376.

Dolan, R.J. & Fletcher P.C. (1997) Dissociating prefrontal and hippocampal function in episodic memory encoding. *Nature* **388**, 582-585.

Eadie, B.D., Cushman, J., Kannangara, T.S., Fanselow, M.S., & Christie, B.R. (2012) NMDA receptor hypofunction in the dentate gyrus and impaired context discrimination in adult Fmr1 knockout mice. *Hippocampus* **22**, 241-254.

Findling, R.L., Kafantaris, V., Pavuluri, M., McNamara, N.K., McClellan, J., Frazier, J.A., Sikich, L., Kowatch, R., Lingler, J., Faber, J., Rowles, B.M., Clemons, T.E., & Taylor-Zapata, P. (2011) Dosing strategies for lithium monotherapy in children and adolescents with bipolar I disorder. *J Child Adolesc Psychopharmacol* **21**,195-205.

Fisch, G.S., Hao, H.K., Bakker, C., & Oostra, B.A. (1999) Learning and memory in the FMR1 knockout mouse. *Am J Med Genet* **84**, 277-282.

Gatto, C.L. & Broadie, K. (2009) The fragile X mental retardation protein in circadian rhythmicity and memory consolidation. *Mol Neurobiol* **39**, 107-129.

Goodrich-Hunsaker, N.J., Hunsaker, M.R., & Kesner, R.P. (2005) Dissociating the role of the parietal cortex and dorsal hippocampus for spatial information processing. *Behav Neurosci* **119**, 1307-1315.

Goodrich-Hunsaker, N.J., Hunsaker, M.R., & Kesner, R.P. (2008) The interactions and dissociations of the dorsal hippocampus subregions: how the dentate gyrus, CA3, and CA1 process spatial information. *Behav Neurosci* **122**, 16-26.

Guo, W., Allan, A.M., Zong, R., Zhang, L., Johnson, E.B., Schaller, E.G., Murthy, A.C., Goggin, S.L., Eisch, A.J., Oostra, B.A., Nelson, D.L., Jin, P., & Zhao, X. (2011) Ablation of Fmrp in adult neural stem cells disrupts hippocampus-dependent learning. *Nat Med* **17**, 559-565.

Hagerman, R.J., Berry-Kravis, E., Kaufmann, W.E., Ono, M.Y., Tartaglia, N., Lachiewicz, A., Kronk, R., Delahunty, C., Hessl, D., Visootsak, J., Picker, J., Gane, L., & Tranfaglia, M. (2009) Advances in the treatment of fragile X syndrome. *Pediatrics* **123,** 378-390.

Hannesson, D.K., Vacca, G., Howland, J.G., & Phillips, A.G. (2004) Medial prefrontal cortex is involved in spatial temporal order memory but not spatial recognition memory in tests relying on spontaneous exploration in rats. *Behav Brain Res* **153**, 273-285.

Hayashi, M.L., Rao, B.S., Seo, J.S., Choi, H.S., Dolan, B.M., Choi, S.Y., Chattarji, S., & Tonegawa, S. (2007) Inhibition of p21-activated kinase rescues symptoms of fragile X syndrome in mice. *Proc Natl Acad Sci U S A* **104**, 11489-11494.

Hoge, J. & Kesner, R.P. (2007) Role of CA3 and CA1 subregions of the dorsal hippocampus on temporal processing of objects. *Neurobiol Learn Mem* **88**, 225-231.

Honey, R.C., Watt, A., & Good, M. (1998) Hippocampal lesions disrupt an associative mismatch process. *J Neurosci* **18**, 2226-2230.

Hooper, C., Markevich, V., Plattner, F., Killick, R., Schofield, E., Engel, T., Hernandez, F., Anderton, B., Rosenblum, K., Bliss, T., Cooke, S.F., Avila, J., Lucas, J.J., Giese, K.P., Stephenson, J., & Lovestone, S. (2007) Glycogen synthase kinase-3 inhibition is integral to long-term potentiation. *Eur J Neurosci* **25**, 81-86.

Hou, L., Antion, M.D., Hu, D., Spencer, C.M., Paylor, R., & Klann, E. (2006) Dynamic translational and proteasomal regulation of fragile X mental retardation protein controls mGluR-dependent long-term depression. *Neuron* **51**, 441-454.

Huber, K.M., Gallagher, S.M., Warren, S.T., & Bear, M.F. (2002) Altered synaptic plasticity in a mouse model of fragile X mental retardation. *Proc Natl Acad Sci U S A* **99**, 7746-7750.

Hunsaker, M.R. & Kesner, R.P. (2008) Evaluating the differential roles of the dorsal dentate gyrus, dorsal CA3, and dorsal CA1 during a temporal ordering for spatial locations task. *Hippocampus* **18**, 955-964.

Hunsaker, M.R., Wenzel, H.J., Willemsen, R., & Berman, R.F. (2009) Progressive spatial processing deficits in a mouse model of the fragile X premutation. *Behav Neurosci* **123**, 1315-1324.

Hunsaker, M.R., Kim, K., Willemsen, R., & Berman, R.F. (2012) CGG trinucleotide repeat length modulates neural plasticity and spatiotemporal processing in a mouse model of the fragile X premutation. *Hippocampus* **22,** 2260-2275.

Jope, R.S. (2011) Glycogen synthase kinase-3 in the etiology and treatment of mood disorders. *Frontiers in Molecular Neuroscience* **4**, 16-26.

Kemper, M.B., Hagerman, R.J., & Altshul-Stark, D. (1988) Cognitive profiles of boys with the fragile X syndrome. *Am J Med Genet* **30**, 191-200.

Klein, P.S. & Melton, D.A. (1996) A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci USA* **93,** 8455-8459.

Knight, R. (1996) Contribution of human hippocampal region to novelty detection. *Nature* **383**, 256-259.

Kooy, R.F., D'Hooge, R., Reyniers, E., Bakker, C.E., Nagels, G., De Boulle, K., Storm, K., Clincke, G., De Deyn, P.P., Oostra, B.A., & Willems, P.J. (1996) Transgenic mouse model for the fragile X syndrome. *Am J Med Genet* **64**, 241-245.

Lisman, J.E. (1999) Relating hippocampal circuitry to function: recall of memory sequences by reciprocal dentate-CA3 interactions. *Neuron* **22**, 233-242.

Liu, Z.H., Chuang, D.M., & Smith, C.B. (2011) Lithium ameliorates phenotypic deficits in a mouse model of fragile X syndrome. *Int J Neuropsychopharmacology* **14**, 618-630.

Liu, Z.H., Huang, T., & Smith, C.B. (2012) Lithium reverses increased rates of cerebral protein synthesis in a mouse model of fragile X syndrome. *Neurobiol Dis* **45**, 1145-1152.

Lu, R., Wang, H., Liang, Z., Ku, L., O'Donnell, W.T., Li, W., Warren, S.T., & Feng, Y. (2004) The fragile X protein controls microtubule-associated protein 1B translation and microtubule stability in brain neuron development. *Proc Natl Acad Sci U S A* **101**:15201-15206.

McBride, S.M., Choi, C.H., Wang, Y., Liebelt, D., Braunstein, E., Ferreiro, D., Sehgal, A., Siwicki, K.K., Dockendorff, T.C., Nguyen, H.T., McDonald, T.V., & Jongens, T.A. (2005) Pharmacological rescue of synaptic plasticity, courtship behavior, and mushroom body defects in a Drosophila model of fragile X syndrome. *Neuron* **45**, 753-764.

Min, W.W., Yuskaitis, C.J., Yan, Q., Sikorski, C., Chen, S., Jope, R.S., & Bauchwitz, R.P. (2009) Elevated glycogen synthase kinase-3 activity in Fragile X mice: key metabolic regulator with evidence for treatment potential. *Neuropharmacology* **56**, 463-472.

Mines, M.A., Yuskaitis, C.J., King, M.K., Beurel, E., & Jope, R.S. (2010) GSK3 influences social preference and anxiety-related behaviors during social interaction in a mouse model of Fragile X syndrome and autism. *PLoS One* **5**, e9706.

Mineur, Y.S., Sluyter, F., de Wit, S., Oostra, B.A., & Crusio, W.E. (2002) Behavioral and neuroanatomical characterization of the Fmr1 knockout mouse. *Hippocampus* **12**, 39-46.

Mitchell, J.B. & Laiacona, J. (1998) The medial frontal cortex and temporal memory: tests using spontaneous exploratory behaviour in the rat. *Behav Brain Res* **97,** 107-113.

Nosyreva, E.D. & Huber, K.M. (2006) Metabotropic receptor-dependent long-term depression persists in the absence of protein synthesis in the mouse model of fragile X syndrome. *J Neurophysiol* **95**, 3291-3295.

O'Brien, W.T., Harper, A.D., Jové, F., Woodgett, J.R., Maretto, S., Piccolo, S., & Klein, P.S. (2004) Glycogen synthase kinase-3β haploinsufficiency mimics the behavioral and molecular effects of lithium. *J Neurosci* **24**, 6791-6798.

Ornstein, P.A., Schaaf, J.M., Hooper, S.R., Hatton, D.D., Mirrett, P., & Bailey, D.B., Jr. (2008) Memory skills of boys with fragile X syndrome. *Am J Ment Retard* **113**, 453-465.

Otto, T. & Eichenbaum, H. (1992) Neuronal activity in the hippocampus during delayed non-match to sample performance in rats: evidence for hippocampal processing in recognition memory. *Hippocampus* **2**, 323-334.

Pacey, L.K., Doss, L., Cifelli, C., van der Kooy, D., Heximer, S.P., & Hampson, D.R. (2011) Genetic deletion of regulator of G-protein signaling 4 (RGS4) rescues a subset of fragile X related phenotypes in the FMR1 knockout mouse. *Mol Cell Neurosci* **46**, 563-572.

Paradee, W., Melikian, H.E., Rasmussen, D.L., Kenneson, A., Conn, P.J., & Warren, S.T. (1999) Fragile X mouse: strain effects of knockout phenotype and evidence suggesting deficient amygdala function. *Neuroscience* **94**, 185-192.

Peier, A.M., McIlwain, K.L., Kenneson, A., Warren, S.T., Paylor, R., & Nelson, D.L. (2000) (Over)correction of FMR1 deficiency with YAC transgenics: behavioral and physical features. *Hum Mol Genet* **9**:1145-1159.

Pieretti, M., Zhang, F.P., Fu, Y.H., Warren, S.T., Oostra, B.A., Caskey, C.T., & Nelson, D.L. (1991) Absence of expression of the FMR-1 gene in fragile X syndrome. *Cell* **66**, 817-822.

Qin, M., Kang, J., & Smith, C.B. (2005) A null mutation for Fmr1 in female mice: effects on regional cerebral metabolic rate for glucose and relationship to behavior. *Neuroscience* **135**, 999-1009.

Rolls, E.T. & Kesner, R.P. (2006) A computational theory of hippocampal function, and empirical tests of the theory. *Prog Neurobiol* **79**, 1-48.

Ryan, N.D., Bhatara, V.S., & Perel, J.M. (1999) Mood stabilizers in children and adolescents. *J Am Acad Child Adolesc Psychiatry* **38**, 529-536.

Save, E., Poucet, B., Foreman, N., & Buhot, M.C. (1992) Object exploration and reactions to spatial and nonspatial changes in hooded rats following damage to parietal cortex or hippocampal formation. *Behav Neurosci* **106**, 447-456.

Shaltiel, G., Maeng, S., Malkesman, O., Pearson, B., Schloesser, R.J., Tragon, T., Rogawski, M., Gasior, M., Luckenbaugh, D., Chen, G., & Manji, H.K. (2008) Evidence for the involvement of the kainate receptor subunit GluR6 (GRIK2) in mediating behavioral displays related to behavioral symptoms of mania. *Mol Psychiatry* **13**, 858-872.

Spencer, C.M., Graham, D.F., Yuva-Paylor, L.A., Nelson, D.L., & Paylor, R (2008) Social behavior in Fmr1 knockout mice carrying a human FMR1 transgene. *Behav Neurosci* **122,** 710-715.

Tassone, F., Long, K.P., Tong, T.H., Lo, J., Gane, L.W., Berry-Kravis, E., Nguyen, D., Mu, L.Y., Laffin, J., Bailey, D.B., & Hagerman, R.J. (2012) FMR1 CGG allele size and prevalence ascertained through newborn screening in the United States. *Genome Med* **4**, 100.

Tsien, J.Z., Huerta, P.T., & Tonegawa, S. (1996) The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. *Cell* **87**, 1327-1338.

Ventura, R., Pascucci, T., Catania, M.V., Musumeci, S.A., & Puglisi-Allegra, S. (2004) Object recognition impairment in Fmr1 knockout mice is reversed by amphetamine: involvement of dopamine in the medial prefrontal cortex. *Behav Pharmacol* **15**, 433-442.

Verkerk, A.J., Pieretti, M., Sutcliffe, J.S., Fu, Y.H., Kuhl, D.P., Pizzuti, A., Reiner, O., Richards, S., Victoria, M.F., Zhang, F.P., Eussen, B.E., van Ommen, G.B., Blonden, L.A.J., Riggins, G.J., Chastain, J.L., Kunst, C.B., Galjaard, H., Caskey, C.T., Nelson, D.L., Oostra, B.A., & Warren, S.T. (1991) Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell* **65**, 905-914.

Wallenstein, G.V., Eichenbaum, H., & Hasselmo, M.E. (1998) The hippocampus as an associator of discontiguous events. *Trends Neurosci* **21**, 317-323.

Yan, Q.J., Asafo-Adjei, P.K., Arnold, H.M., Brown, R.E., & Bauchwitz, R.P. (2004) A phenotypic and molecular characterization of the fmr1-tm1Cgr fragile X mouse. *Genes Brain Behav* **3**, 337-359.

Yuskaitis, C.J., Mines, M.A., King, M.K., Sweatt, J.D., Miller, C.A., & Jope, R.S. (2010a) Lithium ameliorates altered glycogen synthase kinase-3 and behavior in a mouse model of Fragile X Syndrome. *Biochemical Pharmacology* **79**, 632-646.

Yuskaitis, C., Beurel, E., & Jope, R.S. (2010b) Evidence of reactive astrocytes but not peripheral immune system activation in a mouse model of Fragile X syndrome. *Biochim Biophys Acta* **1802**, 1006-1012.

Zhao, M.G., Toyoda, H., Ko, S.W., Ding, H.K., Wu, L.J., & Zhuo, M. (2005) Deficits in trace fear memory and long-term potentiation in a mouse model for fragile X syndrome. *J Neurosci* **25**, 7385-7892.

Zhu, L.Q., Wang, S.H., Liu, D., Yin, Y.Y., Tian, Q., Wang, X.C., Wang, Q., Chen, J.G., & Wang, J.Z. (2007) Activation of glycogen synthase kinase-3 inhibits long-term potentiation with synapse-associated impairments. *J Neurosci* **27,** 12211-12220

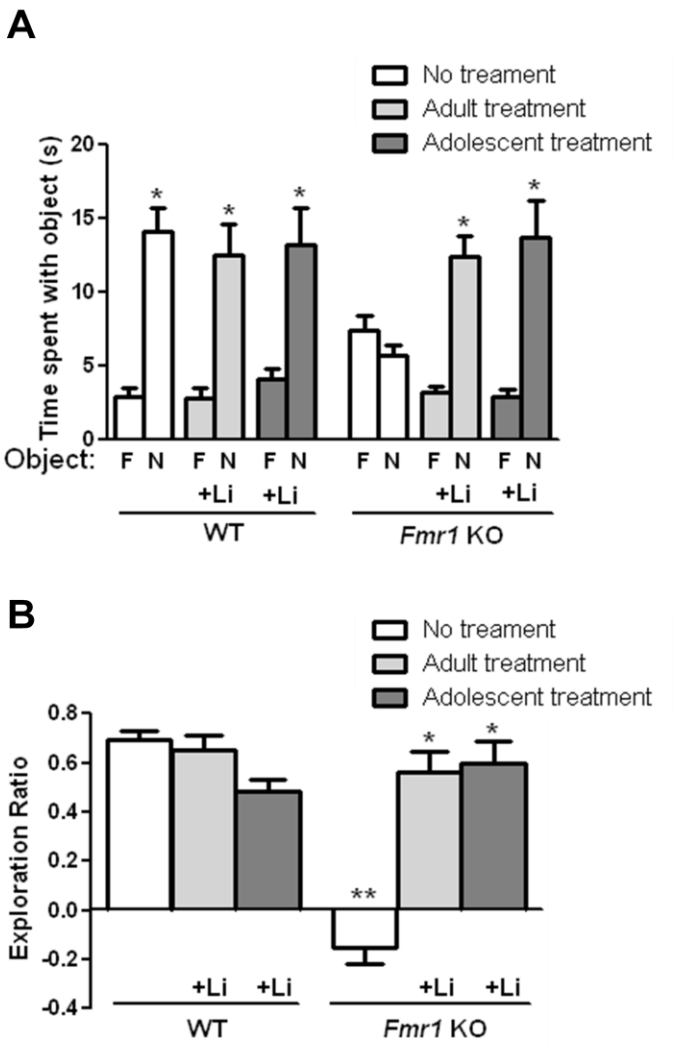


Figure 1**.** Chronic lithium treatment of adult or adolescent *Fmr1* KO mice reverses impaired discrimination in the object novelty detection task.
Lithium was administered for four weeks to adult (from 8 to 12 weeks of age) and adolescent (from 4 to 8 weeks of age) male *Fmr1* knockout (KO) and wild-type (WT) mice prior to testing. (A) Times spent exploring the novel (N) and familiar (F) object. (Student's t-test; *$p<0.05$ compared to time spent with familiar object; WT no treatment: $n=20$, $t(46)=6.51$, $p<0.05$; WT adult lithium treatment: $n=10$, $t(18)=4.29$, $p<0.05$; WT adolescent lithium treatment: $n=9$, $t(10)=3.47$, $p<0.05$; *Fmr1* KO no treatment: $n=20$, $t(48)=1.42$, $p>0.05$; *Fmr1* KO adult lithium treatment: $n=10$, $t(18)=6.20$, $p<0.05$; *Fmr1* KO adolescent lithium treatment: $n=9$, $t(16)=4.19$, $p<0.05$). (B) Exploration ratio. (two-way ANOVA (genotype x treatment) followed by post hoc Bonferroni's multiple comparison test; $F(2,72)=33.02$, $p<0.05$; **$p<0.05$ compared to untreated WT mice; *$p<0.05$ compared to same genotype without treatment).

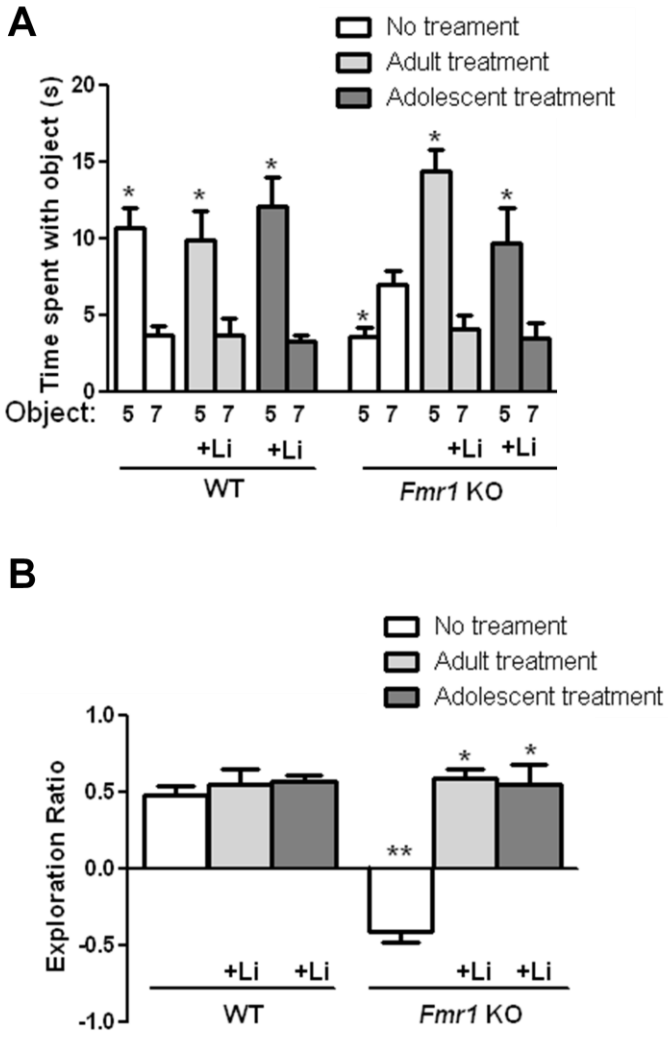


Figure 2. Chronic lithium treatment of adult or adolescent *Fmr1* KO mice ameliorates temporal order memory deficits.

Adult and adolescent male *Fmr1* KO and WT mice were treated with lithium for 4 weeks prior to testing. (A) Times spent exploring the first object presented (Object 5) and the object most recently explored (Object 7). (Student's t-test; *p<0.05 compared to time spent with Object 7; WT no treatment: n=20, t(38)=4.82, p<0.05; WT adult lithium treatment: n=10, t(18)=2.74, p<0.05; WT adolescent lithium treatment: n=9, t(12)=4.56, p<0.05; *Fmr1* KO no treatment: n=20, t(38)=3.16, p<0.05; *Fmr1* KO adult lithium treatment: n=9, t(16)=6.21, p<0.05; *Fmr1* KO adolescent lithium treatment: n=9, t(16)=2.38, p<0.05). (B) Exploration ratio. (two-way ANOVA (genotype x treatment) followed by post hoc Bonferroni's multiple comparison test; F(2,75)=27.48, p<0.05; **p<0.05 compared to untreated WT mice; *p<0.05 compared to same genotype without treatment).

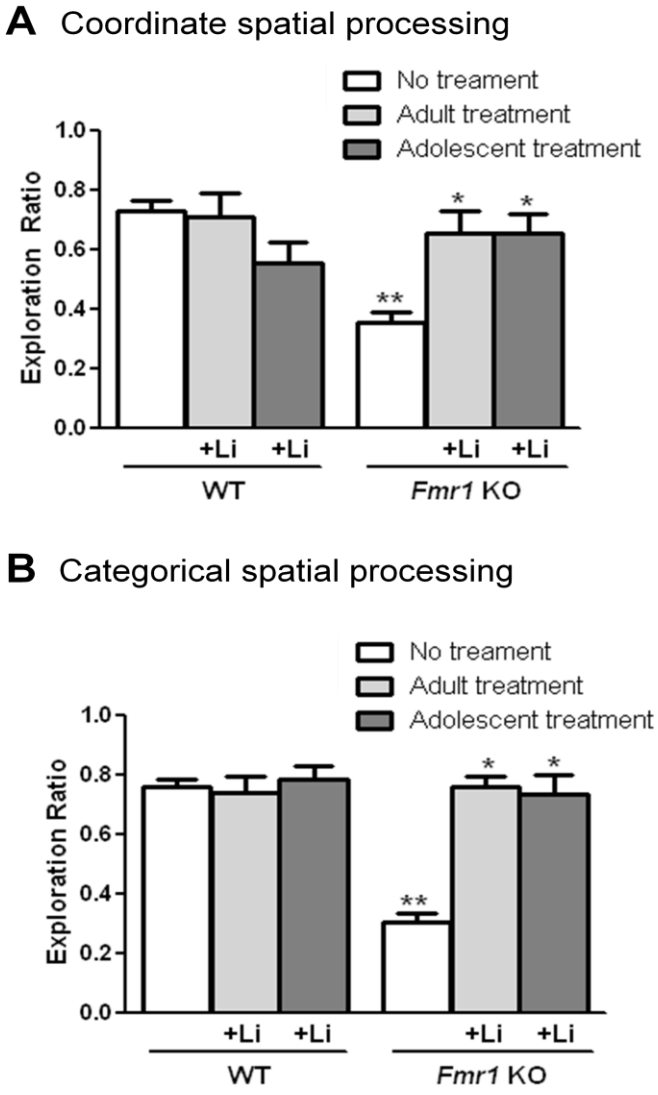
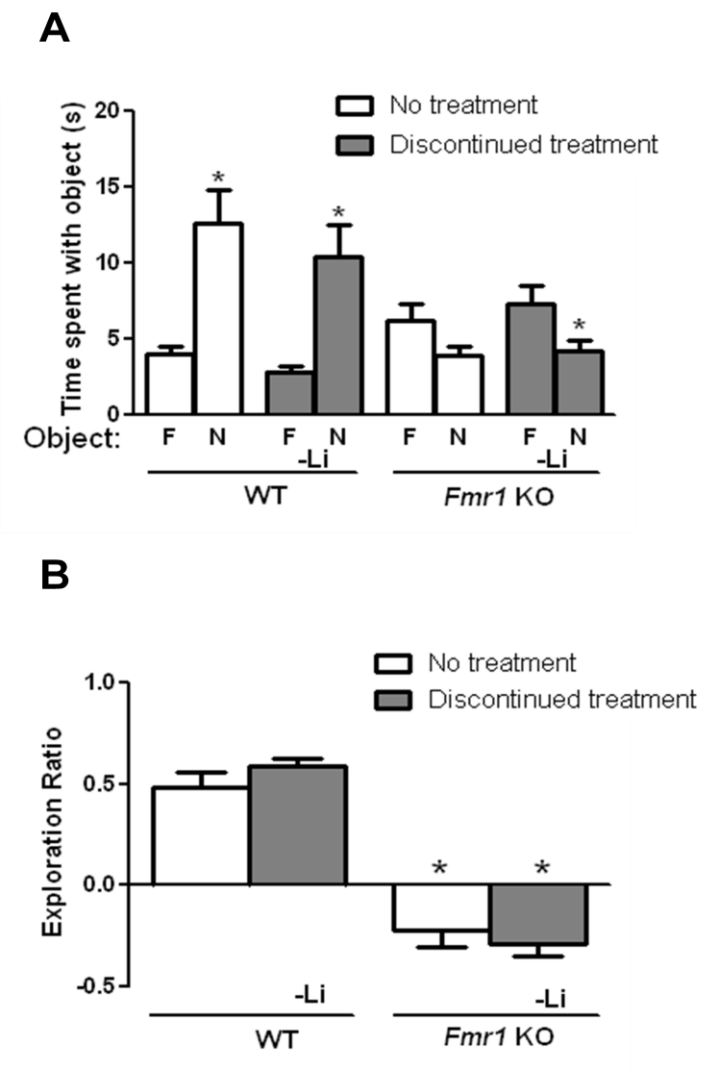
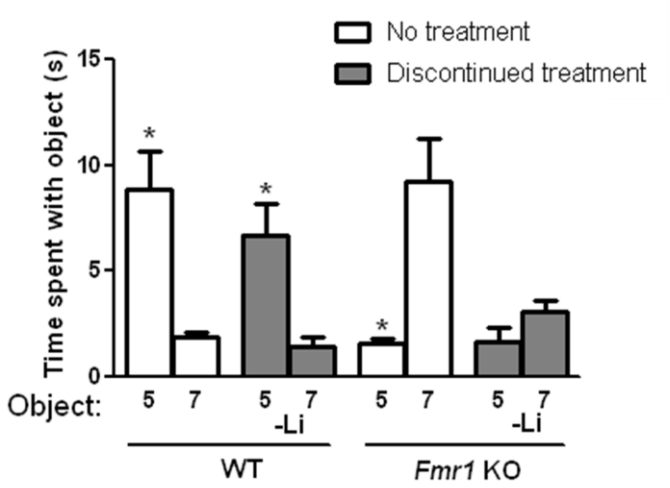


Figure 3. Chronic lithium treatment of adult or adolescent *Fmr1* KO mice alleviates spatial processing impairments in *Fmr1* KO mice.
Adult and adolescent male *Fmr1* KO and WT mice were treated with lithium for 4 weeks prior to testing. (A) Exploration ratio in the coordinate spatial processing task (two-way ANOVA (genotype x treatment) followed by post hoc Bonferroni's multiple comparison test; $F(2,68)=10.68$, $p<0.05$). (B) Exploration ratio in the categorical spatial processing task (two-way ANOVA (genotype x treatment) followed by post hoc Bonferroni's multiple comparison test; $F(2,69)=24.93$, $p<0.05$). **$p<0.05$ compared to untreated WT mice; *$p<0.05$ compared to same genotype without treatment; n=20 WT no treatment; n=10 WT adult treatment; n=9 WT adolescent treatment; n=20 *Fmr1* KO no treatment; n=10 *Fmr1* KO adult treatment; n=9 *Fmr1* KO adolescent treatment.


A
No treatment
Discontinued treatment
Time spent with object (s)
20
15
10
5
0
*
*
*
Object:
F N F N F N F N
-Li
-Li
WT
Fmr1 KO
B
No treatment
Discontinued treatment
Exploration Ratio
1.0
0.5
0.0
-0.5
*
*
-Li
-Li
WT
Fmr1 KO

C


No treatment
Discontinued treatment
Time spent with object (s)
15
10
5
0
*
*
*
Object:
5
7
5
7
5
7
5
7
-Li
-Li
WT
Fmr1 KO

D

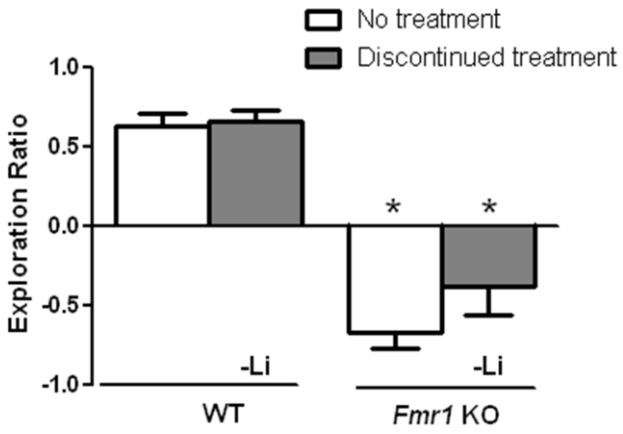

No treatment
Discontinued treatment
Exploration Ratio
1.0
0.5
0.0
-0.5
-1.0
*
*
-Li
-Li
WT
Fmr1 KO

Figure 4. Impaired cognitive deficits are reinstated in *Fmr1* KO mice following discontinuation of lithium in the object novelty task and temporal ordering for objects task.

Adolescent male *Fmr1* KO and WT mice were treated with lithium for 4 weeks. After testing, lithium treatment was discontinued for 4 weeks and the mice were retested. Prior testing in the same paradigms had no effect on re-test performance in untreated WT mice or *Fmr1* KO mice (Student's t-test, $p>0.05$ compared to retest; object novelty detection task: WT no treatment: $n=7$, $t(12)=0.78$, $p>0.05$; FX no treatment: $n=9$, $t(16)=0.29$, $p>0.05$; temporal ordering for objects task: WT no treatment: $n=7$, $t(12)=0.68$, $p>0.05$; FX no treatment: $n=9$, $t(16)=1.42$, $p>0.05$). (A,B) Performance in the object novelty detection task. (A) Times spent exploring the novel (N) and familiar (F) object. (Student's t-test; *$p<0.05$ compared to time spent with familiar object; WT no treatment: $n=7$, $t(12)=3.78$, $p<0.05$; WT discontinued lithium treatment: $n=7$, $t(12)=3.57$, $p<0.05$; *Fmr1* KO no treatment: $n=9$, $t(16)=1.87$, $p>0.05$; *Fmr1* KO discontinued lithium treatment: $n=9$, $t(16)=2.28$, $p<0.05$) (B) Exploration ratio. (one-way ANOVA followed by post hoc Bonferroni's multiple comparison test; $F(3,28)=47.41$). *$p<0.05$ compared to matched WT mice. (C,D) Performance in the temporal ordering for objects task. (C) Times spent exploring the first object presented (Object 5) and the object most recently explored (Object 7). (Student's t-test; *$p<0.05$ compared to time spent with Object 7; WT no treatment: $n=7$, $t(12)=3.87$, $p<0.05$; WT discontinued lithium treatment: $n=9$, $t(12)=3.48$, $p<0.05$; *Fmr1* KO no treatment: $n=9$, $t(16)=3.75$, $p<0.05$; *Fmr1* KO discontinued lithium treatment: $n=9$, $t(16)=1.75$, $p>0.05$). (D) Exploration ratio. (one-way ANOVA followed by post hoc Bonferroni's multiple comparison test; $F(3,28)=28.80$, $p<0.05$). *$p<0.05$ compared to matched WT mice.

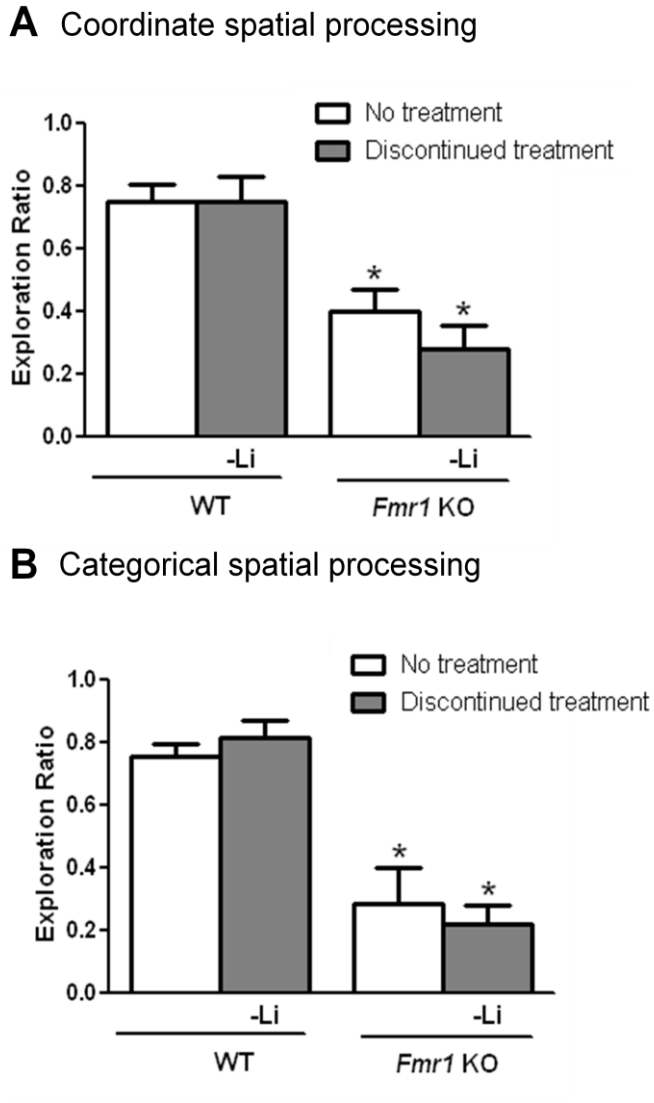


Figure 5. Spatial processing impairments return in *Fmr1* KO mice following discontinuation of lithium.

Adolescent male *Fmr1* KO and WT mice were treated with lithium for 4 weeks and then following testing, lithium treatment was discontinued for 4 weeks and the mice were retested. Prior cognitive testing in the same paradigms had no effect on re-test performance in untreated WT mice or *Fmr1* KO mice (Student's t-test, $p>0.05$ compared to retest; coordinate spatial processing task: WT no treatment: n=7, $t(12)=0.32$, $p>0.05$; FX no treatment: n=9, $t(16)=0.27$, $p>0.05$; categorical spatial processing task: WT no treatment: n=7, $t(12)=0.24$, $p>0.05$; FX no treatment: n=9, $t(16)=0.28$, $p>0.05$). (A) Exploration ratio in the coordinate spatial processing task. (one-way ANOVA followed by post hoc Bonferroni's multiple comparison test; $F(3,29)=11.34$, $p<0.05$). (B) Exploration ratio in the categorical spatial processing task. (one-way ANOVA followed by post hoc Bonferroni's multiple comparison test; $F(3,27)=17.29$, $p<0.05$). *$p<0.05$ compared to matched WT mice. n=7 WT no treatment; n=7 discontinued lithium treatment; n=9 *Fmr1* KO no treatment; n=9 *Fmr1* KO discontinued lithium treatment.

GLYCOGEN SYNTHASE KINASE-3 INHIBITORS REVERSE DEFICITS IN COGNITION IN FRAGILE X MICE

by

MARGARET K. KING, VALLE PALOMO, ANA MARTINEZ, AND RICHARD S. JOPE

Abstract

Impairments in the inhibition of glycogen synthase kinase-3 (GSK3) have been linked to cognitive deficits in several disorders of the central nervous system, including the most common form of inherited intellectual disability, Fragile X Syndrome (FXS). Not only does FXS cause intellectual disability, but it is the leading monogenetic cause of autism, therefore it is crucial to identify therapeutic interventions to alleviate the cognitive deficits and other afflictions of FXS. FXS is caused by loss of function of the *fragile X mental retardation 1* (*FMR1*) gene on the X chromosome, and mice that lack *Fmr1* (*Fmr1* KO mice) exhibit hyperactive GSK3 in the hippocampus. Inhibition of GSK3 significantly reduces locomotor hyperactivity and audiogenic seizure susceptibility in *Fmr1* KO mice, raising the possibility that specific GSK3 inhibitors may also improve cognitive processes. The potential therapeutic utility of GSK3 inhibitors was tested on hippocampus-dependent cognitive behaviors. Administration of either of two specific GSK3 inhibitors, TDZD-8 or VP0.7, completely reversed impairments in four cognitive tasks, including novel object detection, coordinate and categorical spatial processing, and temporal order memory in *Fmr1* KO mice. These results establish that cognitive deficits in *Fmr1* KO mice can be ameliorated by treatment with inhibitors of GSK3, which may prove therapeutically beneficial in FXS.

Introduction

Glycogen synthase kinase-3 (GSK3) is a ubiquitous enzyme that regulates many functions in the central nervous system (CNS). GSK3 is a serine/threonine kinase that exists in two isoforms, GSK3α and GSK3ß (Woodgett, 1990). The main way GSK3 is regulated is by inhibitory phosphorylation on serine-21 of GSK3α and serine-9 of GSK3ß, thus reducing its activity (Jope and Johnson 2004). Hyperactive GSK3 due to impairments in the inhibition of GSK3 has been linked to several common diseases of the CNS that include impaired cognition, and this has led many laboratories to evaluate the therapeutic role of GSK3 inhibitors (King et al., 2013). The first identified inhibitor of GSK3 is lithium (Klein and Melton 1996). Lithium has been used as a primary treatment for bipolar mood disorder, but the therapeutically relevant level of lithium is only about 1 mM in human serum, which only inhibits GSK3 by ~35% (Klein and Melton, 1996). Higher levels of lithium are toxic, so higher doses cannot be used to cause greater inhibition of GSK3. Therefore, in order to test the validity of GSK3 as the therapeutic target of lithium in animal models of diseases, other selective, small molecule inhibitors of GSK3 need to be used.

One disease that exhibits impairments in the inhibition of GSK3 is fragile X syndrome (FXS) (Min et al. 2009; Yuskaitis et al. 2010a; Mines and Jope 2011), the most common form of inherited intellectual disability and the leading monogenetic cause of autism (Garber et al., 2006; Bhakar et al., 2012). FXS is caused by loss of function of the *fragile X mental retardation 1* (*FMR1*) gene on the X chromosome (Pieretti et al., 1991), causing loss of its gene product, Fragile X Mental Retardation Protein (FMRP). FMRP acts as a brake upon translation of individual target mRNAs (Zalfa et al., 2003). The

absence of FMRP is believed to cause the abnormal behavioral and physiological symptoms of FXS, primarily including intellectual disability, but also social anxiety, attention deficit, speech and language impairments, seizures, and increased sensitivity to sensory stimuli (Errijgers et al., 2004; Ornstein et al., 2008; Dissanayake et al., 2009; Hernandez et al., 2009; Bagni et al., 2012).

In order to study the physiological and behavioral manifestations that result from loss of FMRP, a transgenic mouse model of FXS was generated by interrupting the *Fmr1* gene (Bakker et al., 1994). *Fmr1* knockout (KO) mice display several FXS- and autism-related behaviors, including increased audiogenic seizure susceptibility, hyperactivity, abnormal social behavior, and cognitive deficits. Using the *Drosophila* model of FXS, treatment with lithium was found to rescue some aberrant behaviors, including alterations in courtship behavior and defects in cognition (McBride et al., 2005), but this effect may have been due to inhibition of inositol monophosphatase. This seminal finding led to investigations of the role that GSK3 activity and inhibition of GSK3 by lithium plays in the *Fmr1* KO mouse. Inhibitory serine-phosphorylation of GSK3 is decreased in the *Fmr1* KO mouse striatum, hippocampus, and cortex (Min et al., 2009; Yuskaitis et al., 2010a), suggesting that pharmacological therapies to decrease GSK3 activity are a potential target to rescue some behavioral phenotypes of *Fmr1* KO mice. Treatment with lithium to inhibit GSK3 has been reported to increase inhibitory serine-phosphorylation of GSK3 in *Fmr1* KO mouse brain (Min et al., 2009; Yuskaitis et al., 2010a). Lithium treatment reduced susceptibility of *Fmr1* KO mice to audiogenic seizures, decreased locomotor hyperactivity in the open field, improved some of the social behavior deficits (Mines et al., 2010), and ameliorated the passive avoidance

learning deficit in *Fmr1* KO mice (Min et al., 2009; Yuskaitis et al., 2010a; Mines et al., 2010; Liu et al., 2011). In order to test the validity of GSK3 as the therapeutic target of lithium and to address the predominant impairment in FXS, intellectual disability, we tested if acute pharmacological inhibition of GSK3 using two selective, small molecule inhibitors of GSK3, TDZD-8 and VP0.7, reversed cognitive impairments in *Fmr1* KO mice. Using four hippocampus-dependent tasks we report that GSK3 inhibitors, but not mGluR inhibition, ameliorate learning deficits in *Fmr1* KO mice.

Methods and Materials

Animals and in vivo *treatments*

Experiments used adult, male C57Bl/6 mice, with or without a disruption of the *Fmr1* gene, aged 2-3 months. The *Fmr1* knockout mice were generated by breeding male and female C57Bl/6 *Fmr1* heterozygous mice to generate *Fmr1* knockout (KO) and wild-type (WT) littermates. To inhibit GSK3, mice were given an intraperitoneal (ip) injection of 5 mg/kg thiadiazolidindione-8 (TDZD-8), a highly selective ATP non-competitive inhibitor of GSK3 (Martinez et al., 2002), or 5 mg/kg N′-dodecanoyl-1-ethyl-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (VP0.7), an allosteric (not competitive with ATP or substrate) selective GSK3 inhibitor that binds to the C-terminal lobe of the enzyme (Palomo et al., 2011), and results were compared with mice given vehicle (5% Tween-80, 5% DMSO in saline). TDZD-8 and VP0.7 were prepared in the Martinez laboratory (Martinez et al., 2002; Palomo et al., 2011), and these doses have previously been reported to be effective in mice (Ramirez et al., 2010; Kalinichev and Dawson, 2011; Lipina et al., 2011; Lipina et al., 2012; Jones et al., 2012; Beurel et al., 2013). To

inhibit mGluR5, mice were given an ip injection of 30 mg/kg 2-methyl-6-(phenylethynyl)pyridine (MPEP) (Tocris Bioscience, Ellisville, MO), and results were compared to mice given vehicle (0.9% sodium chloride). Mice were housed in light and temperature controlled rooms and treated in accordance with National Institutes of Health and the University of Miami.

Novel object detection task

Novelty detection assesses hippocampal-dependent recognition memory (Otto and Eichenbaum 1992; Knight 1996; Dolan and Fletcher 1997; Lisman 1999), and the novel object detection task is used to assess this general memory function (Hoge and Kesner 2007; Hunsaker and Kesner 2008; Hunsaker and Kesner 2009; Hunsaker et al., 2012). For this and the following task, a Plexiglas box (26 cm long x 20 cm wide x 16 cm tall) and seven objects in duplicate (4-6 cm diameter x 2-6 cm height) were used. For all cognitive assessments, time spent exploring an object was defined as the mouse sniffing or touching an object with its nose, vibrissa, mouth, or forepaws. Time spent near or standing on top of an object without interacting with it was not counted as exploration. As previously described (Hoge and Kesner 2007; Hunsaker et al., 2012) a mouse was allowed to explore two identical copies of Object 1 for 5 min. Subsequently, after 5 min in an opaque holding container, the mouse was allowed to explore two copies of Object 2 for 5 min. After 5 min in the holding container, the mouse was allowed to explore two copies of Object 3 for 5 min. After 5 min in the holding container, the mouse was allowed to explore an unused copy of Object 1 and a novel Object 4 for 5 min. More time spent exploring the novel Object 4 than the familiar Object 1 indicates normal memory

processing. Time spent exploring each object was obtained from videos, and the exploration ratio was calculated as time of: (exploration of Object 4 – exploration of Object 1)/(exploration of Object 1 + exploration of Object 4).

For this and all other behavioral assessments, mice were acclimated to the room containing the behavioral instruments for 30 min before testing, the sessions were filmed, a white noise generator (55 dB) was used, and each apparatus and object was cleaned with 70% ethanol between each test session.

Temporal order for object task

The temporal order for objects task is a hippocampal CA1-dependent task that assesses temporal order memory in rodents (Honey et al., 1998; Wallenstein et al., 1998; Lisman 1999; Hoge and Kesner 2007; Hunsaker and Kesner 2008). As previously described (Hoge and Kesner 2007; Hunsaker et al., 2007; Hunsaker et al., 2012), and equivalent to the novel object detection task, a mouse underwent three sessions to explore three new sets of objects (Objects 5, 6, 7). During the test session, the mouse was allowed to explore an unused copy of Object 5 and an unused copy of Object 7 for 5 min. Normal temporal order memory is exhibited by mice spending more time exploring the first object presented (Object 5) than the most recent object presented (Object 7). The exploration ratio was calculated as time of: (exploration of Object 5 – exploration of Object 7)/(exploration of Object 5 + exploration of Object 7).

Coordinate and categorical spatial processing tasks

The coordinate and categorical spatial processing tasks in mice assess hippocampal-dependent spatial information processing, a cognitive function required to form fine spatial memory (Save et al., 1992; Tsien et al., 1996; Long and Kesner 1996; Lisman 1999; Goodrich-Hunsaker et al., 2005; Goodrich-Hunsaker et al., 2008; Hunsaker and Kesner 2009; Hunsaker et al., 2012). The coordinate spatial processing test was performed according to the method reported by (Hunsaker et al., 2012). The test was conducted in two phases: habituation and test. During the habituation phase each mouse was placed on the edge of the table facing 2 objects that were 45 cm apart. The mouse was allowed to explore the table and objects during a 15 min habituation session. The mouse was then placed in an opaque holding container for 5 min, and the mouse was returned to the table with the objects moved closer together (30 cm) and allowed to explore for a 5 min test session. Mice that remember the distance between objects display increased exploration of the objects during the test session compared with the last 5 min of the habituation phase.

For the categorical spatial processing task 2 novel objects, different from those used for the coordinate spatial processing task, were used. A mouse was placed on the edge of the table facing the 2 objects that were 45 cm apart and allowed to explore the table and objects during a 15 min habituation session. The mouse was then placed in an opaque holding container for 5 min, and the mouse was returned to the table with the positions of the objects transposed and allowed to explore for a 5 min test session. Mice that remember the object positions display increased exploration of the objects during the test session compared with the last 5 min of the habituation phase. The exploration ratio

was calculated as: [(exploration time during the 5 min test session)/(exploration time during the 5 min test session + exploration time during the last 5 min of the habituation session)]. Increased exploration during the 5 min test session compared to the last 5 min of the habituation session reflected a ratio >0.5, and decreased exploration reflected a ratio <0.5.

Data are expressed as mean ± SEM. Student's t test, one-way ANOVA followed by Bonferroni's post-hoc multiple comparison, and Kruskall-Wallis with Dunn's multiple comparison test were used as noted. Significance was taken as $p<0.05$.

Results

Cognitive deficits in Fmr1 *KO mice are rescued by in vivo GSK3 inhibition.*

The novel object detection task, which requires the dentate gyrus (Hunsaker and Kesner 2008; Goodrich-Hunsaker et al., 2008; Hunsaker et al., 2007) and assesses the ability to discriminate between a familiar and novel object, was used to evaluate the potential benefits of GSK3 inhibition on learning deficits in *Fmr1* KO mice. WT mice spent significantly more time exploring the novel versus familiar object (20±3 sec vs 4±1 sec, $p<0.01$), demonstrating learning (Fig. 1A). In contrast, *Fmr1* KO mice spent equivalent amounts of time exploring the novel versus familiar object (8±2 sec vs 7±1 sec), indicating that *Fmr1* KO mice are unable to learn the task. The exploration ratio (calculated by dividing the difference between the time spent with the novel object versus the familiar object divided by total time exploring) was significantly different between WT and *Fmr1* KO mice (exploration ratio WT: 0.64±0.09; *Fmr1* KO: -0.07±0.06, $p<0.05$) (Fig. 1B).

GSK3 inhibition *in vivo* was achieved using two selective GSK3 inhibitors with CNS bioavailability, TDZD-8 (5 mg/kg; ip), a highly selective ATP non-competitive inhibitor (Martinez et al., 2002), and VP0.7 (5 mg/kg; ip), an allosteric (not competitive with ATP or substrate) selective GSK3 inhibitor (Palomo et al., 2011). The GSK3 inhibitors did not alter the performance of WT mice, which spent more time investigating the novel versus familiar object (TDZD-8: 16±3 sec vs 3±1 sec, $p<0.01$; VPO.7: 21±2 sec vs 6±1 sec, $p<0.01$) (Fig. 1A). However, *Fmr1* KO mice treated with TDZD-8 or VPO.7 spent significantly more time exploring the novel versus familiar object (TDZD-8: 20±2 sec vs 2±1 sec, $p<0.01$; VPO.7: 19±2 sec vs 3±1 sec, $p<0.01$), indicating that under conditions of GSK3 inhibition *Fmr1* KO mice are capable of learning the task. Furthermore, the exploration ratio was significantly increased in *Fmr1* KO mice treated with TDZD-8 or VP0.7, but had no effect in WT mice (*Fmr1* KO exploration ratio: TDZD-8: 0.79±0.03; $p<0.05$; VP0.7: 0.72±0.04; $p<0.05$) (WT exploration ratio: TDZD-8: 0.69±0.05; VP0.7: 0.56±0.03) (Fig. 1B), indicating that GSK3 inhibition completely reverses the learning deficit in *Fmr1* KO mice.

Next, we assessed whether *Fmr1* KO mice displayed deficits in pattern separation using coordinate and categorical tasks, which require the dentate gyrus (Goodrich-Hunsaker et al., 2005; Goodrich-Hunsaker et al., 2008; Hunsaker et al., 2012). In the coordinate spatial learning task, the distance between two identical objects is altered between the habituation and testing periods. Pattern separation is indicated when significantly more time is spent exploring objects during the 5 min testing period after repositioning the objects compared to the last 5 min of the habituation phase. WT mice displayed increased object exploration time during testing compared to the last 5 min of

the habituation phase (WT exploration ratio: 0.61±0.05) (Fig. 1C), indicating successful pattern separation. In contrast, *Fmr1* KO mice spent significantly less time than WT exploring the objects during the test period, indicating impaired behavior in this task (*Fmr1* KO exploration ratio: 0.31±0.05, $p<0.05$). While neither TDZD-8 nor VP0.7 altered behavior of WT mice (WT exploration ratio TDZD-8: 0.60±0.05; VP0.7: 0.65±0.07), both drugs reversed the deficit in *Fmr1* KO mice, as they spent significantly more time exploring the objects during testing compared to habituation (*Fmr1* KO exploration ratio TDZD-8: 0.62±0.06, $p<0.05$; VP0.7: 0.66±0.08, $p<0.05$). The categorical spatial learning task involves interchanging the positions of two identical objects following the habituation phase, while maintaining the same distance between them. *Fmr1* KO mice spent significantly less time than WT mice exploring the objects after they had been transposed (*Fmr1* KO exploration ratio: 0.36±0.03; WT exploration ratio: 0.66±0.05, $p<0.05$) (Fig 1D), again revealing impaired spatial pattern separation in *Fmr1* KO mice. Administration of GSK3 inhibitors did not alter the amount of time WT mice spent exploring the objects after they were transposed (WT exploration ratio: TDZD-8: 0.70±0.05; VP0.7: 0.80±0.03), but significantly increased the exploration times of *Fmr1* KO mice (*Fmr1* KO exploration ratio: TDZD-8: 0.63±0.06, $p<0.05$; VP0.7: 0.75 ± 0.04, $p<0.05$), demonstrating a reversal of the deficit. Thus, the results of the coordinate and categorical spatial learning tests demonstrate impaired function of the dentate gyrus in *Fmr1* KO mice that is normalized by the administration of GSK3 inhibitors.

Finally, we assessed whether *Fmr1* KO mice have deficits in temporal ordering of objects, a dorsal and ventral hippocampal CA1-dependent task in which rodents spend less time exploring the object most recently presented during a previous habituation

period (Honey et al., 1998; Wallenstein et al., 1998; Rolls and Kesner 2006; Hoge and Kesner 2007; Hunsaker et al., 2012; Hunsaker and Kesner 2013). In this task, we exposed mice to a series of 3 pairs of objects and then measured the time spent with the initial object when it was reintroduced along with the most recent object. Successful temporal ordering is evident when more time is spent exploring the initial object. WT mice displayed successful temporal ordering because more time was spent exploring the initial object (13±1 sec vs 7±2 sec, $p<0.05$), whereas *Fmr1* KO mice spent significantly less time exploring the initial object presented (7±1 sec vs 16±2 sec, $p<0.01$) (Fig. 1E). Thus, the object exploration ratio (calculated by dividing the difference between the time spent with the initial object (object 5) versus the more recent object (object 7) by total time exploring), differed significantly between *Fmr1* KO and WT mice, revealing a temporal order deficit (WT exploration ratio: 0.32±0.10; *Fmr1* KO exploration ratio: -0.41±0.05, $p<0.05$) (Fig. 1F). *Fmr1* KO mice treated with either GSK3 inhibitor, TDZD-8 or VP0.7, spent significantly more time exploring the first object compared to the most recent object presented (TDZD-8: 12±2 sec vs 4±2 sec, $p<0.01$; VP0.7: 9±3 sec vs 3±1 sec, $p<0.01$) (Fig. 1E). Similarly, WT mice treated with TDZD-8 or VP0.7 spent significantly more time exploring the first versus the recent object (TDZD-8: 10±1 sec vs 5±1 sec, $p<0.01$; VP0.7: 11±1 sec vs 3±1 sec, $p<0.01$). Thus, administration of TDZD-8 or VP0.7 significantly increased the exploration ratio in *Fmr1* KO mice (*Fmr1* KO exploration ratio: TDZD-8: 0.47±0.07, $p<0.05$; VP0.7: 0.56±0.07; $p<0.05$), (Figure 1F), eliminating the impairment in temporal ordering. TDZD-8 or VP0.7 treatment also tended to improve the behavior of wild-type mice in this task (WT exploration ratio: TDZD-8: 0.37±0.08; VP0.7: 0.60±0.06) (Figure 1F). These results demonstrate that temporal ordering of

visual objects is impaired in *Fmr1* KO mice and that this deficit is corrected by inhibition of GSK3.

Cognitive deficits in Fmr1 *KO mice are not rescued by mGluR5 inhibition.*

Because inhibition of mGluR reverses many of the synaptic and behavioral phenotypes in *Fmr1* KO mice (Dolen et al., 2007; Michalon et al., 2012), we investigated whether MPEP, an mGluR5 antagonist, would also reverse the dentate gyrus associated deficits in learning and memory. Treatment of *Fmr1* KO mice with MPEP did not affect the impairments in novel object detection (*Fmr1* KO: 6±3 sec vs 8±3 sec vs *Fmr1* KO + MPEP: 13±3 sec vs 15±2 sec) (Fig. 2A) (*Fmr1* KO exploration ratio: -0.48±0.23; *Fmr1* KO + MPEP: -0.06±0.13) (Fig. 2B), coordinate spatial processing (*Fmr1* KO exploration ratio: 0.31±0.09; *Fmr1* KO + MPEP: 0.18±0.03) (Fig. 2C), categorical spatial processing (*Fmr1* KO exploration ratio: 0.35±0.04; *Fmr1* KO + MPEP: 0.43±0.07) (Fig. 2D), or temporal order memory (*Fmr1* KO: 8±2 sec vs 9±1 sec vs *Fmr1* KO + MPEP: 11±2 sec vs 10±1 sec) (Fig 2E) (*Fmr1* KO exploration ratio: -0.15±0.11; *Fmr1* KO + MPEP: 0.02±0.08) (Fig 2F) . Administration of MPEP did not alter the performance of WT mice in any of these tasks. Thus, cognitive deficits in *Fmr1* KO mice are not rescued by acute inhibition of mGluR5.

Discussion

Impairments in the inhibition of GSK3 have been reported in the *Fmr1* KO mouse hippocampus (Min et al., 2009; Yuskaitis et al., 2010a), and here we report severe impairments in four hippocampus-dependent cognitive tasks. No current treatments are

known that improve impairment in these cognitive tasks in *Fmr1* KO mice, even though intellectual disability is the most prevalent symptom of FXS. Here we found that pharmacological inhibition of GSK3, but not an inhibitor of mGluR5, reversed cognitive deficits, strongly suggesting that GSK3 is a potential therapeutic target of cognitive and behavioral impairments in FXS.

Cognitive deficits were originally difficult to identify in the *Fmr1* KO mouse model. *Fmr1* KO mice behave normally or display only modest cognitive deficits in several hippocampus-dependent tasks, including the Morris water maze, radial arm maze, and operant conditioning paradigms (Bakker, 1994; Kooy et al., 1996; D'Hooge et al., 1997; Fisch et al., 1999; Paradee et al., 1999; Peier et al., 2000; Mineur et al., 2002). However, *Fmr1* KO mice exhibit deficits in fear motivated learning tasks, including passive and active avoidance behaviors, and contextual, conditioned and trace fear memory (Yan et al., 2004; Qin et al., 2005; Zhao et al., 2005; Brennan et al., 2006; Hayashi et al., 2007; Baker et al., 2010; Guo et al., 2011). Recently, severe deficits in non-aversive learning and memory tasks that are dependent upon the dentate gyrus, including novel object recognition and context discrimination, have been identified in *Fmr1* KO mice (Pacey et al., 2011; Eadie et al., 2012; Bhattacharya and Klann, 2012). We extended these findings by demonstrating significant deficits, not only in novel object recognition, but also in coordinate and categorical spatial processing tasks and temporal order memory.

We tested if reducing GSK3 activity improves impaired cognition in *Fmr1* KO mice. This idea was based on the multiple beneficial effects following administration of the GSK3 inhibitor lithium to *Fmr1* KO mice (Mines and Jope 2011). Chronic lithium

treatment of *Fmr1* KO mice ameliorates locomotor hyperactivity, audiogenic seizure hypersensitivity, increased spine density, enhanced mGluR-mediated LTD, reactive astrocytes, macroorchidism, excess protein synthesis, and social behavior deficits (Min et al., 2009; Yuskaitis et al., 2010a; Yuskaitis et al., 2010b; Mines et al., 2010; Choi et al., 2011; Liu et al., 2011; Liu et al. 2012). Additionally, lithium treatment improved passive avoidance learning deficits in *Fmr1* KO mice, the only reported behavioral test of the effect of lithium treatment on cognitive impairments in *Fmr1* KO mice (Liu et al., 2011). Other GSK3 inhibitors besides lithium have been reported to significantly reduce locomotor hyperactivity, susceptibility to audiogenic seizures, trace conditioning, delayed non-matching-to-place radial arm maze and neurogenesis in *Fmr1* KO mice (Min et al., 2009; Guo et al., 2012). These results suggest that the therapeutic actions of lithium are due to inhibition of GSK3 in *Fmr1* KO mice leading us to test if cognitive impairments in *Fmr1* KO mice may be alleviated by administration of specific inhibitors of GSK3. GSK3 was inhibited pharmacologically using two small molecule selective inhibitors of GSK3, TDZD-8 and VP0.7, followed by measuring behavior in novel object detection, coordinate and categorical spatial learning, and temporal ordering for objects. This determined if GSK3 inhibition increases learning in *Fmr1* KO mice. Impaired novel object detection, coordinate and categorical spatial processing, and temporal order memory in *Fmr1* KO mice were repaired by administration of GSK3 inhibitors, suggesting that GSK3 inhibition reversed these cognitive deficits.

Several phenotypes of *Fmr1* KO mice are known to be regulated by aberrant mGluR5 function, and acute administration of the mGluR5 antagonist MPEP to *Fmr1* KO mice reverses deficits in several behaviors (Yan et al., 2004; de Vrij et al., 2008; Min et al.,

2009; Suvrathan et al., 2010; Gross et al., 2012; Thomas et al., 2012), therefore we tested if acute treatment with MPEP also reversed impairments in cognition. Acute administration of MPEP was completely ineffective in repairing cognitive deficits in *Fmr1* KO mice. These results indicate that although mGluR5 is a promising therapeutic target (Dolen et al., 2007; Michalon et al., 2012), GSK3 inhibitors must also be considered for treatment of FXS.

Currently there are no adequate therapies for the treatment of FXS (Garber et al., 2006; Bhakar et al., 2012), although some symptoms can be alleviated by anticonvulsants, antidepressants, stimulants and antipsychotics (Hagerman et al., 2012). No current treatments improve cognitive impairment in *Fmr1* KO mice, even though intellectual disability is the most prevalent symptom of FXS. It is notable that lithium is the only drug that has been used in FXS patients that improved any measure of cognition (Berry-Kravis et al., 2008). The cognitive assessments we studied in *Fmr1* KO mice may model the nonverbal measures of intelligence used in FXS patients. Patients with FXS display impaired recognition memory, spatial memory, working memory and short-term memory (Kemper et al., 1988; Cornish et al., 1999; Ornstein et al., 2008; Gatto and Broadie 2009). FXS patients also have difficulty with inhibition and attentional control that is consistent with the memory deficits (Cornish et al., 2001). The novel object detection task and the temporal order memory task were used to assess recognition memory, working memory, and short-term memory in *Fmr1* KO mice. The coordinate and categorical spatial learning tasks were used to assess spatial memory in *Fmr1* KO mice. All of these cognitive abilities were impaired in *Fmr1* KO mice and were corrected by administration of GSK3 inhibitors. Thus, abnormally active GSK3 in the hippocampus

of *Fmr1* KO mice (Min et al., 2009; Yuskaitis et al., 2010a; Liu et al., 2011; Guo et al., 2011) appears to play an important role in these cognitive deficits, further supporting the possibility that GSK3 inhibitors may be beneficial for multiple aspects of FXS, including intellectual disability. It will be of great interest in future studies to elucidate the precise mechanisms by which hyperactive GSK3 decreases learning and memory, as these mechanisms represent novel targets for therapeutic development for the treatment of intellectual disability in FXS.

Acknowledgements

This research was supported by grants from the NIMH (MH038752) and from the FRAXA Foundation.

References

Bhattacharya A, Klann E (2012): Fragile X syndrome therapeutics S(C)TEP through the developmental window. *Neuron*. 74:1-3.

Bagni C, Tassone F, Neri G, Hagerman R (2012): Fragile X syndrome: causes, diagnosis, mechanisms, and therapeutics. *J Clin Invest*. 122:4314-4322.

Baker KB, Wray SP, Ritter R, Mason S, Lanthorn TH, Savelieva KV (2010): Male and female Fmr1 knockout mice on C57 albino background exhibit spatial learning and memory impairments. *Genes Brain Behav*. 9:562-574.

Bakker DB et al. (1994): Fmr1 knockout mice: a model to study fragile X mental retardation. The Dutch-Belgian Fragile X Consortium. *Cell*. 78:23-33.

Berry-Kravis E, Sumis A, Hervey C, Nelson M, Porges SW, Weng N, et al. (2008): Open-label treatment trial of lithium to target the underlying defect in fragile X syndrome. *J Dev Behav Pediatr*. 29:293-302.

Beurel E, Kaidanovich-Beilin O, Yeh WI, Song L, Palomo V, Michalek SM, et al. (2013): Regulation of Th1 cells and experimental autoimmune encephalomyelitis by glycogen synthase kinase-3. *J Immunol*. 190:5000-5011.

Bhakar AL, Dolen G, Bear MF (2012): The pathophysiology of fragile X (and what it teaches us about synapses). *Annu Rev Neurosci*. 35:417-443.

Brennan, F. X., Albeck, D. S., & Paylor, R. (2006). Fmr1 knockout mice are impaired in a leverpress escape/avoidance task. *Genes Brain Behav 5*, 467-471.

Choi CH, Schoenfeld BP, Bell AJ, Hinchey P, Kollaros M, Gertner MJ, et al. (2011): Pharmacological reversal of synaptic plasticity deficits in the mouse model of Fragile X syndrome by group II mGluR antagonist or lithium treatment. *Brain Res*. 1380:106-119.

Cornish KM, Munir F, Cross G (1999): Spatial cognition in males with Fragile-X syndrome: evidence for a neuropsychological phenotype. *Cortex*. 35:263-271.

Cornish K, Munir F, Wilding J (2001): [A neuropsychological and behavioural profile of attention deficits in fragile X syndrome]. *Rev Neurol*. 33 Suppl 1:S24-29.

D'Hooge R, Nagels G, Franck F, Bakker CE, Reyniers E, Storm K, et al. (1997): Mildly impaired water maze performance in male Fmr1 knockout mice. *Neuroscience*. 76:367-376.

de Vrij FM, Levenga J, van der Linde HC, Koekkoek SK, De Zeeuw CI, Nelson DL, et al. (2008): Rescue of behavioral phenotype and neuronal protrusion morphology in Fmr1 KO mice. *Neurobiol Dis*. 31:127-132.

Dissanayake C, Bui Q, Bulhak-Paterson D, Huggins R, Loesch DZ (2009): Behavioural and cognitive phenotypes in idiopathic autism versus autism associated with fragile X syndrome. *J Child Psychol Psychiatry*. 50:290-299.

Dolan RJ, Fletcher PC (1997): Dissociating prefrontal and hippocampal function in episodic memory encoding. *Nature*. 388:582-585.

Dolen G, Osterweil E, Rao BS, Smith GB, Auerbach BD, Chattarji S, et al. (2007): Correction of fragile X syndrome in mice. *Neuron*. 56:955-962.

Eadie BD, Cushman J, Kannangara TS, Fanselow MS, Christie BR (2012): NMDA receptor hypofunction in the dentate gyrus and impaired context discrimination in adult Fmr1 knockout mice. *Hippocampus*. 22:241-254.

Errijgers V, Kooy RF (2004): Genetic modifiers in mice: the example of the fragile X mouse model. *Cytogenet Genome Res*. 105:448-454.

Fisch GS, Hao HK, Bakker C, Oostra BA (1999): Learning and memory in the FMR1 knockout mouse. *Am J Med Genet*. 84:277-282.

Garber K, Smith KT, Reines D, Warren ST (2006): Transcription, translation and fragile X syndrome. *Curr Opin Genet Dev*. 16:270-275.

Gatto CL, Broadie K (2009): The fragile X mental retardation protein in circadian rhythmicity and memory consolidation. *Mol Neurobiol*. 39:107-129.

Goodrich-Hunsaker NJ, Hunsaker MR, Kesner RP (2005): Dissociating the role of the parietal cortex and dorsal hippocampus for spatial information processing. *Behav Neurosci*. 119:1307-1315.

Goodrich-Hunsaker NJ, Hunsaker MR, Kesner RP (2008): The interactions and dissociations of the dorsal hippocampus subregions: how the dentate gyrus, CA3, and CA1 process spatial information. *Behav Neurosci*. 122:16-26.

Gross C, Berry-Kravis EM, Bassell GJ (2012): Therapeutic strategies in fragile X syndrome: dysregulated mGluR signaling and beyond. *Neuropsychopharmacology*. 37:178-195.

Guo W, Allan AM, Zong R, Zhang L, Johnson EB, Schaller EG, et al. (2011): Ablation of Fmrp in adult neural stem cells disrupts hippocampus-dependent learning. *Nat Med*. 17:559-565.

Guo W, Murthy AC, Zhang L, Johnson EB, Schaller EG, Allan AM, et al. (2012): Inhibition of GSK3beta improves hippocampus-dependent learning and rescues neurogenesis in a mouse model of fragile X syndrome. *Hum Mol Genet*. 21:681-691.

Hagerman R, Lauterborn J, Au J, Berry-Kravis E (2012): Fragile X syndrome and targeted treatment trials. *Results Probl Cell Differ*. 54:297-335.

Hayashi, M. L., Rao, B. S., Seo, J. S., Choi, H. S., Dolan, B. M., Choi, S. Y., et al. (2007). Inhibition of p21-activated kinase rescues symptoms of fragile X syndrome in mice. *Proc Natl Acad Sci U S A 104*, 11489-11494.

Hernandez RN, Feinberg RL, Vaurio R, Passanante NM, Thompson RE, Kaufmann WE (2009): Autism spectrum disorder in fragile X syndrome: a longitudinal evaluation. *Am J Med Genet A*. 149A:1125-1137.

Hoge J, Kesner RP (2007): Role of CA3 and CA1 subregions of the dorsal hippocampus on temporal processing of objects. *Neurobiol Learn Mem*. 88:225-231.

Honey RC, Watt A, Good M (1998): Hippocampal lesions disrupt an associative mismatch process. *J Neurosci*. 18:2226-2230.

Hunsaker MR, Mooy GG, Swift JS, Kesner RP (2007): Dissociations of the medial and lateral perforant path projections into dorsal DG, CA3, and CA1 for spatial and nonspatial (visual object) information processing. *Behav Neurosci*. 121:742-750.

Hunsaker MR, Kesner RP (2008): Evaluating the differential roles of the dorsal dentate gyrus, dorsal CA3, and dorsal CA1 during a temporal ordering for spatial locations task. *Hippocampus*. 18:955-964.

Hunsaker MR, Kesner RP (2009): Transecting the dorsal fornix results in novelty detection but not temporal ordering deficits in rats. *Behav Brain Res*. 201:192-197.

Hunsaker MR, Kim K, Willemsen R, Berman RF (2012): CGG trinucleotide repeat length modulates neural plasticity and spatiotemporal processing in a mouse model of the fragile X premutation. *Hippocampus*. 22:2260-2275.

Hunsaker MR, Kesner RP (2013): The operation of pattern separation and pattern completion processes associated with different attributes or domains of memory. *Neurosci Biobehav Rev*. 37:36-58.

Jones K, Wei C, Iakova P, Bugiardini E, Schneider-Gold C, Meola G, et al. (2012): GSK3beta mediates muscle pathology in myotonic dystrophy. *J Clin Invest*. 122:4461-4472.

Jope RS, Johnson GV (2004): The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci*. 29:95-102.

Kalinichev M, Dawson LA (2011): Evidence for antimanic efficacy of glycogen synthase kinase-3 (GSK3) inhibitors in a strain-specific model of acute mania. *Int J Neuropsychopharmacol*. 14:1051-1067.

Kemper MB, Hagerman RJ, Altshul-Stark D (1988): Cognitive profiles of boys with the fragile X syndrome. *Am J Med Genet*. 30:191-200.

King, M. K., Pardo, M., Cheng, Y., Downey, K., Jope, R. S., & Beurel, E. (2013). Glycogen synthase kinase-3 inhibitors: rescuers of cognitive impairments. *Pharmacol and Therapeutics* (Epub ahead of print).

Klein PS, Melton DA (1996). A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci U S A.* 93:8455-8459.

Knight R (1996): Contribution of human hippocampal region to novelty detection. *Nature*. 383:256-259.

Kooy RF, D'Hooge R, Reyniers E, Bakker CE, Nagels G, De Boulle K, et al. (1996): Transgenic mouse model for the fragile X syndrome. *Am J Med Genet*. 64:241-245.

Lipina TV, Kaidanovich-Beilin O, Patel S, Wang M, Clapcote SJ, Liu F, et al. (2011): Genetic and pharmacological evidence for schizophrenia-related Disc1 interaction with GSK-3. *Synapse*. 65:234-248.

Lipina TV, Wang M, Liu F, Roder JC (2012): Synergistic interactions between PDE4B and GSK-3: DISC1 mutant mice. *Neuropharmacology*. 62:1252-1262.

Lisman JE (1999): Relating hippocampal circuitry to function: recall of memory sequences by reciprocal dentate-CA3 interactions. *Neuron*. 22:233-242.

Liu ZH, Chuang DM, Smith CB (2011): Lithium ameliorates phenotypic deficits in a mouse model of fragile X syndrome. *Int J Neuropsychopharmacol*. 14:618-630.

Liu ZH, Huang T, Smith CB (2012): Lithium reverses increased rates of cerebral protein synthesis in a mouse model of fragile X syndrome. *Neurobiol Dis*. 45:1145-1152.

Long JM, Kesner RP (1996): The effects of dorsal versus ventral hippocampal, total hippocampal, and parietal cortex lesions on memory for allocentric distance in rats. *Behav Neurosci*. 110:922-932.

Martinez A, Alonso M, Castro A, Perez C, Moreno FJ (2002): First non-ATP competitive glycogen synthase kinase 3 beta (GSK-3beta) inhibitors: thiadiazolidinones (TDZD) as potential drugs for the treatment of Alzheimer's disease. *J Med Chem*. 45:1292-1299.

McBride SM, Choi CH, Wang Y, Liebelt D, Braunstein E, Ferreiro D, et al. (2005): Pharmacological rescue of synaptic plasticity, courtship behavior, and mushroom body defects in a Drosophila model of fragile X syndrome. *Neuron*. 45:753-764.

Michalon A, Sidorov M, Ballard TM, Ozmen L, Spooren W, Wettstein JG, et al. (2012): Chronic pharmacological mGlu5 inhibition corrects fragile X in adult mice. *Neuron*. 74:49-56.

Min WW, Yuskaitis CJ, Yan Q, Sikorski C, Chen S, Jope RS, et al. (2009): Elevated glycogen synthase kinase-3 activity in Fragile X mice: key metabolic regulator with evidence for treatment potential. *Neuropharmacology*. 56:463-472.

Mines MA, Yuskaitis CJ, King MK, Beurel E, Jope RS (2010): GSK3 influences social preference and anxiety-related behaviors during social interaction in a mouse model of fragile X syndrome and autism. *PLoS One*. 5:e9706.

Mines MA, Jope RS (2011): Glycogen synthase kinase-3: a promising therapeutic target for fragile x syndrome. *Front Mol Neurosci*. 4:35.

Mineur YS, Sluyter F, de Wit S, Oostra BA, Crusio WE (2002): Behavioral and neuroanatomical characterization of the Fmr1 knockout mouse. *Hippocampus*. 12:39-46.

Ornstein PA, Schaaf JM, Hooper SR, Hatton DD, Mirrett P, Bailey DB, Jr. (2008): Memory skills of boys with fragile X syndrome. *Am J Ment Retard*. 113:453-465.

Otto T, Eichenbaum H (1992): Neuronal activity in the hippocampus during delayed non-match to sample performance in rats: evidence for hippocampal processing in recognition memory. *Hippocampus*. 2:323-334.

Pacey LK, Doss L, Cifelli C, van der Kooy D, Heximer SP, Hampson DR (2011): Genetic deletion of regulator of G-protein signaling 4 (RGS4) rescues a subset of fragile X related phenotypes in the FMR1 knockout mouse. *Mol Cell Neurosci*. 46:563-572.

Palomo V, Soteras I, Perez DI, Perez C, Gil C, Campillo NE, et al. (2011): Exploring the binding sites of glycogen synthase kinase 3. Identification and characterization of allosteric modulation cavities. *J Med Chem*. 54:8461-8470.

Paradee W, Melikian HE, Rasmussen DL, Kenneson A, Conn PJ, Warren ST (1999): Fragile X mouse: strain effects of knockout phenotype and evidence suggesting deficient amygdala function. *Neuroscience*. 94:185-192.

Peier AM, McIlwain KL, Kenneson A, Warren ST, Paylor R, Nelson DL (2000): (Over)correction of FMR1 deficiency with YAC transgenics: behavioral and physical features. *Hum Mol Genet*. 9:1145-1159.

Pieretti, M., Zhang, F. P., Fu, Y. H., Warren, S. T., Oostra, B. A., Caskey, C. T., et al. (1991). Absence of expression of the FMR-1 gene in fragile X syndrome. *Cell 66*, 817-822.

Qin, M., Kang, J., & Smith, C. B. (2005). A null mutation for *Fmr1* in female mice: effects on regional cerebral metabolic rate for glucose and relationship to behavior. *Neuroscience 135*, 999-1009.

Ramirez SH, Fan S, Zhang M, Papugani A, Reichenbach N, Dykstra H, et al. (2010): Inhibition of glycogen synthase kinase 3beta (GSK3beta) decreases inflammatory responses in brain endothelial cells. *Am J Pathol*. 176:881-892.

Rolls ET, Kesner RP (2006): A computational theory of hippocampal function, and empirical tests of the theory. *Prog Neurobiol*. 79:1-48.

Save E, Poucet B, Foreman N, Buhot MC (1992): Object exploration and reactions to spatial and nonspatial changes in hooded rats following damage to parietal cortex or hippocampal formation. *Behav Neurosci*. 106:447-456.

Suvrathan A, Hoeffer CA, Wong H, Klann E, Chattarji S (2010): Characterization and reversal of synaptic defects in the amygdala in a mouse model of fragile X syndrome. *Proc Natl Acad Sci U S A*. 107:11591-11596.

Thomas AM, Bui N, Perkins JR, Yuva-Paylor LA, Paylor R (2012): Group I metabotropic glutamate receptor antagonists alter select behaviors in a mouse model for fragile X syndrome. *Psychopharmacology (Berl)*. 219:47-58.

Tsien JZ, Chen DF, Gerber D, Tom C, Mercer EH, Anderson DJ, et al. (1996): Subregion- and cell type-restricted gene knockout in mouse brain. *Cell*. 87:1317-1326.

Wallenstein GV, Eichenbaum H, Hasselmo ME (1998): The hippocampus as an associator of discontiguous events. *Trends Neurosci*. 21:317-323.

Woodgett, J. R. (1990). Molecular cloning and expression of glycogen synthase kinase-3/factor A. *EMBO J 9*, 2431–2438.

Yan QJ, Asafo-Adjei PK, Arnold HM, Brown RE, Bauchwitz RP (2004): A phenotypic and molecular characterization of the fmr1-tm1Cgr fragile X mouse. *Genes Brain Behav*. 3:337-359.

Yuskaitis CJ, Mines MA, King MK, Sweatt JD, Miller CA, Jope RS (2010a): Lithium ameliorates altered glycogen synthase kinase-3 and behavior in a mouse model of fragile X syndrome. *Biochem Pharmacol*. 79:632-646.

Yuskaitis CJ, Beurel E, Jope RS (2010b): Evidence of reactive astrocytes but not peripheral immune system activation in a mouse model of Fragile X syndrome. *Biochim Biophys Acta*. 1802:1006-1012.

Zalfa, F., Giorgi, M., Primerano, B., Moro, A., Di Penta, A., Reis, S., et al. (2003). The fragile X syndrome protein FMRP associates with BC1 RNA and regulates the translation of specific mRNAs at synapses. *Cell 112,* 317-327.

Zhao, M. G., Toyoda, H., Ko, S. W., Ding, H. K., Wu, L. J., & Zhuo, M. (2005). Deficits in trace fear memory and long-term potentiation in a mouse model for fragile X syndrome. *J Neurosci 25*, 7385-7892.

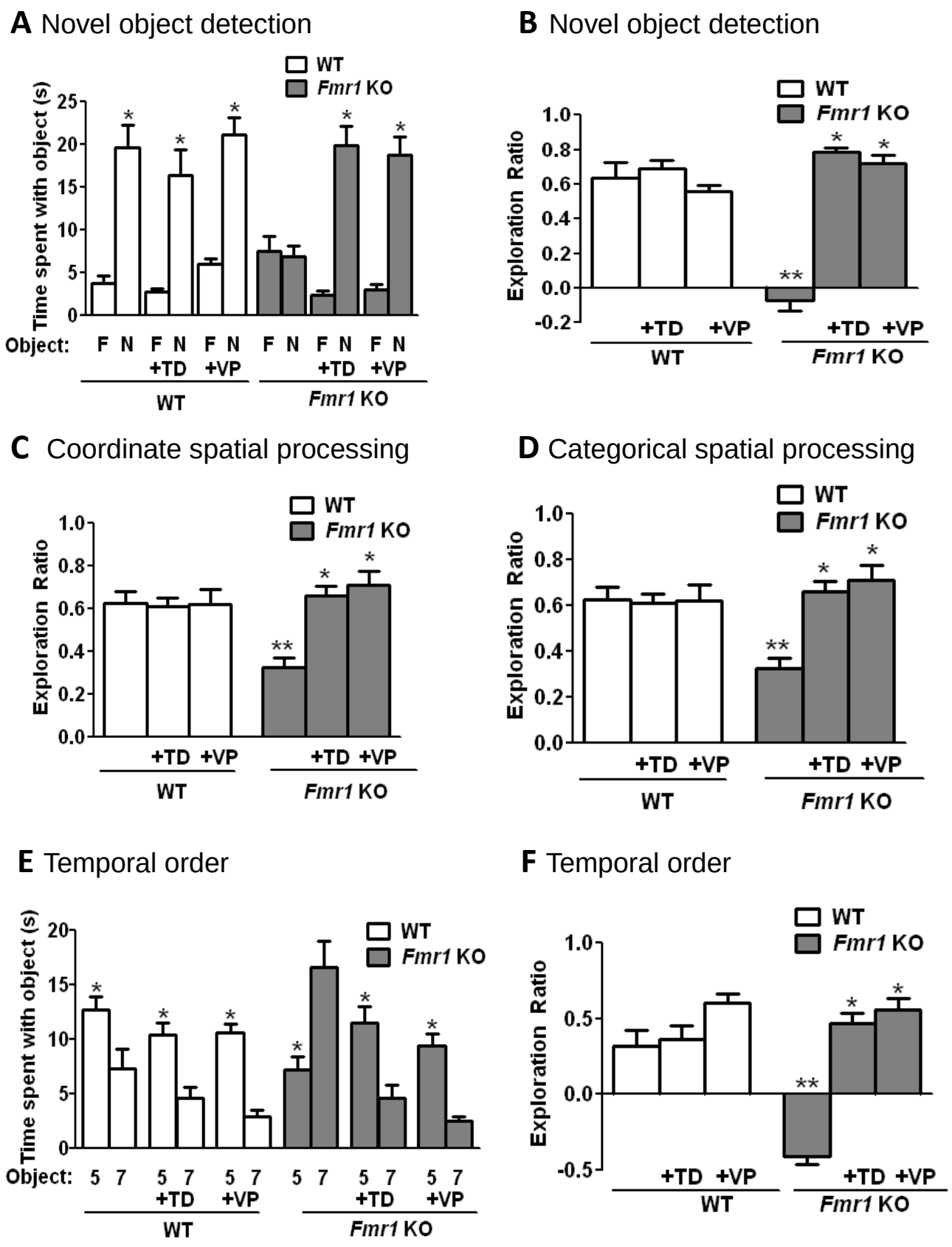

A Novel object detection
WT
Fmr1 KO
Time spent with object (s)
Object: F N F N F N F N F N F N
+TD +VP
WT
Fmr1 KO
B Novel object detection
Exploration Ratio
C Coordinate spatial processing
D Categorical spatial processing
E Temporal order
Object: 5 7 5 7 5 7 5 7 5 7 5 7
F Temporal order

Figure 1. Inhibition of GSK3 ameliorates cognitive impairments in *Fmr1* KO mice. *Fmr1* KO and WT mice were treated with 5 mg/kg of TDZD-8 (TD) or VP0.7 (VP) 1 hr prior to cognitive assessments. (A,B) Performance in the novel object detection task. (A) Times spent exploring the novel (N) and familiar (F) object. **$p<0.01$ compared to time spent with familiar object (Student's t test). (B) Exploration ratio. (C) Exploration ratio in the coordinate spatial processing task. (D) Exploration ratio in the categorical spatial processing task. (E,F) Performance in the temporal order for objects task. (E) Times spent exploring Object 5 and Object 7 (most recently explored). **$p<0.01$, *$p<0.05$ compared to time spent with Object 7 (Student's t test). (F) Exploration ratio. B, C, D and F: **$p<0.05$ compared to untreated wild-type mice; *$p<0.05$ compared to same genotype without treatment (Kruskall-Wallis [genotype x treatment] with Dunn's multiple comparison test). n=10-20 mice per group.

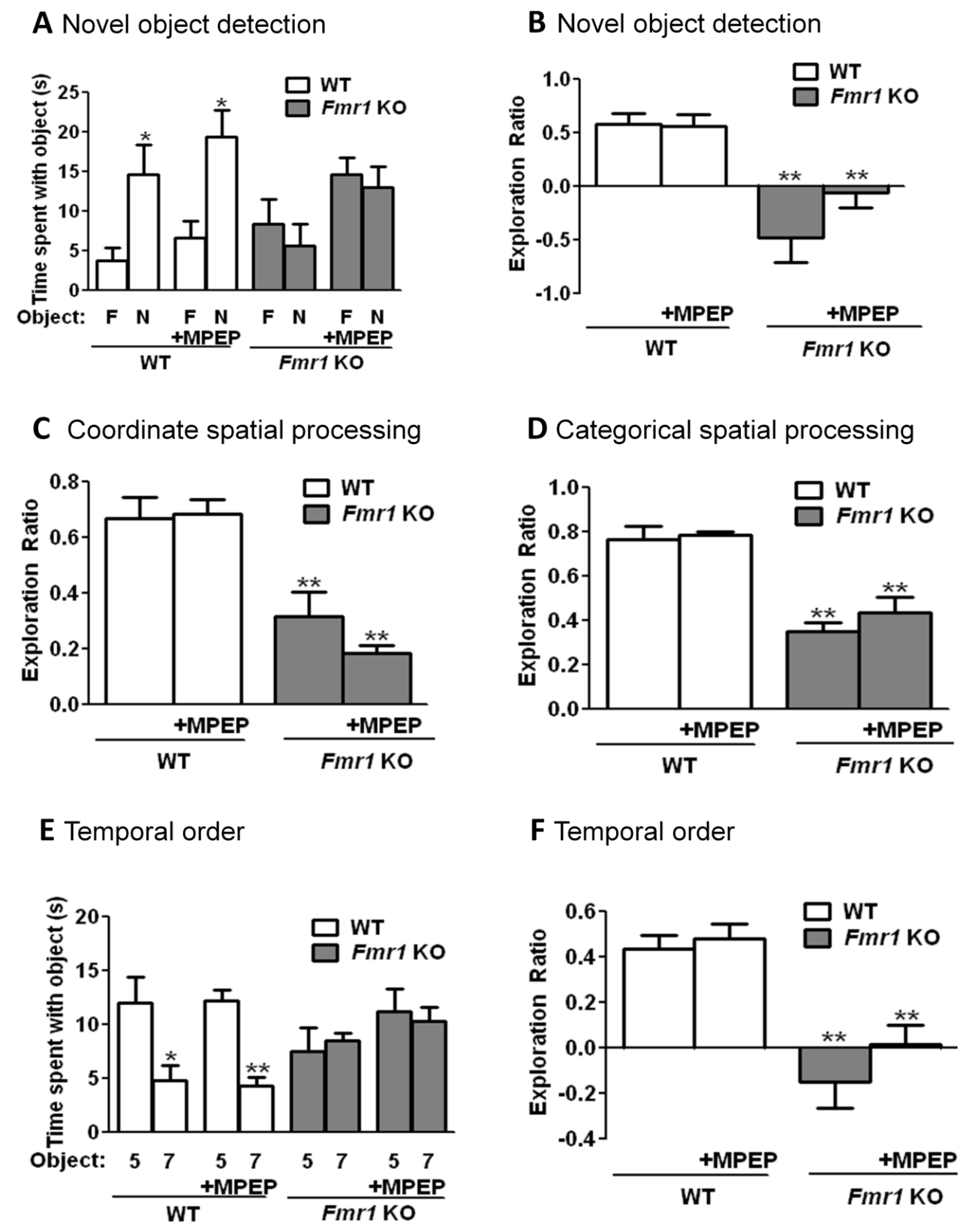

A Novel object detection
Time spent with object (s)
WT
Fmr1 KO
Object: F N F N F N F N
+MPEP
+MPEP
WT
Fmr1 KO
B Novel object detection
Exploration Ratio
WT
Fmr1 KO
+MPEP
+MPEP
WT
Fmr1 KO
C Coordinate spatial processing
Exploration Ratio
WT
Fmr1 KO
+MPEP
+MPEP
WT
Fmr1 KO
D Categorical spatial processing
Exploration Ratio
WT
Fmr1 KO
+MPEP
+MPEP
WT
Fmr1 KO
E Temporal order
Time spent with object (s)
WT
Fmr1 KO
Object: 5 7 5 7 5 7 5 7
+MPEP
+MPEP
WT
Fmr1 KO
F Temporal order
Exploration Ratio
WT
Fmr1 KO
+MPEP
+MPEP
WT
Fmr1 KO

Figure 2. Cognitive deficits in *Fmr1* KO mice are not altered by mGluR inhibition. *Fmr1* KO and WT mice were treated with 30 mg/kg of MPEP 1 hr prior to cognitive assessments. (A,B) Performance in the novel object detection task. (A) Times spent exploring the novel (N) and familiar (F) object. *$p<0.05$ compared to time spent with familiar object (Student's t test) (B) Exploration ratio. (C) Exploration ratio in the coordinate spatial processing task. (D) Exploration ratio in the categorical spatial processing task. (E,F) Performance in the temporal order for objects task. (E) Times spent exploring Object 5 and Object 7 (most recently explored). **$p<0.01$, *$p<0.05$ compared to time spent with Object 7 (Student's t test). (F) Exploration ratio. B, C, D and F: **$p<0.05$ compared to untreated wild-type mice. (one-way ANOVA [genotype x treatment] followed by post hoc Bonferroni's multiple comparison test). n=6 mice per group.

CONCLUSIONS

From depressive behaviors and impaired cognition to adult neurogenesis and neurodevelopmental disorders, GSK3 plays an immense role in functions of the CNS. Impaired inhibitory control of GSK3 has been linked to numerous disorders of the CNS including FXS, Alzheimer's disease, multiple sclerosis, depression, and bipolar disorder, among many others. Lithium, the seminal inhibitor of GSK3, protects cognitive processes or promotes their repair in a robust number of conditions that cause cognitive impairments in rodents. Furthermore, lithium is the only drug that has been found to improve cognition in an open label treatment trial with FXS patients (Berry-Kravis et al., 2008a). In addition, hippocampal neurogenesis may be impaired in mood disorders, and GSK3 has been linked to the regulation of neurogenesis that may be involved in mood regulation. Given these previous findings, the present study examined the regulatory role of GSK3 in adult mouse hippocampal neurogenesis and impaired cognition in the mouse model of FXS.

Hyperactive GSK3 appears to contribute to impaired adult hippocampal neurogenesis (Eom and Jope 2009), so we tested how hyperactive GSK3 might contribute to the regulation of neurogenesis, specifically changes in neurogenesis elicited by alterations in the environment: EE and CRS. Because the regulation of GSK3 contributes to multiple aspects of many neurological processes, we first tested if these environmental manipulations affected GSK3 in mouse hippocampus. EE and CRS increased and decreased, respectively, inhibition of GSK3 in wild-type male hippocampus, but neither EE nor CRS altered GSK3 activity in female mice. This led us to test if the basal levels of

GSK3 inhibition were different in wild-type male and female hippocampus, and we found that basal inhibition of GSK3 is higher in female than male mice, raising the possibility that inhibition of GSK3 may protect female mice from fluctuations caused by environmental manipulations.

To evaluate this hypothesis and how it affects adult hippocampal neurogenesis, we examined how the combinatorial effects of environmental changes (EE, CRS) and genetics (sex, hyperactive GSK3) affect neurogenesis in mice. To do this, we used male and female GSK3 knockin mice that have constitutively active GSK3, because the inhibitory serines have been mutated to alanines, and their wild-type littermates. In wild-type male mice, EE increased GSK3 inhibition and NPC proliferation, survival and differentiation. GSK3 knockin mice exhibited decreased neurogenesis compared to wild-type mice, but unlike common antidepressants (Eom and Jope, 2009), EE was able to reverse the deficit in NPC proliferation in GSK3 knockin male mice. EE did not alter survival or differentiation in GSK3 knockin mice, suggesting the EE-induced increased phosphorylation of GSK3 may be necessary for EE-induced NPC survival and differentiation but not proliferation in male mice. Due to the divergent response to EE-induced changes in neurogenesis in male mice and because GSK3 knockin male mice are susceptible to stress-induced depression (Polter et al., 2010), it would be interesting to compare the effects of EE to depression-like behaviors after the NPC proliferation experimental paradigm and following the NPC survival and differentiation paradigm. For example, does EE-induced increased NPC proliferation in male GSK3 knockin mice correlate with reduced depression-like behaviors? Or is NPC survival and differentiation responsible for changes in depression-like behaviors following EE?

CRS decreased the inhibitory serine-phosphorylation of GSK3 and NPC proliferation in wild-type male mice, but CRS did not alter NPC proliferation in GSK3 knockin mice. Thus, CRS and GSK3 activity may share common mechanisms to decrease NPC proliferation in male wild-type mice. We found that male GSK3 knockin mice did not exhibit decreased NPC proliferation which may be due to a floor effect of neurogenesis in these mice. One weakness in this study is that we were unable to evaluate NPC survival and differentiation in mice following CRS, but the significant deficit in hippocampal NPC proliferation suggests that survival and differentiation of NPCs following CRS would also be decreased in wild-type mice and unaltered in GSK3 knockin mice.

EE and CRS did not alter inhibitory serine phosphorylation of GSK3 in female wild-type mice or NPC proliferation in female wild-type or GSK3 knockin mice. EE did increase NPC survival and differentiation in female wild-type mice, thus increased inhibitory phosphorylation of GSK3 is not required for the long term effects of EE-induced increased neurogenesis in female mice. Female GSK3 knockin exhibited impaired NPC survival and differentiation, and unlike male GSK3 knockin mice, EE rescued impaired NPC survival and differentiation in the female mice. These results support gender-specific differences in response to environmental manipulation (Walker and Mason, 2011). It has been shown that estrogen increases neurogenesis (Gould et al., 2000; Banasr et al., 2001; Perez-Martin et al., 2003), and that estrogen is neuroprotective in many types of brain injury (Lang and McCullough, 2008). Future studies should determine if estrogen mediates the difference in neurogenesis plasticity in male and female wild-type and GSK3 knockin mice, and how this might relate to

depressive like behavior. To do so, wild-type and GSK3 knockin female mice can be ovariectomized and subjected to EE or CRS and compared to each other and to male wild-type and GSK3 knockin mice. Advances in our knowledge of how estrogen exerts its effect on the brain may lead to targeted therapies for neuronal dysfunction (Barha and Galea, 2010).

Although it has been shown that the regulation of hippocampal neurogenesis is important for brain function in rodents, only recently was it demonstrated that hippocampal neurogenesis occurs throughout the lifetime in human subjects (Spalding et al., 2013). By measuring the concentration of nuclear bomb test-derived ^{14}C in genomic DNA, that study concluded that neurons are generated throughout adulthood and the rates are comparable in middle-aged humans and mice. The results suggest that hippocampal neurogenesis may contribute to human brain function. Because GSK3 knockin mice have decreased neurogenesis which correlates with stress-induced depression (Polter et al., 2009), it would be interesting if, using the same ^{14}C technique, adult neurogenesis was measured in human subjects with different psychiatric illnesses and compared to controls. Additionally, it would be beneficial to identify the specific neurochemicals that NPCs express following environmental manipulation in order to evaluate the positive and negative influences on neurogenesis plasticity. This could lead to potential targeted therapies in humans with cognitive deterioration related to increasing age, learning, and memory disorders, such as Alzheimer's disease (Lazorov and Marr, 2013), and psychiatric disorders, such as depression and schizophrenia (Benarroch, 2013; Déry et al., 2013). In any event, confirmation of hippocampal neurogenesis in adult humans

(Spalding et al., 2013) is an exciting finding because it implies that neurogenesis research is not in vain because the mouse does model humans.

In addition to the regulation of adult neurogenesis, hyperactive GSK3 contributes to cognitive impairments in animal models of numerous diseases of the CNS, as we reviewed in this project. To specifically evaluate impaired cognition in one disease model with impaired GSK3 regulation, we assessed the FXS mouse model. FXS is the most common inherited cause of intellectual disability, affecting 1 in 4000-5000 males and 1 in 2500-8000 females (Tassone et al., 2012). Currently there are no adequate therapies for the treatment of FXS (Garber et al., 2006; Bhakar et al., 2012), although some symptoms can be alleviated by anticonvulsants, antidepressants, stimulants and antipsychotics (Hagerman et al., 2012). FXS is the result of a single *FMR1* gene mutation, and so it was one of the first neurodevelopmental disorders to be studied in a transgenic mouse model (Bakker et al., 1994). In the *Fmr1* knockout mouse brain, impairments in the inhibition of GSK3 have been reported (Min et al., 2009; Yuskaitis et al., 2010a; Liu et al., 2011; Guo et al., 2011). Inhibition of GSK3 with chronic lithium treatment and other GSK3 inhibitors ameliorate physiological and behavioral impairments in *Fmr1* knockout mice, including locomotor hyperactivity, audiogenic seizure hypersensitivity, macroorchidism, social behavior deficits, and impairments in trace conditioning and delayed non-matching-to-place radial arm maze (Min et al., 2009; Yuskaitis et al., 2010a; Mines et al., 2010; Mines and Jope, 2011; Liu et al. 2011). Furthermore, CNS abnormalities in *Fmr1* knockout mice including increased spine density, enhanced mGlu-R mediated LTD, reactive astrocytes, excess protein synthesis and impaired neurogenesis are all repaired by treatment with lithium and other GSK3

inhibitors (Min et al., 2009; Yuskaitis et al., 2010b; Choi et al., 2011; Liu et al., 2012; Guo et al., 2012). Thus, these results suggest that the therapeutic actions of lithium are due to inhibition of GSK3 in *Fmr1* knockout mice.

Prior to our study, no treatments were identified that improve non-aversive learning impairments in *Fmr1* knockout mice, even though intellectual disability is the most prevalent symptom of FXS. Severe cognitive deficits were initially difficult to identify in the *Fmr1* knockout mouse. Modest deficits have been reported in several hippocampus-dependent tasks, such as the Morris water maze, radial arm maze, and operant conditioning paradigms (Bakker, 1994; Kooy et al., 1996; D'Hooge et al., 1997; Fisch et al., 1999; Paradee et al., 1999; Peier et al., 2000; Mineur et al., 2002). *Fmr1* knockout mice exhibit deficits in fear motivated learning tasks, including passive and active avoidance behaviors, and contextual, conditioned and trace fear memory (Yan et al., 2004; Qin et al., 2005; Zhao et al., 2005; Brennan et al., 2006; Hayashi et al., 2007; Baker et al., 2010; Guo et al., 2011), and recently, severe deficits in non-aversive learning and memory tasks, including novel object recognition and context discrimination, have been identified in *Fmr1* knockout mice (Pacey et al., 2011; Eadie et al., 2012; Bhattacharya and Klann 2012). Therefore, the goal of this study was to investigate other cognitive tests in *Fmr1* knockout mice that might better model the FXS patient, and then to determine if we could improve cognition with chronic GSK3 inhibition using lithium. To determine if a therapeutic benefit of lithium was due to GSK3 inhibition, we assessed cognitive performance following acute inhibition of GSK3 using two specific GSK3 inhibitors, TDZD-8 and VP0.7.

We found that *Fmr1* knockout mice exhibited significant impairments in recognition memory, working memory, and short-term memory that were assessed in the novel object detection task and the temporal ordering for objects task, and spatial memory measured in the coordinate and categorical spatial processing tasks (Goodrich-Hunsaker et al., 2005; Hoge and Kesner, 2007; Hunsaker and Kesner, 2008; Goodrich-Hunsaker et al., 2008; Hunsaker et al., 2009; Hunsaker et al., 2012). Chronic lithium treatment proved to be remarkably effective in essentially normalizing severe deficits in *Fmr1* knockout mice in all four cognitive tasks. We hypothesized that treatment of younger *Fmr1* knockout mice would be more effective than treatment of adult *Fmr1* knockout mice because FMRP is more highly expressed in young than adult mouse brain (Lu et al., 2004). The absence of FMRP might cause irreversible deficits in adult *Fmr1* knockout mice. However, lithium treatment was equally effective in adolescent and adult *Fmr1* knockout mice in reversing cognitive deficits.

Next we tested if reducing GSK3 activity using two small molecule selective inhibitors of GSK3, TDZD-8 and VP0.7, improves impaired cognition in *Fmr1* knockout mice. We found that acute pharmacological inhibition of GSK3 reversed impaired novel object detection, temporal order memory, and coordinate and categorical spatial processing in *Fmr1* knockout mice, strongly suggesting that GSK3 is a potential therapeutic target of the cognitive impairments in FXS.

Improvement of cognition by GSK3 inhibition treatment correlates well with previous findings in *Fmr1* knockout mice of altered synaptic plasticity, measured as LTP and LTD. *Fmr1* knockout mice display enhanced mGluR-dependent LTD at hippocampal CA1 synapses (Huber et al., 2002; Hou et al., 2006; Nosyreva and Huber, 2006) and

deficient LTP at medial perforant path synapses in the dentate gyrus (Eadie et al., 2012). Hyperactive GSK3 impairs LTP and promotes LTD (Hooper et al., 2007; Zhu et al., 2007). Lithium treatment in adolescent or adult *Fmr1* knockout mice normalized mGluR-dependent LTD in the hippocampus (Choi et al., 2011). mGluR5 is inhibited by its antagonist MPEP, and acute administration of MPEP to *Fmr1* knockout mice reverses deficits in several behaviors (Yan et al., 2004; de Vrij et al., 2008; Min et al., 2009; Suvrathan et al., 2010; Gross et al., 2012; Thomas et al., 2012), therefore we compared the beneficial effects of GSK3 inhibition on cognition in *Fmr1* knockout mice to acute treatment with MPEP. Acute administration of MPEP was completely ineffective in repairing cognitive deficits in *Fmr1* knockout mice. These results indicate that although mGluR5 is a promising therapeutic target (Dolen et al., 2007; Michalon et al., 2012), inhibition of GSK3 contributes to both the normalization of synaptic plasticity and cognition in *Fmr1* knockout mice.

The novel object detection task and the coordinate and categorical spatial processing tasks are dependent upon normal function of the dentate gyrus (Honey et al., 1998; Wallenstein et al., 1998; Goodrich-Hunsaker et al., 2005; Rolls and Kesner 2006; Goodrich-Hunsaker et al., 2008; Hunsaker et al., 2008; Hunsaker and Kesner, 2013) and the temporal order memory task is dependent upon area Cornu Ammonis 1 (CA1) (Hoge and Kesner, 2007; Hunsaker and Kesner, 2008). Our collaborators found that GSK3 is hyperactive in the dentate gyrus but not area CA1 in the *Fmr1* knockout hippocampus (Franklin et al., 2013). Additionally, they found LTP deficits at medial perforant path synapses onto dentate granule cells (MPP-DGC) in *Fmr1* knockout mice, which were rescued by treatment with lithium or another GSK3 inhibitor, but not by inhibition of

mGluR5. They found no difference in LTP magnitude at CA3-CA1 synapses between *Fmr1* knockout mice and wild-type mice, but despite these findings, we observed a deficit in temporal order memory, which was reversed by GSK3 inhibition (Godfraind et al., 1996; Huber et al., 2002). This may be because behavioral tasks require normal function of the hippocampal trisynaptic circuit. The hippocampal trisynaptic circuit consists of three connected pathways: the entorhinal cortex projects to the dentate granule cells via the MPP; the MPP-DGC then project to the pyramidal cells of area CA3; then the CA3 pyramidal cells project to the pyramidal cells of are CA1 (Amaral, 1978; Amaral and Witter, 1989). Deficits at MPP-DGC synapses are likely propagated to downstream CA3-CA1 synapses, impacting CA1 dependent behavior, even though LTP at CA3-CA1 synapses is normal in slices from *Fmr1* knockout mice. In support of this concept, GSK3 inhibition, which reverses LTP deficits at MPP-DGC synapses, also normalizes temporal ordering deficits indicating that systemic GSK3 inhibition improves the overall function of the hippocampal trisynaptic circuit. Another possibility is that there are deficits in plasticity present at the temporoammonic pathway, the monosynaptic projection from entorhinal cortex directly onto distal dendrites of CA1, that contribute to deficits in temporal order memory. The results indicate that if such deficits exist and underlie termporal order learning, inhibition of GSK3 will also rescue deficits at temporoammonic synapses although this has not yet been explored.

To fully understand how hyperactive GSK3 leads to synaptic and cognitive impairments in FXS, identification of downstream targets of GSK3 is needed. Both LTP and pattern separation require proper *N*-methyl-D-aspartate receptor (NMDAR) function. Hyperactive GSK3 may diminish NMDAR transmission which can be reversed by GSK3

inhibition, an idea consistent with GSK3-dependent NMDAR internalization in cortical neurons (Chen et al., 2007). Alternatively, inhibition of GSK3 may rescue LTP and cognition through effector targets downstream of NMDAR activation. GSK3 regulates AMPAR trafficking (Wei et al., 2010), however this mechanism is unlikely since there are no deficits in baseline AMPAR transmission in *Fmr1* knockout mice.

In future studies clarification of the mechanisms underlying the cognitive enhancing actions of GSK3 inhibitors in FXS and other diseases with a cognitive impairment component would provide a better understanding of the causes of cognitive decline and of the mechanisms that may be exploited in the development of improved interventions.

The cognitive assessments we studied in *Fmr1* knockout mice may model the nonverbal measures of intelligence used in FXS patients. Patients with FXS display impaired recognition memory, spatial memory, working memory and short-term memory (Kemper et al., 1988; Cornish et al., 1999; Ornstein et al., 2008; Gatto and Broadie 2009). FXS patients also have difficulty with inhibition and attentional control that is consistent with the memory deficits (Cornish et al., 2001). The novel object detection task and the temporal order memory task were used to assess recognition memory, working memory, and short-term memory in *Fmr1* knockout mice. The coordinate and categorical spatial learning tasks were used to assess spatial memory in *Fmr1* knockout mice. All of these cognitive abilities were impaired in *Fmr1* knockout mice and were corrected by administration of GSK3 inhibitors. These findings are encouraging because we found that some cognitive impairments may be pharmacologically reversible even with post-adolescent administration in FXS. Thus, abnormally active GSK3 in the hippocampus of

Fmr1 knockout mice (Min et al., 2009; Yuskaitis et al., 2010a; Liu et al., 2011; Guo et al., 2011) appears to play an important role in these cognitive deficits, further supporting the possibility that GSK3 inhibitors may be beneficial for multiple aspects of FXS, including intellectual disability.

In summary, this study provides novel insights regarding the function of GSK3 in two neurological processes, neurogenesis and cognition. First, neurogenesis plasticity may be regulated by GSK3 activity in male mice, but female mice exhibit differences in the contribution of GSK3 to neurogenesis. These results open the possibility to examine the role of GSK3 and male and female differences in the regulation of neurogenesis and its potential relationship to depression-like behavior and treatment for mood disorders. Furthermore, neurogenesis appears to support certain forms of learning and memory and may be defective in some conditions associated with impairments in cognition (van Praag et al., 2005; Leuner et al., 2006; Deng et al., 2010; Massa et al., 2011). Thus, in diseases such as depression, Alzheimer's disease, and FXS, in which GSK3 in the CNS is abnormally active, an outcome may be diminished neurogenesis and consequently cognitive impairments. Conversely, neurogenesis is increased by treatment with lithium or other drugs that inhibit GSK3 (Chen et al., 2000: Hashimoto et al., 2003; Silva et al., 2008; Wexler et al., 2008; Kim et al., 2009; Morales-Garcia et al., 2012), and treatment with the GSK3 inhibitor SB216763 increased neurogenesis that is impaired in mice expressing DISC1 mutations (Mao et al., 2009). Thus, administration of GSK3 inhibitors may improve cognition in part by restoring impairments in neurogenesis.

Second, the FXS studies provide strong initial evidence that GSK3 inhibitors have the potential to ameliorate cognitive impairments in the *Fmr1* knockout mouse. Our

results support the finding that in a small open-label trial of lithium treatment in children and young adults with FXS, lithium treatment significantly improved cognition in the Repeatable Battery for the Assessment of Neuropsychological Status List Learning measure (Berry-Kravis et al., 2008a). Thus, lithium administration to *Fmr1* KO mice at a variety of ages improves cognitive abilities in several tasks, and preliminary evidence indicates that lithium also may be effective in patients. These results provide important progress in the discovery of potential therapies, specifically GSK3 inhibitors, for patients with FXS and will hopefully stimulate experiments to characterize more fully the function of GSK3 in FXS. Most importantly, considering the prevalence and devastating consequences of loss of cognitive functions, and the dearth of efficacious interventions, our research emphasizes the importance of greater development and utilization of GSK3 inhibitors to treat conditions causing cognitive impairments. The current study provides further evidence that GSK3 plays a substantial role in important neurological processes.

GENERAL LIST OF REFERENCES

Abitbol, M., Menini, C., Delezoide, A., Rhyner, T., Vehemans, M., & Mallet, J. (1993). Nucleus basalis magnocellularis and hippocampus are the major sites of *FMR-1* expression in the human fetal brain. *Nature 4*, 147-153.

Amaral, D. G. (1978). A Golgi study of cell types in the hilar region of the hippocampus in the rat. *J Comp Neurol 182*, 851-914.

Amaral D. G. & Witter, M. P. (1989) The three-dimensional organization of the hippocampal formation: a review of anatomical data. *Neuroscience 31*, 571-591.

Antar, L. N., Afroz, R., Dictenberg, J. B., Carroll, R. C., & Bassell, G. J. (2004). Metabotropic glutamate receptor activation regulates fragile x mental retardation protein and FMR1 mRNA localization differentially in dendrites and at synapses. *J Neurosci 24*, 2648-2455.

Baker, K. B., Wray, S. P., Ritter, R., Mason, S., Lanthorn, T. H., & Savelieva, K.V. (2010). Male and female Fmr1 knockout mice on C57 albino background exhibit spatial learning and memory impairments. *Genes Brain Behav 9*, 562-574.

Bakker, C. E., Verheij, C., Willemsen, R., van der Helm, R., Oerlemans, F., Vermeij, M., et al. (1994). Fmr1 knockout mice: a model to study Fragile X mental retardation. *Cell 78*, 23-33.

Barha C. K. & Galea L. A. (2010) Influence of different estrogens on neuroplasticity and cognition in the hippocampus. *Biochim Biophys Acta 1800*, 1056-1067.

Banasr, M., Hery, M., Brezun, J. M., & Daszuta, A. (2001). Serotonin mediates oestrogen stimulation of cell proliferation in the adult dentate gyrus. *Eur J Neurosci 14*, 1417-1424.

Banasr, M., Hery, M., Brezun, J. M., & Daszuta, A. (2001). Serotonin mediates oestrogen stimulation of cell proliferation in the adult dentate gyrus. *Eur J Neurosci 14*, 1417-1424.

Bear, M. F., Huber, K. M., & Warren, S. T. (2004). The mGluR theory of fragile X mental retardation. *Trends Neurosci 27*, 370-377.

Beaulieu, J. M., Sotnikova, T. D., Yao, W. D., Kockeritz, L., Woodgett, J. R., Gainetdinov, R. R., et al. (2004). Lithium antagonizes dopamine-dependent behaviors mediated by an AKT/glycogen synthase kinase 3 signaling cascade. *Proc Natl Acad Sci USA 101*, 5099-5104.

Beaulieu, J. M., Zhang, X., Rodriguiz, R. M., Sotnikova, T. D., Cools, M. J., Wetsel, W. C., et al. (2008a). Role of GSK3 beta in behavioral abnormalities induced by serotonin deficiency. *Proc Natl Acad Sci U S A 105*, 1333-1338.

Beaulieu, J. M., Marion, S., Rodriguiz, R. M., Medvedev, I. O., Sotnikova, T. D., Ghis, V., et al. (2008b) A beta-arrestin 2 signaling complex mediates lithium action on behavior. *Cell 132*, 125-136.

Bechara, E. G., Didiot, M. C., Melko, M., Davidovic, L., Bensaid, M., Martin, P., et al. (2009). A novel function for fragile X mental retardation protein in translational activation. *PLoS Biol 7*, e16.

Benarroch E. E. (2013) Adult neurogenesis in the dentate gyrus: General concepts and potential implications. *Neurology 81,* 1443-1452.

Bernardet, M., & Crusio, W. E. (2006). Fmr1 KO mice as a possible model of autistic features. *Scientific World Journal 6*, 1164-1176.

Berry-Kravis, E. (2002). Epilepsy in fragile X syndrome. *Dev Med Child Neurology 44*, 724-728.

Berry-Kravis, E., & Potanos, K. (2004). Psychopharmacology in fragile X syndrome—present and future. *Ment Retard Dev Disabil Res Rev 10*, 42-48.

Berry-Kravis, E., Abrams, L., Coffey, S. M., Hall, D. A., Greco, C., Gane, L. W., et al. (2007). Fragile X-associated tremor/ataxia syndrome: clinical features, genetics, and testing guidelines. *Mov Disord 22*, 2018-2030.

Berry-Kravis, E., Sumis, A., Hervey, C., Nelson, M., Porges, S. W., Weng, N., et al. (2008a). Open-label treatment trial of lithium to target the underlying defect in fragile X syndrome. *J Dev Behav Pediatr 29*, 293-302.

Berry-Kravis, E., Sumis, A., Kim, O. K., Lara, R., & Wuu, J. (2008b). Characterization of potential outcome measures for future clinical trials in fragile X syndrome. *J Autism Dev Disord 38*, 1751-1757.

Berry-Kravis, E., Raspa, M., Loggin-Hester, L., Bishop, E., Holiday, D., & Bailey, D. B. (2010). Seizures in fragile X syndrome: characteristics and comorbid diagnoses. *Am J Intellect Dev Disabil 115*, 461-472.

Berry-Kravis, E., Knox, A., & Hervey, C. (2011). Targeted treatments for fragile X syndrome. *J Neurodev Disord 3*, 193-210.

Beurel, E., & Jope, R. S. (2009). Lipopolysaccharide-induced interleukin-6 production is controlled by glycogen synthase kinase-3 and STAT3 in the brain. *J Neuroinflammation 6*, 9.

Beurel, E., Kaidanovich-Beilin, O., Yeh, W. I., Song, L., Palomo, V., Michalek, S. M., et al. (2013). Regulation of Th1 cells and experimental autoimmune encephalomyelitis by glycogen synthase kinase-3. *J Immunol 190,* 5000-5011.

Bhakar A. L., Dölen G., & Bear M. F. (2012) The pathophysiology of fragile X (and what it teaches us about synapses). *Annu Rev Neurosci 35,* 417-443.

Bhattacharya, A., & Klann, E. (2012). Fragile X syndrome therapeutics S(C)TEP through the developmental window. *Neuron 74*, 1-3.

Brenes Sáenz, J. C., Villagra, O. R., & Fornaguera Trías, J. (2006). Factor analysis of Forced Swimming test, Sucrose Preference test and Open Field test on enriched, social and isolated reared rats. *Behav Brain Res 169,* 57-65.

Brennan, F. X., Albeck, D. S., & Paylor, R. (2006). Fmr1 knockout mice are impaired in a leverpress escape/avoidance task. *Genes Brain Behav 5,* 467-471.

Brown, J., Cooper-Kuhn, C. M., Kempermann, G., Van Praag, H., Winkler, J., Gage, F. H., et al. (2003). Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. *Eur J Neurosci 17,* 2042-2046.

Brown, V., Jin, P., Ceman, S., Darnell, J. C., O'Donnell, W. T., Tenenbaum, S. A., et al. (2001) Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. *Cell 107,* 477-487.

Cade, J. F. (1949). Lithium salts in the treatment of psychotic excitement. *Med J Aust 2,* 349-352.

Cameron, H. A., & Gould, E. (1994). Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. *Neuroscience 61*, 203-209.

Chakrabarti, L., Scafidi, J., Gallo, V., & Haydar, T. F. (2011). Environmental enrichment rescues postnatal neurogenesis defect in the male and female Ts65Dn mouse model of Down syndrome. *Dev Neurosci 33,* 428-441.

Charney, D. S., & Manji, H. K. (2004). Life stress, genes, and depression: multiple pathways lead to increased risk and new opportunities for intervention. *Sci STKE 16,* re5.

Chen, G., Rajkowska, G., Du, F., Seraji-Bozorgzad, N., & Manji, H. K. (2000). Enhancement of hippocampal neurogenesis by lithium. *J Neurochem 75,* 1729-1734.

Chen, L., Toth, M. (2001). Fragile X mice develop sensory hyperreactivity to auditory stimuli. *Neuroscience 103*, 1043-1050.

Chen P, Gu Z, Liu W, Yan Z (2007): Glycogen synthase kinase 3 regulates N-methyl-D-aspartate receptor channel trafficking and function in cortical neurons. *Mol Pharmacol*. 72:40-51.

Chiba, S., Numakawa, T., Ninomiya, M., Richards, M. C., Wakabayashi, C., & Kunugi, H. (2012). Chronic restraint stress causes anxiety- and depression-like behaviors, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex. *Progress in Neuro-psychopharmacology & Biological Psychiatry 39*, 112-119.

Choi, C. H., Schoenfeld, B. P., Bell, A. J., Hinchey, P., Kollaros, M., Gertner, M. J., Woo, N. H., Tranfaglia M. R., Bear, M. F., Zukin, R. S., McDonald, T. V., Jongens, T. A., McBride, S. M. (2011). Pharmacological reversal of synaptic plasticity deficits in the mouse model of fragile X syndrome by group II mGluR antagonist or lithium treatment. *Brain Res 1380*, 106-119.

Cornish, K. M., Munir, F., Cross, G. (1999). Spatial cognition in males with Fragile-X syndrome: evidence for a neuropsychological phenotype. *Cortex 35*, 263-271.

Cornish, K., Munir, F., Wilding, J. (2001). A neuropsychological and behavioural profile of attention deficits in fragile X syndrome. *Rev Neurol 33*, Suppl 1:S24-29.

Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., & Hemmings, B. A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature 378*, 785–789.

Czeh, B., Michaelis, T., Watanabe, T., Frahm, J., de Biurrun, G., van Kampen, M., et al. (2001). Stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation are prevented by antidepressant treatment with tianeptine. *Proc Natl Acad Sci USA 98*, 12796-12801.

D'Hooge, R., Nagels, G., Franck, F., Bakker, C. E., Reyniers, E., Storm, K., et al. (1997). Mildly impaired water maze performance in male Fmr1 knockout mice. *Neuroscience 76*, 367-376.

David, D. J., Samuels, B. A., Rainer, Q., Wang, J. W., Marsteller, D., Mendez, I., et al. (2009). Neurogenesis-dependent and -independent effects of fluoxetine in an animal model of anxiety/depression. *Neuron 62*, 479-493.

De Sarno, P., Li, X., & Jope, R. S. (2002). Regulation of Akt and glycogen synthase kinase-3β phosphorylation by sodium valproate and lithium. *Neuropharmacol 43*, 1158-1164.

del Rio, J. A., & Soriano, E. (1989). Immunocytochemical detection of 5'-bromodeoxyuridine incorporation in the central nervous system of the mouse. *Brain Res Dev Brain Res 49*, 311-377.

Deng, W., Aimone, J. B., & Gage, F. H. (2010). New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? *Nat Rev Neurosci 11,* 339-350.

Déry, N., Pilgrim, M., Gibala, M., Gillen, J., Wojtowicz, J. M., Macqueen, G., Becker, S. (2013). Adult hippocampal neurogenesis reduces memory interference in humans: opposing effects of aerobic exercise and depression. *Front Neurosci 7*, 66.

Devys, D., Lutz, Y., Rouyer, N., Bellocq, J. P., & Mandel, J. L. (1993). The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. *Nat Genet 4*, 335-340.

Ding, Q., Vaynman, S., Akhavan, M., Ying, Z., & Gomez-Pinilla, F. (2006). Insulin-like growth factor interfaces with brain-derived neurotrophic factor-mediated synaptic plasticity to modulate aspects of exercise-induced cognitive function. *Neuroscience 140*, 823-833.

Dolen G, Osterweil E, Rao BS, Smith GB, Auerbach BD, Chattarji S, et al. (2007). Correction of fragile X syndrome in mice. *Neuron*. 56:955-962.

Dranovsky, A., & Hen, R. (2006). Hippocampal neurogenesis: regulation by stress and antidepressants. *Biol Psychiatry 59*, 1136-1143.

Duman, C. H., Schlesinger, L., Russell, D. S., & Duman, R. S. (2008). Voluntary exercise produces antidepressant and anxiolytic behavioral effects in mice. *Brain Research 1199*, 148-158.

Duman, R. S., Malberg, J., & Nakagawa, S. (2001). Regulation of adult neurogenesis by psychotropic drugs and stress. *J Pharmacol Exp Ther 299*, 401-407.

Eadie, B. D., Cushman, J., Kannangara, T. S., Fanselow, M. S., & Christie, B. R. (2012). NMDA receptor hypofunction in the dentate gyrus and impaired context discrimination in adult Fmr1 knockout mice. *Hippocampus 22*, 241-254.

Eom, T. Y., & Jope, R. S. (2009). Blocked inhibitory serine-phosphorylation of glycogen synthase kinase-3α/β impairs in vivo neural precursor cell proliferation. *Biological Psychiatry 66*, 494-502.

Fabel, K., Fabel, K., Tam, B., Kaufer, D., Baiker, A., Simmons, N., et al. (2003). VEGF is necessary for exercise-induced adult hippocampal neurogenesis. *Eur J Neurosci 18*, 2803-2812.

Falconer, E. M., & Galea, L. A. (2003). Sex differences in cell proliferation, cell death and defensive behavior following acute predator odor stress in adult rats. *Brain Res 975*, 22-36.

Fang, X., Yu, S. X., Lu, Y., Bast, R. C. Jr., Woodgett, J. R., & Mills, G. B. (2000). Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. *Proc Natl Acad Sci USA 97,* 11960–11965.

Farmer, J., Zhao, X., van Praag, H., Wodtke, K., Gage, F. H., & Christie, B. R. (2004). Effects of voluntary exercise on synaptic plasticity and gene expression in the dentate gyrus of adult male Sprague-Dawley rats in vivo. *Neuroscience 124*, 71-79.

Fisch, G. S., Hao, H. K., Bakker, C., & Oostra, B. A. (1999). Learning and memory in the FMR1 knockout mouse. *Am J Med Genet 84*, 277-282.

Franklin, A. V., King, M. K., Palomo, V., Martinez, A., McMahon, L. L., & Jope RS. (2013). Glycogen synthase kinase-3 inhibitors reverse deficits in long-term potentiation and cognition in fragile X mice. *Biol Psychiatry*. [Epub ahead of print].

Garber, K., Smith, K. T., Reines, D., & Warren, S. T. (2006). Transcription, translation and fragile X syndrome. *Curr Opin Genet Dev 16*, 270-275.

Gatto, C. L. & Broadie, K. (2009). The fragile X mental retardation protein in circadian rhythmicity and memory consolidation. *Mol Neurobiol 39*, 107-129.

Glasper, E. R., Llorens-Martin, M. V., Leuner, B., Gould, E., & Trejo, J. L. (2010). Blockade of insulin-like growth factor-I has complex effects on structural plasticity in the hippocampus *Hippocampus 20*, 706-712.

Godfraind, J. M., Reyniers, E., De Boulle, K., D'Hooge, R., De Deyn, P. P., Bakker, C. E., et al. (1996). Long-term potentiation in the hippocampus of fragile X knockout mice. *Am J Med Genet 64*, 246-251.

Goode, N., Hughes, K., Woodgett, J. R., & Parker, P. J. (1992). Differential regulation of glycogen synthase kinase-3ß by protein kinase C isotypes. *J Bio Chem 267*, 16878–16882.

Goodrich-Hunsaker, N.J., Hunsaker, M.R., & Kesner, R.P. (2005) Dissociating the role of the parietal cortex and dorsal hippocampus for spatial information processing. *Behav Neurosci* 119, 1307-1315.

Goodrich-Hunsaker, N.J., Hunsaker, M.R., & Kesner, R.P. (2008) The interactions and dissociations of the dorsal hippocampus subregions: how the dentate gyrus, CA3, and CA1 process spatial information. *Behav Neurosci* 122, 16-26.

Gould, E., Cameron, H. A., Daniels, D. C., Woolley, C. S., & McEwen, B. S. (1992). Adrenal hormones suppress cell division in the adult rat dentate gyrus. *J Neurosci 12*, 3642-3650.

Gould, E., Tanapat, P., Rydel, T., & Hastings, N. (2000) Regulation of hippocampal neurogenesis in adulthood. *Biol Psychiatry 48*, 715-720.

Green, T. A., Alibhai, I. N., Roybal, C. N., Winstanley, C. A., Theobald, D. E., Birnbaum, S. G., et al. (2010). Environmental enrichment produces a behavioral phenotype mediated by low cyclic adenosine monophosphate response element binding (CREB) activity in the nucleus accumbens. *Biological Psychiatry 67*, 28-35.

Gross, C., Yao, X., Pong, D. L., Jeromin, A., & Bassell, G. J. (2011). Fragile X mental retardation protein regulates protein expression and mRNA translation of the potassium channel Kv4.2. *J Neurosci 31*, 5693-5698.

Gross, C., Berry-Kravis, E. M., & Bassell, G. J. (2012). Therapeutic strategies in fragile X syndrome: dysregulated mGluR signaling and beyond. *Neuropsychopharmacology 37*, 178-195.

Guo, W., Allan, A. M., Zong, R., Zhang, L., Johnson, E. B., Schaller, E. G., et al. (2011). Ablation of Fmrp in adult neural stem cells disrupts hippocampus-dependent learning. *Nat Med 17*, 559-565.

Guo, W., Murthy, A. C., Zhang, L., Johnson, E. B., Schaller, E. G., Allan, A. M., & Zhao X. (2012). Inhibition of GSK3β improves hippocampus-dependent learning and rescues neurogenesis in a mouse model of fragile X syndrome. *Hum Mol Genet 21*, 681-691.

Haenisch, B., Bilkei-Gorzo, A., Caron, M. G., & Bonisch, H. (2009). Knockout of the norepinephrine transporter and pharmacologically diverse antidepressants prevent behavioral and brain neurotrophin alterations in two chronic stress models of depression. *Journal of Neurochemistry 111*, 403-416.

Hagerman, R., Hoem, G., & Hagerman, P. (2010). Fragile X and autism: Intertwined at the molecular level leading to targeted treatments. *Mol Autism 1*, 12.

Hagerman, R., Lauterborn, J., Au, J., & Berry-Kravis, E. (2012). Fragile X syndrome and targeted treatment trials. *Results Probl Cell Differ 54*, 297-335.

Hashimoto, R., Senatorov, V., Kanai, H., Leeds, P., & Chuang, D. M. (2003). Lithium stimulates progenitor proliferation in cultured brain neurons. *Neuroscience 117*, 55-61.

Hayashi, M. L., Rao, B. S., Seo, J. S., Choi, H. S., Dolan, B. M., Choi, S. Y., et al. (2007). Inhibition of p21-activated kinase rescues symptoms of fragile X syndrome in mice. *Proc Natl Acad Sci U S A 104*, 11489-11494.

Hessl, D., Tassone, F., Cordeiro, L., Koldewyn, K., McCormick, C., Green, C., et al. (2008). Brief report: aggression and stereotypic behavior in males with fragile X syndrome—moderating secondary genes in a "single gene" disorder. *J Autism Dev Disord 38*, 184-189.

Hillerer, K. M., Neumann, I. D., Couillard-Despres, S., Aigner, L., & Slattery, D. A. (2013) Sex-dependent regulation of hippocampal neurogenesis under basal and chronic stress conditions in rats. *Hippocampus 23*, 476-487.

Hinds, H. L., Ashley, C. T., Sutcliffe, J. S., Nelson, D. L., Warren, S. T., Housman, D. E., et al. (1993). Tissue specific expression of FMR-1 provides evidence for a functional role in fragile X syndrome. *Nat Genet 3*, 36-43.

Hoge, J. & Kesner, R.P. (2007) Role of CA3 and CA1 subregions of the dorsal hippocampus on temporal processing of objects. *Neurobiol Learn Mem* 88, 225-231.

Honey R. C., Watt, A., & Good, M. (1998). Hippocampal lesions disrupt an associative mismatch process. *J Neurosci 18*, 2226-2230.

Hooper, C., Markevich, V., Plattner, F., Killick, R., Schofield, E., Engel, T., et al. (2007). Glycogen synthase kinase-3 inhibition is integral to long-term potentiation. *Eur J Neurosci 25*, 81-86

Hou, L., Antion, M.D., Hu, D., Spencer, C.M., Paylor, R., & Klann, E. (2006) Dynamic translational and proteasomal regulation of fragile X mental retardation protein controls mGluR-dependent long-term depression. *Neuron* 51, 441-454.

Hu, Y. S., Xu, P., Pigino, G., Brady, S. T., Larson, J., & Lazarov, O. (2010). Complex environment experience rescues impaired neurogenesis, enhances synaptic plasticity, and attenuates neuropathology in familial Alzheimer's disease-linked APPswe/PS1DeltaE9 mice. *FASEB J 24*, 1667-1681.

Huang, G. J., Ben-David, E., Tort Piella,, A., Edwards, A., Flint, J., & Shifman, S. (2012a). Neurogenomic evidence for a shared mechanism of the antidepressant effects of exercise and chronic fluoxetine in mice. *PloS one 7*, e35901.

Huang, Y. F., Yang, C. H., Huang, C. C., & Hsu, K. S. (2012b). Vascular Endothelial Growth Factor-Dependent Spinogenesis Underlies Antidepressant-Like Effects of Enriched Environment. *The Journal of Biological Chemistry 287*, 40938-40955.

Huber, K. M., Gallagher, S. M., Warren, S. T., & Bear, M. F. (2002). Altered synaptic plasticity in a mouse model of fragile X mental retardation. *Proc Natl Acad Sci U S A. 99*, 7746-7750.

Hunsaker, M.R. & Kesner, R.P. (2008) Evaluating the differential roles of the dorsal dentate gyrus, dorsal CA3, and dorsal CA1 during a temporal ordering for spatial locations task. *Hippocampus* 18, 955-964.

Hunsaker, M.R., Wenzel, H.J., Willemsen, R., & Berman, R.F. (2009) Progressive spatial processing deficits in a mouse model of the fragile X premutation. *Behav Neurosci* 123, 1315-1324.

Hunsaker, M.R., Kim, K., Willemsen, R., & Berman, R.F. (2012) CGG trinucleotide repeat length modulates neural plasticity and spatiotemporal processing in a mouse model of the fragile X premutation. *Hippocampus* 22, 2260-2275.

Hunsaker, M. R. & Kesner, R. P. (2013). The operation of pattern separation and pattern completion processes associated with different attributes or domains of memory. *Neurosci Biobehav Rev 37*, 36-58.

Iosif, R. E., Ahlenius, H., Ekdahl, C. T., Darsalia, V., Thored, P., Jovinge, S., et al. (2008). Suppression of stroke-induced progenitor proliferation in adult subventricular zone by tumor necrosis factor receptor 1. *J Cereb Blood Flow Metab 28*, 1574-1587.

Jope, R. S., & Roh, M. S. (2006). Glycogen synthase kinase-3 (GSK3) in psychiatric diseases and therapeutic interventions. *Curr Drug Targets 7*, 1421-1434.

Kalinichev, M., & Dawson, L. A. (2011). Evidence for antimanic efficacy of glycogen synthase kinase-3 (GSK3) inhibitors in a strain-specific model of acute mania. Int J *Neuropsychopharmacol 14*, 1051-1067.

Karege, F., Perroud, N., Burkhardt, S., Schwald, M., Ballmann, E., La Harpe, R., et al. (2007). Alteration in kinase activity but not in protein levels of protein kinase B and glycogen synthase kinase-3beta in ventral prefrontal cortex of depressed suicide victims. *Biol Psychiatry 61*, 240-245.

Kemper, M. B., Hagerman, R. J., & Altshul-Stark, D. (1988). Cognitive profiles of boys with the fragile X syndrome. *Am J Med Genet 30*,191-200.

Kempermann, G., Kuhn, H. G., & Gage, F. H. (1997). More hippocampal neurons in adult mice living in an enriched environment. *Nature 386*, 493-495.

Kempermann, G. (2002). Regulation of adult hippocampal neurogenesis - implications for novel theories of major depression. *Bipolar Disord 4*, 17-33.

Kendler, K. S., Karkowski, L. M., & Prescott, C. A. (1999). Causal relationship between stressful life events and the onset of major depression. *Am J Psychiatry 156*, 837-841.

Kim, K. S., & Han, P. L. (2006). Optimization of chronic stress paradigms using anxiety- and depression-like behavioral parameters. *Journal of Neuroscience Research 83*, 497-507.

Kim, W. Y., Wang, X., Wu, Y., Doble, B. W., Patel, S., Woodgett, J. R., et al. (2009). GSK-3 is a master regulator of neural progenitor homeostasis. *Nat Neurosci 12*, 1390-1397.

King, M. K., Pardo, M., Cheng, Y., Downey, K., Jope, R. S., & Beurel, E. (2013). Glycogen synthase kinase-3 inhibitors: rescuers of cognitive impairments. *Pharmacol and Therapeutics* (Epub ahead of print).

Klein, P. S., & Melton, D. A. (1996). A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci USA 93*, 8455-8459.

Kobilo, T., Liu, Q. R., Gandhi, K., Mughal, M., Shaham, Y., & van Praag, H. (2011). Running is the neurogenic and neurotrophic stimulus in environmental enrichment. *Learn Mem 18*, 605-609

Komitova, M., Mattsson, B., Johansson, B. B., & Eriksson, P. S. (2005). Enriched environment increases neural stem/progenitor cell proliferation and neurogenesis in the subventricular zone of stroke-lesioned adult rats. *Stroke 36*, 1278-1282.

Koo, J. W., Russo, S. J., Ferguson, D., Nestler, E. J., & Duman, R. S. (2010) Nuclear factor-kappaB is a critical mediator of stress-impaired neurogenesis and depressive behavior. *Proc Natl Acad Sci U S A 107*, 2669-2674.

Kooy, R. F., D'Hooge, R., Reyniers, E., Bakker, C. E., Nagels, G., De Boulle, K., et al. (1996). Transgenic mouse model for the fragile X syndrome *Am J Med Genet 64*, 241-245.

Komitova, M., Mattsson, B., Johansson, B. B., & Eriksson, P. S. (2005). Enriched environment increases neural stem/progenitor cell proliferation and neurogenesis in the subventricular zone of stroke-lesioned adult rats. *Stroke 36*, 1278-1282.

Lang J. T., & McCullough, L. D. (2008) Pathways to ischemic neuronal cell death: are sex differences relevant? *J Transl Med 6*, 33.

Lazarov, O., & Marr, R. A. (2013). Of mice and men: neurogenesis, cognition and Alzheimer's disease. *Front Aging Neurosci 5*, 43.

Leal-Galicia, P., Saldívar-González, A., Morimoto, S., & Arias, C. (2007). Exposure to environmental enrichment elicits differential hippocampal cell proliferation: role of individual responsiveness to anxiety. *Dev Neurobiol 67*, 395-405.

Leuner, B., Gould, E., & Shors, T. J. (2006). Is there a link between adult neurogenesis and learning? *Hippocampus 16*, 216-224.

Li, M., Wang, X., Meintzer, M. K., Laessig, T., Birnbaum, M. J., & Heidenreich, K. A. (2000) Cyclic AMP promotes neuronal survival by phosphorylation of glycogen synthase kinase 3ß. *Mol Cell Biol 20*, 9356–9363.

Li, X., Zhu, W., Roh, M. S., Friedman, A. B., Rosborough, K., & Jope, R. S. (2004). In vivo regulation of glycogen synthase kinase-3β (GSK3β) by serotonergic activity in mouse brain. *Neuropsychopharmacology 29*, 1426-1431.

Li, X., & Jope, R. S. (2010). Is glycogen synthase kinase-3 a central modulator in mood regulation? *Neuropsychopharmacology 35*, 2143-2154.

Li, Y., Luikart, B. W., Birnbaum, S., Chen, J., Kwon, C. H., Kernie, S. G., et al. (2008). TrkB regulates hippocampal neurogenesis and governs sensitivity to antidepressive treatment. *Neuron 59*, 399-412.

Lipina, T. V., Kaidanovich-Beilin, O., Patel, S., Wang, M., Clapcote, S. J., Liu, F., et al. (2011). Genetic and pharmacological evidence for schizophrenia-related Disc1 interaction with GSK-3. *Synapse 65*, 234-248.

Lipina, T. V., Wang, M., Liu, F., & Roder, J. C. (2012). Synergistic interactions between PDE4B and GSK-3: DISC1 mutant mice. *Neuropharmacology 62*, 1252-1262.

Liu, Z. H., & Smith, C. B. (2009). Dissociation of social and nonsocial anxiety in a mouse model of fragile X syndrome. *Neurosci Lett 454*, 62-66.

Liu, Z. H., Chuang, D. M., & Smith, C. B. (2011). Lithium ameliorates phenotypic deficits in a mouse model of fragile X syndrome. *Int J Neuropsychopharmacol 14*, 618-630.

Liu, Z. H., Huang, T., & Smith, C. B. (2012). Lithium reverses increased rates of cerebral protein synthesis in a mouse model of fragile X syndrome. *Neurobiol Dis 45*, 1145-1152.

Lu, R., Wang, H., Liang, Z., Ku, L., O'donnell, W. T., Li, W., et al. (2004). The fragile X protein controls microtubule-associated protein 1B translation and microtubule stability in brain neuron development. *Proc Natl Acad Sci USA 101*, 15201-15206.

Mai, L., Jope, R. S., & Li, X. (2002). BDNF-mediated signal transduction is modulated by GSK3β and mood stabilizing agents. *J Neurochem 82*, 75-83.

Malberg, J. E., Eisch, A. J., Nestler, E. J., & Duman, R. S. (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci 20*, 9104-9110.

Manev, H., Uz, T., Smalheiser, N. R., & Manev, R. (2001). Antidepressants alter cell proliferation in the adult brain in vivo and in neural cultures in vitro. *Eur J Pharmacol 411*, 67-70.

Martin, M., Rehani, K., Jope, R. S., & Michalek, S. M. (2005). Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat Immunol 6*, 777-784.

Martinez, A., Alonso, M., Castro, A., Perez, C., & Moreno, F. J. (2002). First non-ATP competitive glycogen synthase kinase 3 beta (GSK-3beta) inhibitors: thiadiazolidinones (TDZD) as potential drugs for the treatment of Alzheimer's disease. *J Med Chem 45*, 1292-1299.

Martinez, A. (2006). TDZD: Selective GSK-3 inhibitor with great potential for Alzheimer disease. *Neurobiol Aging 27*, S13.

Massa, F., Koehl, M., Wiesner, T., Grosjean, N., Revest, J. M., Piazza, P. V., et al. (2011). Conditional reduction of adult neurogenesis impairs bidirectional hippocampal synaptic plasticity. *Proc Natl Acad Sci U S A 108*, 6644-6649.

McBride, S. M., Choi, C. H., Wang, Y., Liebelt, D., Braunstein, E., Ferreiro, D., et al. (2005). Pharmacological rescue of synaptic plasticity, courtship behavior, and mushroom body defects in a Drosophila model of fragile X syndrome. *Neuron 45*, 753-764.

McManus, E. J., Sakamoto, K., Armit, L. J., Ronaldson, L., Shpiro, N., Marquez, R., et al. (2005). Role that phosphorylation of GSK3 plays in insulin and Wnt signalling defined by knockin analysis. *EMBO J 24*, 1571-1583.

McNaughton, C. H., Moon, J., Strawderman, M. S., Maclean, K. N., Evans, J., & Strupp, J. (2008). Evidence or social anxiety and impaired social cognition in a mouse model of fragile X syndrome. *Behav Neurosci 122*, 292-300.

Michalon A, Sidorov M, Ballard TM, Ozmen L, Spooren W, Wettstein JG, et al. (2012): Chronic pharmacological mGlu5 inhibition corrects fragile X in adult mice. *Neuron*. 74:49-56.

Min, W. W., Yuskaitis, C. J., Yan, Q., Sikorski, C., Chen, S., Jope, R. S., et al. (2009). Elevated glycogen synthase kinase-3 activity in Fragile X mice: key metabolic regulator with evidence for treatment potential. *Neuropharmacology 56*, 463-472.

Mines, M. A., Yuskaitis, C. J., King, M. K., Beurel, E., & Jope, R. S. (2010). GSK3 influences social preference and social anxiety in a mouse model of Fragile X syndrome and autism. *PLoS One 5*: e9706.

Mines, M. A. & Jope, R. S. (2011). Glycogen synthase kinase-3: a promising therapeutic target for fragile x syndrome. *Front Mol Neurosci 4*, 35.

Mineur, Y. S., Sluyter, F., de Wit, S., Oostra, B. A., & Crusio, W. E. (2002). Behavioral and neuroanatomical characterization of the Fmr1 knockout mouse. *Hippocampus 12*, 39-46.

Mineur, Y. S., Juynh, L. X., & Crusio, W. E. (2006). Social behavior deficits in the *Fmr1* mutant mouse. *Behav Brain Res 168*, 172-175.

Mineur, Y. S., Belzung, C., & Crusio, W. E. (2007). Functional implications of decreases in neurogenesis following chronic mild stress in mice. *Neuroscience 150*, 251-259.

Mirescu, C., Peters, J. D., Noiman, L., & Gould, E. (2006). Sleep deprivation inhibits adult neurogenesis in the hippocampus by elevating glucocorticoids. *Proc Natl Acad Sci USA 103*, 19170-19175.

Monje, M. L., Toda, H., & Palmer, T. D. (2003). Inflammatory blockade restores adult hippocampal neurogenesis. *Science 302*, 1760-1765.

Morales-Garcia, J. A., Luna-Medina, R., Alonso-Gil, S., Sanz-Sancristobal, M., Palomo, V., Gil, C., et al. (2012). Glycogen synthase kinase 3 inhibition promotes adult hippocampal neurogenesis in vitro and in vivo. *ACS Chem Neurosci 3*, 963-971.

Murray, F., Smith, D. W., & Hutson, P. H. (2008). Chronic low dose corticosterone exposure decreased hippocampal cell proliferation, volume and induced anxiety and depression like behaviours in mice. *Eur J Pharmacol 583*, 115-127.

Musumeci, S. A., Hagerman, R. J., Ferri, R. Bosco, P., Dalla Bernardina, B., Tassinari, C. A., et al. (1999). Epilepsy, and EEG findings in males with fragile X syndrome. *Epilepsia 40*, 1092-1099.

Nosyreva, E. D. & Huber, K. M. (2006). Metabotropic receptor-dependent long-term depression persists in the absence of protein synthesis in the mouse model of fragile X syndrome. *J Neurophysiol 95*, 3291-3295.

O'Brien, W. T., Harper, A. D., Jove, F., Woodgett, J. R., Maretto, S., Piccolo, S., et al. (2004). Glycogen synthase kinase-3β haploinsufficiency mimics the behavioral and molecular effects of lithium. *J Neurosci 24*, 6791-6798.

Ornstein PA, Schaaf JM, Hooper SR, Hatton DD, Mirrett P, Bailey DB, Jr. (2008): Memory skills of boys with fragile X syndrome. *Am J Ment Retard*. 113:453-465.

Pacey, L. K., Doss, L., Cifelli, C., van der Kooy, D., Heximer, S. P., & Hampson, D. R. (2011). Genetic deletion of regulator of G-protein signaling 4 (RGS4) rescues a subset of fragile X related phenotypes in the FMR1 knockout mouse. *Mol Cell Neurosci 46*, 563-572.

Palomo, V., Soteras, I., Perez, D. I., Perez, C., Gil, C., Campillo, N. E., et al. (2011) Exploring the binding sites of glycogen synthase kinase 3. Identification and characterization of allosteric modulation cavities. *J Med Chem 54*, 8461-8470.

Paradee, W., Melikian, H. E., Rasmussen, D. L., Kenneson, A., Conn, P. J., & Warren, S. T. (1999). Fragile X mouse: strain effects of knockout phenotype and evidence suggesting deficient amygdala function. *Neuroscience 94*, 185-192.

Peier, A. M., McIlwain, K. L., Kenneson, A., Warren, S. T., Paylor, R., & Nelson, D. L. (2000), (Over)correction of FMR1 deficiency with YAC transgenics: behavioral and physical features. *Hum Mol Genet 9*, 1145-1159.

Perez-Martin, M., Azcoitia, I., Trejo, J. L., Sierra, A., & Garcia-Segura, L. M. (2003). An antagonist of estrogen receptors blocks the induction of adult neurogenesis by insulin-like growth factor-I in the dentate gyrus of adult female rat. *Eur J Neurosci. 18*, 923-930.

Pham, K., Nacher, J., Hof, P. R., & McEwen, B. S. (2003). Repeated restraint stress suppresses neurogenesis and induces biphasic PSA-NCAM expression in the adult rat dentate gyrus. *Eur J Neurosci 17*, 879-886.

Pieretti, M., Zhang, F. P., Fu, Y. H., Warren, S. T., Oostra, B. A., Caskey, C. T., et al. (1991). Absence of expression of the FMR-1 gene in fragile X syndrome. *Cell 66*, 817-822.

Polter, A., Beurel, E., Garner, R., Song, L., Miller, C., Sweatt, J. D., et al. (2010). Deficiency in the inhibitory serine-phosphorylation of glycogen synthase kinase-3 increases sensitivity to mood disturbances. *Neuropsychopharmacology 35*, 1761-1774.

Qin, M., Kang, J., & Smith, C. B. (2005). A null mutation for *Fmr1* in female mice: effects on regional cerebral metabolic rate for glucose and relationship to behavior. *Neuroscience 135*, 999-1009.

Renoir, T., Pang, T. Y., Mo, C., Chan, G., Chevarin, C., Lanfumey, L., et al. (2013). Differential effects of early environmental enrichment on emotionality related behaviours in Huntington's disease transgenic mice. *J Physiol 591*, 41-55.

Rolls, E. T. & Kesner, R. P. (2006). A computational theory of hippocampal function, and empirical tests of the theory. *Prog Neurobiol 79*, 1-48.

Ryves, W. J., & Harwood, A. J. (2001). Lithium inhibits glycogen synthase kinase-3 by competition for magnesium. *Biochem Biophys Res Commun 280*, 720-725.

Salam, J. N., Fox, J. H., Detroy, E. M., Guignon, M. H., Wohl, D. F., & Falls, W. A. (2009). Voluntary exercise in C57 mice is anxiolytic across several measures of anxiety. *Behavioral Brain Research 197*, 31-40.

Schmidt, H. D., & Duman, R. S. (2007). The role of neurotrophic factors in adult hippocampal neurogenesis, antidepressant treatments and animal models of depressive-like behavior. *Behav Pharmacol 18*, 391-418.

Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., et al. (2003). Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science 301*, 805-809.

Seo, J. S., Park, J. Y., Choi, J., Kim, T. K., Shin, J. H., Lee, J. K., et al. (2012). NADPH oxidase mediates depressive behavior induced by chronic stress in mice. *Journal of Neuroscience 32*, 9690-9699.

Sherman, S. L. (2000). Premature ovarian failure in the fragile X syndrome. *Am J Med Genet 97*, 189-194.

Shors, T. J., Mathew, J., Sisti, H. M., Edgecomb, C., Beckoff, S., & Dalla, C. (2007). Neurogenesis and helplessness are mediated by controllability in males but not in females. *Biol Psychiatry 62*, 487-495.

Shorter E. (2009). The history of lithium therapy. *Bipolar Disord 11*, Suppl 2:4-9.

Silva, R., Mesquita, A. R., Bessa, J., Sousa, J. C., Sotiropoulos, I., Leão, P., et al. (2008). Lithium blocks stress-induced changes in depressive-like behavior and hippocampal cell fate: the role of glycogen-synthase-kinase-3beta. *Neuroscience 152*, 656-669.

Smith, M. A., Makino, S., Kvetnansky, R., & Post, R. M. (1995). Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J Neurosci 15*, 1768-1777.

Spalding, K. L., Bergmann, O., Alkass, K., Bernard, S., Salehpour, M., Huttner, H. B., Boström, E., Westerlund, I., Vial, C., Buchholz, B. A., Possnert, G., Mash, D. C., Druid, H., Frisén, J. (2013). Dynamics of hippocampal neurogenesis in adult humans. *Cell 153*, 1219-27.

Spencer, C. M., Alekseyenko, O., Serysheva, E., Yuva-Paylor, L. A., & Paylor, R. (2005). Altered anxiety-related and social behaviors in the *Fmr1* knockout mouse of fragile X syndrome. *Genes Brain Behav 4*, 420-430.

Stranahan, A. M., Khalil, D., Gould, E. (2006). Social isolation delays the positive effects of running on adult neurogenesis. *Nat Neurosci 9*, 526-533.

Suvrathan, A., Hoeffer, C. A., Wong, H., Klann, E., Chattarji, S. (2010) Characterization and reversal of synaptic defects in the amygdala in a mouse model of fragile X syndrome. *Proc Natl Acad Sci U S A 107*, 11591-11596.

Tassone, F., Long, K. P., Tong, T. H., Lo, J., Gane, L. W., Berry-Kravis, E., et al. (2012). FMR1 CGG allele size and prevalence ascertained through newborn screening in the United States. *Genome Med 4*, 100.

Thomas, A. M., Bui, N., Perkins, J. R., Yuva-Paylor, L. A., & Paylor, R. (2012). Group I metabotropic glutamate receptor antagonists alter select behaviors in a mouse model for fragile X syndrome. *Psychopharmacology (Berl) 219*, 47-58.

Trejo, J. L., Llorens-Martin, M. V., & Torres-Aleman, I. (2008). The effects of exercise on spatial learning and anxiety-like behavior are mediated by an IGF-I-dependent mechanism related to hippocampal neurogenesis. *Molecular and Cellular Neurosciences 37*, 402-411.

van Praag, H., Kempermann, G., & Gage, F. H. (1999). Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat Neurosci 2*, 266-270.

van Praag, H., Shubert, T., Zhao, C., & Gage, F. H. (2005). Exercise enhances learning and hippocampal neurogenesis in aged mice. *J Neurosci 25*, 8680-8685.

Verkerk, A. J., Pieretti, M., Sutcliffe, J. S., Fu, Y. H., Kuhl, D. P., Pizzuti, A., et al. (1991). Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell 65*, 905-914.

Walker, M. D. & Mason, G. (2011). Female C57BL/6 mice show consistent individual differences in spontaneous interaction with environmental enrichment that are predicted by neophobia. *Behav Brain Res 224*, 207-212.

Wallenstein, G. V., Eichenbaum, H., & Hasselmo, M. E. (1998). The hippocampus as an associator of discontiguous events. *Trends Neurosci 21*, 317-323.

Wang, L. W., Berry-Kravis, E., & Hagerman, R. J. (2010). Fragile X: leading the way for targeted treatments in autism. *Neurotherapeutics 7*, 264-274.

Warner-Schmidt, J. L., & Duman, R. S. (2007). VEGF is an essential mediator of the neurogenic and behavioral actions of antidepressants. *Proc Natl Acad Sci USA 104*, 4647-4652.

Wei J, Liu W, Yan Z (2010): Regulation of AMPA receptor trafficking and function by glycogen synthase kinase 3. *J Biol Chem*. 285:26369-26376.

Westenbroek, C., Den Boer, J. A., Veenhuis, M., & Ter Horst, G. J. (2004). Chronic stress and social housing differentially affect neurogenesis in male and female rats. *Brain Res Bull 64*, 303-308.

Wexler, E. M., Geschwind, D. H., & Palmer, T. D. (2008). Lithium regulates adult hippocampal progenitor development through canonical Wnt pathway activation. *Mol Psychiatry 13*, 285-292.

Woodgett, J. R. (1990). Molecular cloning and expression of glycogen synthase kinase-3/factor A. *EMBO J 9*, 2431–2438.

Yan, Q. J., Asafo-Adjei, P. K., Arnold, H. M., Brown, R. E, & Bauchwitz, R. P. (2004). A phenotypic and molecular characterization of the fmr1-tm1Cgr fragile X mouse. *Genes Brain Behav 3*, 337-359.

Yuskaitis, C. J. & Jope, R. S. (2009). Glycogen synthase kinase-3 regulates microglial migration, inflammation, and inflammation-induced neurotoxicity. *Cell Signal 21*, 264-273.

Yuskaitis, C. J., Mines, M. A., King, M. K., Sweatt, J. D., Miller, C. A., & Jope, R. S. (2010a). Lithium ameliorates altered glycogen synthase kinase-3 and behavior in a mouse model of Fragile X Syndrome. *Biochemical Pharmacology 79*, 632-646.

Yuskaitis, C. J., Beurel, E., & Jope, R. S. (2010b). Evidence of reactive astrocytes but not peripheral immune system activation in a mouse model of Fragile X syndrome. *Biochim Biophys Acta 1802*, 1006-1012.

Zalfa, F., Giorgi, M., Primerano, B., Moro, A., Di Penta, A., Reis, S., et al. (2003). The fragile X syndrome protein FMRP associates with BC1 RNA and regulates the translation of specific mRNAs at synapses. *Cell 112*, 317-327.

Zhao, C., Deng, W., & Gage, F. H. (2008). Mechanisms and functional implications of adult neurogenesis. *Cell 132*, 645-660.

Zhao, M. G., Toyoda, H., Ko, S. W., Ding, H. K., Wu, L. J., & Zhuo, M. (2005). Deficits in trace fear memory and long-term potentiation in a mouse model for fragile X syndrome. *J Neurosci 25*, 7385-7892.

Zhu, L. Q., Wang, S. H., Liu, D., Yin, Y. Y., Tian, Q., Wang, X. C., et al. (2007). Activation of glycogen synthase kinase-3 inhibits long-term potentiation with synapse-associated impairments. *J Neurosci 27*, 12211-12220.

APPENDIX A

IACUC APPROVAL

MILLER
SCHOOL OF MEDICINE

Protocol Approval

06-Oct-2011

Dear Dr. JOPE,

The following animal use application was reviewed and approved by the University of Miami Institutional Animal Care and Use Committee (IACUC). If conditional approval was issued, please address the additional approval requirements outlined below.

Protocol Number:	11-236 NEW
Protocol Title:	Glycogen synthase kinase-3 in Fragile X mice
Protocol Sponsor:	FRAXA Research Foundation
Protocol PI:	JOPE, RICHARD
Institution:	University of Miami
Date of Approval:	06-Oct-2011
Duration of Approval:	06-Oct-2011 to 05-Oct-2012
Prior Protocol, if any:	
Additional Approval Requirements, if any:	

The University of Miami has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health. The assurance number is #A-3224-01, effective July 11, 2007. Additionally, as of July 20, 2010, the Council on Accreditation of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International) has continued the University of Miami's full accreditation.

Sincerely,

Sari Izenwasser, PhD

Chair, **Institutional Animal Care and Use (IACUC)**
Professor of Psychiatry and Behavioral Sciences
University of Miami Miller School of Medicine
1600 NW 10th Ave., Room 4113A (D-80)
Miami, FL 33136

Email Address: sizenwasser@med.miami.edu
Tel: 305-243-2032; Fax: 305-243-5475

APPENDIX B

IACUC RENEWAL APPROVAL

MILLER
SCHOOL OF MEDICINE

Annual Renewal Approval

06-Sep-2012

Dear Dr. JOPE,

The following annual renewal was reviewed and approved by the University of Miami Institutional Animal Care and Use Committee (IACUC) as of the date below:

Protocol Number:	11-236 RENEWAL 02
Protocol Title:	Glycogen synthase kinase-3 in Fragile X mice
Protocol Sponsor:	FRAXA RESEARCH FOUNDATION
Protocol PI:	JOPE, RICHARD
Institution:	University of Miami
Date of Approval:	06-Sep-2012
Approval Period:	06-Oct-2012 to 05-Oct-2013

The University of Miami has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health. The assurance number is #A-3224-01, effective July 11, 2007. Additionally, as of July 20, 2010, the Council on Accreditation of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International) has continued the University of Miami's full accreditation.

Sincerely,

Sari Izenwasser, PhD

Chair, **Institutional Animal Care and Use (IACUC)**
Professor of Psychiatry and Behavioral Sciences
University of Miami Miller School of Medicine
1600 NW 10th Ave., Room 4113A (D-80)
Miami, FL 33136

Email Address: sizenwasser@med.miami.edu
Tel: 305-243-2032; Fax: 305-243-5475