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Glycogen Synthase Kinase-3β: An Investigation Of The Novel Serine 389 Phosphorylation Site

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GLYCOGEN SYNTHASE KINASE-3β: AN INVESTIGATION OF THE NOVEL SERINE 389 PHOSPHORYLATION SITE

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ABSTRACT

Stress associated psychiatric disorders such as depression, anxiety, and post-traumatic stress disorder affect a large proportion of the population. Reductions in the complexity of neuronal morphology and reduced neurogenesis are commonly observed outcomes following stress exposure in rodent models and may represent a mechanism for the reduced brain volume in stress sensitive regions such as the hippocampus observed in individuals diagnosed with stress associated disorders. Multiple lines of evidence suggest that glycogen synthase kinase (GSK) 3β may play a role in the neurodegenerative phenotype observed following stress exposure. GSK3β is atypical in that it is inhibited by phosphorylation. This inhibitory phosphorylation has typically been studied by examining the phosphorylation state of the serine 9 (S^9) site. Inhibition of GSK3β is implicated in synaptic stabilization, increased expression of trophic factors that support dendritic complexity and neurogenesis, reduced apoptosis, and the antidepressive effects of currently implemented therapeutics. It is surprising then that little research has examined the regulation of GSK3β by stress. A novel GSK3β phosphorylation site, serine 389 (S^{389}), has recently been described that is regulated by p38 mitogen activated protein kinase (MAPK) and is independent of S^9 phosphorylation by AKT. p38 MAPK is implicated in the behavioral effects of stress exposure making an understanding of its interaction with GSK3β S^{389} phosphorylation during stress a compelling research target. The current studies examine GSK3β regulation following variate stress exposure in stress reactive brain regions, describe the anatomical specificity of GSK3β S^{389} phosphorylation in the brain, and detail the behavioral phenotype of a novel mutant mouse that cannot inhibit GSK3β by S^{389} phosphorylation (GSK3β KI). Region specific changes in GSK3β phosphorylation were observed following stress exposure, as well as voluntary exercise, a behavior that confers stress resistance. Elevated GSK3β S^{389} phosphorylation was associated with increased levels of phosphorylated p38 MAPK. This pathway is implicated in the response to DNA damage, and, surprisingly, we observed that histone H2A-variant-X (ϒH2A.X), a marker of DNA damage, was elevated following stress and exercise. Accumulated DNA damage is a proposed driver of neurodegeneration suggesting that the pathway activated by stress may be engaged to protect against such decline. Consistent with a role in the response to DNA damage, we observed a primarily nuclear localization of GSK3β S^{389} phosphorylation in the brain while S^9 phosphorylation was found in nuclear and cytosolic compartments. Further, we observed neurodegeneration in hippocampal and cortical regions of GSK3β KI mice supporting the idea that the inhibition of GSK3β by S^{389} phosphorylation observed following stress and exercise may be protective. Though largely similar to wild type mice in behavioral tests, increased auditory fear conditioning was evident in GSK3β KI mice. Contextual and cued freezing was prolonged in GSK3β KI mice, a phenotype that is commonly observed in stress models. Together these findings suggest that GSK3β S^{389} phosphorylation is playing a critical role in neuronal integrity that is independent of GSK3β S^9 phosphorylation, and that the subset of neurons protected by GSK3β S^{389} phosphorylation may play an important role in preventing a portion of the maladaptive behavioral changes observed following stress exposure.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1: LITERATURE REVIEW

Depression and anxiety are commonly comorbid psychiatric disorders that affect a broad portion of society. Recent estimates suggest that 16-25% of the population suffers from depression, with as many as 60% having a comorbid anxiety disorder (Kessler et al., 2003; Kessler et al., 2005). It is estimated that nearly 4% of the population seeks treatment for depression, and that 75% of those with severe or very severe depression receive inadequate treatment (Kessler et al., 2003). For those seeking treatment, remission rates with typical pharmacological interventions are high, and comorbid anxiety disorders predict negative outcomes (Trivedi et al., 2006). Inadequate mental health treatment represents a social and fiscal burden, and the lack of adequate treatment suggests that further research is necessary to understand the underlying causes of such disorders as well as the changes occurring during successful treatment. Stress, an actual or perceived challenge to homeostasis, has been associated with precipitating and augmenting psychiatric disorders (Kendler et al., 1999; Holsboer, 2001; Gold and Chrousos, 2002; Hettema et al., 2006) and can be utilized in rodents to model some of the features of psychiatric disorders.

The Stress Response

The stereotypical stress response involves rapid activation of the sympathetic nervous system followed by activation of the hypothalamic pituitary adrenal (HPA)-axis (Sapolsky et al., 2000). HPA-axis activation leads to release of corticotropin releasing hormone from the parvocellular nucleus of the hypothalamus. Within the anterior pituitary, corticotropin releasing hormone activity causes release of adrenocorticotropic

hormone (ACTH). ACTH activation of fascicular cells in the adrenal cortex leads to release of the glucocorticoid cortisol in humans, corticosterone in rodents. Glucocorticoids act at receptor targets throughout the peripheral and central nervous system. The central nervous system possesses both the high affinity mineralocorticoid receptor (MR) and the lower affinity glucocorticoid receptor (GR). GRs are widely expressed throughout the brain, while co-expression of MRs and GRs occurs mainly in limbic structures (Reul and de Kloet, 1985). The MR has a higher affinity for corticosterone than the GR and is thought to play a role in neuronal integrity and basal excitability (Joels, Karst, DeRijk, & de Kloet, 2008). GR occupancy is associated with stress levels of corticosterone and promotes termination of the HPA-axis response through negative feedback initiation in limbic structures, as well as through direct action in the hypothalamus and the pituitary (de Kloet et al., 2005; Herman, Ostrander, Mueller, & Figueiredo, 2005).

The immediate actions of corticosterone are directed at surviving stress exposure and include increased cardiovascular tone, energy mobilization, immune activation, memory consolidation, and cerebral blood flow (Sapolksy et. al., 2000). The negative impacts of stress exposure are largely attributed to failed negative feedback and associated increased exposure to hormones associated with the stress response (Gold and Chrousos, 2002). Beyond increased incidence of psychiatric disorders these effects include immunosuppression (Dhabhar, 2009), visceral fat deposition (Peeke and Chrousos, 1995), and impaired declarative memory (Conrad et al., 1996). Given the necessity of mounting a stress response when met with homeostatic challenge, and the

negative consequences of exposure to stress hormones, a brain circuitry is in place to regulate activation, and termination, of the stress response.

Limbic Brain Regions Involved in Stress Regulation

Brain regions that play an important role in emotional regulation are also involved in control of the stress response. Research suggests that the amygdala, a brain region implicated in fear regulation (Walker et al., 2003), activates the HPA-axis (Herman et al., 2005). Acute stress induces immediate early gene expression in the amygdala (Cullinan et al., 1995), and lesions of the central amygdala reduce ACTH levels during restraint stress (Beaulieu et al., 1986). The bed nucleus of the stria terminalis (BNST) is implicated in anxiety regulation and the anxiogenic response to corticotropin releasing hormone (Lee and Davis, 1997). The BNST receives projections from brain regions implicated in activation as well as inhibition of the HPA-axis and is therefore positioned to integrate opposing signals (Herman et al., 2005). Consistent with this, a regional specificity has been proposed for BNST regulation of the stress response where anterior portions of the structure are associated with activation of the HPA-axis, and the posterior portions are associated with inhibition (Herman et al., 2005; Radley and Sawchenko, 2011). For example, lesions of the anterior-dorsal BNST decrease corticotropin releasing hormone expression in the paraventricular nucleus, while lesions of the posterior BNST increase corticotropin releasing hormone expression (Herman et al., 1994). A similar specificity is observed in the corticosterone response to restraint stress as anterior BNST lesions reduce and posterior BNST lesions augment corticosterone output (Choi et al., 2007). The hippocampus, a key area for memory encoding, especially that of the context

in which stimuli are occurring, is associated with inhibitory control over HPA-axis activity. Lesions of the ventral subiculum portion of the hippocampus, a region that sends projections to the BNST and amygdala, result in an increased corticosterone response to restraint and open field stress (Herman et al., 1998). The medial prefrontal cortex, a brain region involved in processing emotional stimuli and regulating the subsequent response, is also implicated in inhibitory control over HPA-axis activity. Medial prefrontal cortex immediate early gene activation in deep cortical layers (i.e., V and VI) is observed in glucocorticoid receptor positive cells after restraint stress (Ostrander et al., 2003), and lesions of this region augment, while corticosterone implants reduce, the response to restraint (Diorio et al., 1993). As evidenced above, a complex interplay exists between limbic brain structures that results in control over the stress response. It is interesting then that some of the most robust findings in response to stress exposure involve changes in the morphology and physiology of limbic brain regions, particularly those involved with inhibitory control over HPA-axis responding.

Stress Effects on Neuronal Structure, Neurogenesis, and Trophic Support

Broadly, neuroimaging studies have shown increased activity in the amygdala, decreased volume in hippocampus, and hypoactivation and decreased volume in the medial prefrontal cortex in depressed individuals (Savitz and Drevets, 2009). Notably, volume loss in the hippocampus has been negatively correlated with depression duration (Sheline et al., 1999), as well as symptom severity (Vakili et al., 2000). Additionally, post-mortem analyses have revealed decreased neuron size in the frontal cortex of depressed patients (Rajkowska et al., 1999), as well as reduced synapse number, and

synapse related protein expression (Kang et al., 2012). Findings such as these have pushed the focus of stress effects on psychological disorders towards mechanisms that might account for volume loss such as neuronal death, reduced neurogenesis, and dendritic and/or synaptic atrophy (Sapolsky, 2000; Duman and Monteggia, 2006).

Stress models in rodents support the idea that stress can result in degeneration of neurons including impoverished neuronal morphology, and neuronal loss, as well as reduced neurogenesis. Early work in this area focused on the effects of corticosterone and stress on hippocampal neuron loss and vulnerability to damage. For example, corticosterone administration over 30 days was shown to reduce neuron number in the hippocampus (Sapolsky et al., 1985), and adrenalectomized rats provided with high physiological range corticosterone pellets, and those that were exposed to variate stress over three days, suffered more kainate-induced damage to the hippocampus than control animals (Stein-Behrens et al., 1994). Neuronal degradation was also observed in fatally stressed monkeys (Uno et al., 1989), a finding that was replicated with implanted cortisol pellets (Sapolsky et al., 1990). Further work demonstrated that the number of branch points and length of hippocampal dendrites were reduced by repeated restraint (Watanabe et al., 1992) and 21 days of variate stress (Magarinos and McEwen, 1995). More recently, it was demonstrated that escape deficits following inescapable stress were concurrent with loss of hippocampal spine density (Hajszan et al., 2009).

Similar structural changes have been shown to occur in frontal cortex after stress exposure. For example, 14 days of variate stress produced a reduction in dendritic arborization and spine density in unlabeled cortical neurons, as well as those with

retrograde labeling from anterior BNST, an effect that was associated with impaired inhibitory HPA-axis tone after stress (Radley et al., 2013). Similar effects on dendritic branching and spine density are observed after repeated restraint stress (Radley et al., 2006). It has been observed that spine loss typically involves large spines suggesting that stress associated changes in plasticity may reflect a decrease in spine stability as larger spines are thought to represent a more mature variety (Radley et al., 2008; Shansky and Morrison, 2009). Structural changes in the hippocampus and frontal cortex suggest that stress produces an impoverished neuronal architecture at the level of dendritic and synaptic complexity which may underlie some of the behavioral changes observed in stress- associated disease.

Stress can also affect the generation of new neurons. The hippocampal sub-granular zone is one of two identified sites of neurogenesis in the adult brain. New neurons in the sub-granular zone extend axons to the hippocampal CA3 field, a region where stress-associated atrophy is observed, and, like dendrites in the CA3, new neurons are also impacted by stress and stress hormones (Schoenfeld and Gould, 2012). Acute stress is not regularly shown to affect neurogenesis, though repeated inescapable shock exposure, an acute stress that may be particularly intense, has been shown to reduce neurogenesis. Rats receiving 100 inescapable tail shocks had decreased cell proliferation 7, but not 1, day after receiving shock (Fornal et al., 2007). A similar time course and reduction in proliferation was observed in rats that received 60 inescapable shocks (Malberg and Duman, 2003). Exposure to predator odor has also been shown to reduce cell proliferation for up to one week, an effect that was blocked by adrenalectomy

(Tanapat et al., 2001). Exposure to repeated stress is also associated with reduced neurogenesis. Chronic restraint stress of three and six weeks decreased cell proliferation and survival (Pham et al., 2003). Similarly, three and seven weeks of variate stress reduced cell proliferation in mice (Alonso et al., 2004). Further, mice that conditionally lack adult neurogenesis have a prolonged response to stress exposure while also demonstrating impaired glucocorticoid negative feedback (Snyder et al., 2011). Additionally, these animals display a depression-like phenotype in forced swim and sucrose preference tests. Taken together, these findings suggest that stress and associated glucocorticoid exposure may negatively impact generation of new cells in the hippocampus. Impaired generation of new cells may impact regulation of the stress response, as well as stress sensitive behaviors that model anxiety and depression.

Growth factor regulation by stress, particularly expression of brain derived neurotrophic factor (BDNF), became a focus of research based upon the anti-neurogenic and atrophy inducing effects of stress exposure. BDNF expression was reduced in the frontal cortex and hippocampus of suicide victims with prior diagnosis of depression (Karege et al., 2005), and was increased in post-mortem tissue of depressed patients receiving treatment as compared to those that were untreated (Chen et al., 2001). BDNF can increase neuronal growth and complexity (McAllister et al., 1997; Niblock et al., 2000), and haploinsufficient BDNF mice demonstrate dendritic atrophy (Magarinos et al., 2011) suggesting a role in stress associated neuronal changes. Indeed, stress has commonly been found to reduce expression of BDNF in the hippocampus. One and seven days of restraint stress produced marked reductions in BDNF expression (Smith et

al., 1995). Similarly, 10 days of unpredictable stress reduced BDNF expression (Nibuya et al., 1999). Corticosterone administration to adrenalectomized rats is also sufficient to reduce BDNF expression (Schaaf et al., 1998). The ability not only to produce, but also release, BDNF seems to play a critical role in the response to stress. Mice containing a gene polymorphism that limits BDNF release demonstrate increased depressive and anxiety like behaviors, an increased HPA-axis response to stress, and augmented spine loss in the frontal cortex after repeated restraint stress (Yu et al., 2012). Concurrent with anxiety like behaviors, mice with limited BDNF release also show a reduction in hippocampal dendritic complexity (Chen et al., 2006). In contrast to findings examining BDNF insufficiency, hippocampal infusions of BDNF have been shown to reduce immobility in forced swim test, and increase escape behavior following inescapable shock exposure both indicative of an anti-depressant effect (Shirayama et al., 2002). Together these findings suggest that down regulation of BDNF expression, and release, by stress may play a mechanistic role in changes in neuronal morphology, genesis, and behavioral changes observed after stress exposure.

Effects of Antidepressant Treatment, and Exercise, on Neuronal Structure, Neurogenesis, and Trophic Support

That the physiological changes described above may play a role in stress-related pathology is supported by findings with pharmacological manipulations that provide relief for some afflicted individuals. Twenty one days of antidepressant treatment has been shown to increase the expression of hippocampal BDNF and its membrane bound receptor, and to block the restraint stress associated reduction in BDNF (Nibuya et al.,

1995). Similarly, antidepressant treatment increases proliferation and survival of new neurons (Madsen et al., 2000; Malberg et al., 2000), an effect that is reduced when BDNF signaling is knocked down (Sairanen et al., 2005). Reductions in anxiety and depression-like behavior are not evident after chronic antidepressant treatment when BDNF trafficking and release are impaired (Chen et al., 2006) nor are they evident when hippocampal neurogenesis is ablated (Santarelli et al., 2003). Beyond neurogenesis, antidepressant treatment supports synapse formation in the hippocampal regions affected by stress associated atrophy after two weeks of treatment (Hajszan et al., 2009), and protects against bulbectomy associated reductions in spine density (Norrholm and Ouimet, 2001). Taken together, these findings suggest that increased expression of trophic factors, as well as support of structural complexity and neurogenesis, are critical components to the beneficial effects of antidepressant treatment in the hippocampus.

Work in the frontal cortex suggests that antidepressant treatment may also alleviate the symptoms of stress pathology in this brain region. In a double-blind study, ketamine was shown to produce rapid antidepressant effects in human subjects (Berman et al., 2000). Rapid antidepressant behavioral effects were also observed in rodent models (Garcia et al., 2008; Engin et al., 2009). Extensions of this work have now demonstrated that a single dose of ketamine after variate stress induces a rapid reversal of dendritic spine deficits in the frontal cortex (Li et al., 2010; Li et al., 2011), and that ketamine associated increases in apical dendrite spine density are lost in mice that are impaired in their ability to release BDNF (Liu et al., 2012). Consistent with this, using BDNF antibodies to block BDNF signaling after ketamine administration blocks the

antidepressant effects of ketamine in the forced swim test (Lepack et al., 2014). Thus, together with reports of the physiological effects of antidepressant in the hippocampus, there is consistent evidence that treatments effective in treating stress associated disorders regulate neuronal morphology and trophic support in the frontal cortex.

Physical activity may also alleviate the effects of stress exposure. In human and animal models, the effects of exercise regularly oppose those of stress. The incidence of stress associated disorders is reduced following exercise (Blumenthal et al., 1999; De Moor, Beem, Stubbe, Boomsma, & De Geus, 2006; Harvey, Hotopf, Overland, & Mykletun, 2010; Hoffman et al., 2011; Lawlor & Hopker, 2001). As a treatment for stress associated disorders, exercise has been demonstrated to be as efficacious as antidepressant treatment or cognitive behavioral therapy (Blumenthal et al., 1999; Lawlor and Hopker, 2001), and sustain beneficial effects well after intervention (Babyak et al., 2000). Rodent models demonstrate that engaging in wheel running activity results in reduced anxiety and depression-like behaviors (Binder et al., 2004; Duman et al., 2008; Fox et al., 2008; Salam et al., 2009; Sciolino et al., 2012). Startle amplitude, a measure in which increased response magnitude is indicative of a more anxious subject, is reduced in mice after two weeks of exercise (Salam et al., 2009; Hare et al., 2012; Hare et al., 2013). Approach-avoidance anxiety tests also demonstrate an anxiolytic effect of exercise (Binder et al., 2004; Salam et al., 2009). Immobility time in forced swim and tail suspension testing is also reduced by exercise, indicative of an anti-depressant effect of wheel access (Duman et al., 2008). Six weeks of wheel access blocks shuttle box escape deficits and conditioned fear in learned helplessness models, measures of

depression and anxiety respectively (Greenwood et al., 2003). Together, the results from animal and human subject research suggest that exercise has the capacity to regulate mood in a beneficial manner.

There are numerous ways in which exercise may exert its beneficial effects, including enhancing trophic factor expression, neurogenesis, and neuronal complexity. Exercise is associated with increased neurogenesis (van Praag et al., 1999b; van Praag et al., 1999a; Marlatt et al., 2010) and expression of BDNF (Neeper et al., 1996; Fabel et al., 2003; Duman et al., 2008; Trejo et al., 2008) which are typically observed to be reduced after stress, or stress hormone exposure. Increased activation of the BDNF pathway following exercise is associated with neuroprotection (Zhao et al., 2014), memory enhancement (Intlekofer et al., 2013) and resistance to the negative effects of corticosterone on neurogenesis and dendritic arborization (Yau et al., 2011). Exercise has been shown to augment spine density in the frontal cortex (Sciolino et al., 2015), striatum (Toy et al., 2014), and hippocampus (Yau et al., 2011). Increased mitochondrial content in the dendritic portion of hippocampal neurons (Steib et al., 2014), as well as an increase in BDNF transport to dendrites (Baj et al., 2012) may support exercise associated changes in neuronal morphology.

Exercise associated changes in insulin-like growth factor 1 (IGF-1) may also play an important role in neurotrophic support. IGF-1 is a peripherally and centrally expressed growth factor that supports neurogenesis (Aberg et al., 2000). IGF-1 entry into the brain is increased by exercise, and can acutely increase BDNF expression (Carro et al., 2000). Blocking IGF-1 during exercise blocks the exercise associated reduction in

forced swim immobility time (Duman et al., 2009), the anxiolytic effect of exercise on the elevated plus maze, and the exercise associated increase in markers of neurogenesis (Llorens-Martin et al., 2010). There is also evidence supporting a role for IGF-1 in stress-associated disease. Stress associated anhedonia is reduced by IGF-1 administration during stress, and IGF-1 administration over 14 days was shown to reduce depression-like behaviors (Duman et al., 2009). Additionally, dendritic spine density and arborization are reduced in the frontal cortex of IGF-1 knockout mice (Cheng et al., 2003). The role of IGF-1 as an indicator of peripheral, as well as central, metabolic activity positions it well to signal when the energetic demands of neurogenesis or cellular plasticity are feasible (Llorens-Martin et al., 2009) though more work needs to be done to clarify its role, especially in stress-associated disorders.

Exercise may also generate beneficial effects through changes in the hormonal responses to stress. Findings in exercising animals are consistent with a robustly responsive, yet finely controlled, HPA-axis (Droste et al., 2003; Droste et al., 2006; Fediuc et al., 2006; Sasse et al., 2008; Campbell et al., 2009; Campeau et al., 2010; Nyhuis et al., 2010; Hare et al., 2013; Schoenfeld et al., 2013). The corticosterone response to many types of stress is augmented following exercise (Droste et al., 2003; Droste et al., 2006; Droste et al., 2007; Campbell et al., 2009; Hare et al., 2013), an effect that may be driven by increased adrenal sensitivity to adrenocorticotropic hormone (Hare et al., 2013). However, stress associated increases in corticosterone are of shorter duration (Hare et al., 2013) and habituate at a faster rate (Sasse et al., 2008; Nyhuis et al., 2010). Increased hippocampal glucocorticoid receptor expression (Droste et al., 2007)

and GABAergic inhibitory tone (Schoenfeld et al., 2013) following exercise may contribute to efficient stress response termination. As many of the negative effects of stress can be mimicked by stress hormone exposure exercise may serve to limit such detrimental effects, while allowing a robust response when necessary.

The beneficial effects of exercise may also be mediated by changes in neurotransmission. The reduction in learned helplessness in exercising animals is associated with increased 5HT-1A receptor expression in the dorsal raphe nucleus which could serve to limit serotonin release and sensitization associated with uncontrollable stress exposure (Greenwood et al., 2003). Exercise has also been shown to reduce 5HT-1B receptor expression in the raphe (Greenwood et al., 2005b) and attenuate the anxiogenic response to 5HT-2C agonists administered into the amygdala (Greenwood et al., 2012b) and peripherally (Fox et al., 2008). Thus, exercise may produce a brain that is less responsive to stress associated serotonin neurotransmission. Increased expression of markers for GABA neurotransmission are present throughout the brain including the hippocampus and BNST of exercising rats (Hill et al., 2010), and increased GABAergic tone was associated with a reduced response to stress exposure following exercise (Schoenfeld et al., 2013). Exercise is also associated with changes in the noradrenergic system. Increased brain noradrenaline after exercise in areas associated with adrenocorticotropic hormone release may mediate the robust response to stress exposure observed after exercise (Dishman et al., 2000). However, a concurrent increase in expression of galanin, a negative regulator of noradrenaline release, is postulated to reduce noradrenergic tone in the frontal cortex, an area important in the behavioral

response to stress exposure (Soares et al., 1999; Sciolino et al., 2012; Sciolino et al., 2015).

Glycogen Synthase Kinase 3β: A Multifunctional Protein Kinase With Connections to Stress and Exercise Related Outcomes

Stress exposure produces persistent behavioral and physiological changes that are brought about by exposure(s) to stressful stimuli. This suggests changes in gene products occurring due to cellular signals associated with stress exposure. Cellular signals affect gene transcription following signal transduction by second messenger systems. The diversity of cellular signals and pathological outcomes identified as contributing to stress associated disease suggests that dysregulation of a factor tasked with integration of multiple signals may contribute to pathology. Glycogen synthase kinase (GSK) 3β is a serine/threonine protein kinase involved in second messenger systems initiated by signals including growth factors, neurotransmitters, and inflammatory cytokines, placing it in a privileged position to sense environmental stimuli and regulate a broad range of cellular functions. Consistent with this, GSK3β is involved in regulation of cell functions including metabolism, morphology, synaptic plasticity, neurogenesis, and apoptosis. As might be expected based on its role in multiple signaling pathways and cellular processes, dysregulation of GSK3β may play a role in many of the negative effects observed following stress exposure, making understanding the function and regulation of GSK3β critically important.

GSK3 was first identified as a protein that negatively regulates glycogen synthase (Embi et al., 1980), though it is now suggested that GSK3 interacts with well over 50 substrates resulting in both positive and negative regulation of substrate activity (Kaidanovich-Beilin and Woodgett, 2011). GSK3 is expressed in two isoforms, GSK3α and GSK3β, which are not redundant. Deletion of the gene encoding GSK3β, but not that encoding GSK3α, is embryonically lethal due to liver hepatocyte apoptosis (Hoeflich et al., 2000). GSK3β is present in peripheral and central tissues, though the brain is particularly rich in GSK3β (Woodgett, 1990). In contrast to the majority of kinases, GSK3β is constitutively active and inhibited by N- or C-terminus phosphorylation (Fig. 1). Activation is achieved through autophosphorylation of tyrosine 216 as GSK3β passes from an intermediate to a mature, active form (Lochhead et al., 2006). Reversible inhibition of GSK3β is achieved by phosphorylation of the N-terminus serine 9 (S^9) site by several kinases, though it is most commonly associated with protein kinase B/AKT (Stambolic and Woodgett, 1994; Cross et al., 1995). S^9 phosphorylation of GSK3β results in a conformational change that competes with substrates for binding site access and therefore limits GSK3β activity (Frame et al., 2001). Reversal of phosphorylation is mediated by protein phosphatase-1 (PP1) (Hernandez et al., 2010). Recently GSK3β was shown to be a substrate of p38 mitogen

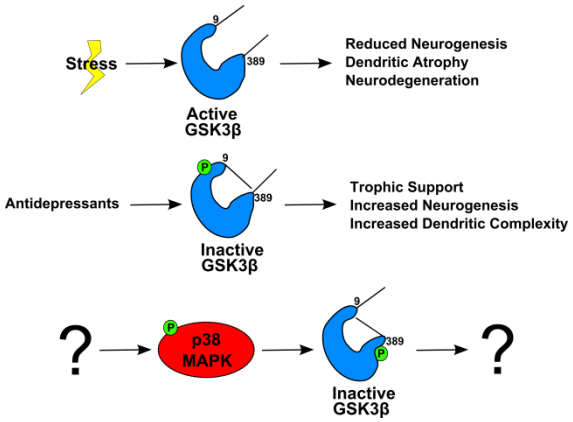

Figure 1. Regulation of GSK3β activity. Stress is proposed to produce neuronal remodeling through GSK3β activation. Antidepressants may produce effects that oppose those of stress by augmenting trophic support through inactivation of GSK3β by S^9 phosphorylation. The upstream signaling, and cellular effects, of a recently described mechanism for GSK3β inactivation by S^{389} phosphorylation are unknown.

activated protein kinase (MAPK) at a novel inhibitory phosphorylation site. p38 MAPK phosphorylation of GSK3β at the C-terminus serine 389 (S^{389}) site was shown to be distinct from AKT mediated phosphorylation at S^{9}, and was demonstrated to be enriched in brain (Thornton et al., 2008). Though convenient, an understanding of GSK3β regulation would be limited by considering only phosphorylation state.

Beyond phosphorylation, regulation of GSK3β activity is achieved by a combination of localization, interaction with protein complexes, and, in many cases, the requirement of substrate priming. This is exemplified in the regulation of β-catenin by GSK3β as part of the canonical WNT signaling pathway. β-catenin regulates aspects of proliferation and cellular plasticity through transcription factor activation (Valvezan and Klein, 2012), and was recently shown to be protective in a social defeat stress model (Wilkinson et al., 2011). GSK3β inhibits the WNT signaling cascade as part of a protein complex that forms between axin, adenomatous polyposis coli, casein kinase-1, and β-catenin. GSK3β is localized to this complex by its association with the cellular scaffolding protein axin and represents a subcellular pool thought to act in isolation from the remaining total fraction of GSK3β in the cell (Ding et al., 2000). In this complex, casein kinase-1 phosphorylation of β-catenin acts as a prime that allows GSK3β to phosphorylate β-catenin, targeting it for destruction (Amit et al., 2002). In the absence of such a prime, or in the presence of WNT ligands, β-catenin is stabilized and can facilitate transcription factor activation (Jho et al., 1999). β-catenin stabilization following activation of the WNT pathway occurs in the absence of changes in inhibitory S^{9} phosphorylation of GSK3β, though a reduction in GSK3β activity is observed (Ding et

al., 2000). Additionally, β-catenin levels are similar in wild type (WT) and mutant animals in which the inhibitory GSK3β S^9 site cannot be phosphorylated (McManus et al., 2005). Further, activation of pathways involving AKT, the prototypical inhibitor of GSK3β, does not produce β-catenin accumulation (Ding et al., 2000; Ng et al., 2009). These findings demonstrate that regulation of GSK3β activity can be achieved by localization within an isolated protein complex, and availability of primed substrates, even in the absence of changes in inhibitory phosphorylation.

The observation that lithium treatment, a mainstay in the treatment of bipolar depression, inhibits GSK3β prompted interest in the therapeutic effects of GSK3β regulation (Klein and Melton, 1996). Further work demonstrated that compounds useful in the treatment of depression and anxiety, selective serotonin reuptake inhibitors and tricyclic antidepressants, also inhibit GSK3β through S^9 phosphorylation (Li et al., 2004). Consistent with the idea that compounds which have therapeutic efficacy may regulate GSK3β, dysregulation of GSK3β has the potential to mediate many of the deleterious effects of stress exposure described above. As a key mediator of apoptotic signaling in peripheral cells and neurons, GSK3β is central to cell survival (Hetman et al., 2002; Beurel and Jope, 2006). However, stress hypotheses that focused on neuronal death as a factor in stress pathology have given way to those suggesting that stress results in regionally distinct changes in neuronal morphology, and reduced neurogenesis, likely through alterations in trophic factor expression (Duman and Monteggia, 2006; McEwen, 2007). GSK3β, through its role in cellular plasticity and neurogenesis, could mediate these aspects of stress exposure.

As described above, stress has been demonstrated to result in regionally distinct alterations in neuronal morphology and synapse number that may underlie stress pathology. There are multiple ways in which GSK3β may play a role in such effects. GSK3β is one of the only serine/threonine protein kinases found to be involved in long term depression (LTD), and also plays a role in long term potentiation (LTP) associated inhibition of LTD (Peineau et al., 2007; Peineau et al., 2009). LTD involves the weakening of AMPAR mediated glutamatergic transmission likely through removal of receptors from the synapse (Collingridge et al., 2010). Inhibition of GSK3β is a critical component in the ~1 hour period after LTP induction in which LTD induction is inhibited (Peineau et al., 2007). In contrast, active GSK3β is necessary for induction of LTD which is achieved through PP1 activation (Peineau et al., 2009). Collingridge and colleagues (Collingridge et al., 2010) propose a "pathological plasticity" process by which loss of GSK3β inhibitory tone impairs AMPA trafficking and, by doing so, may reduce AMPAR mediated glutamatergic transmission as receptors turn over in the synapse, likely through negative regulation of the kinesin cargo system (Du et al., 2010). This "pathological plasticity" would destabilize synapses and could lead to the synaptic reduction observed in chronic stress models.

The "pathological plasticity" proposed by Collingridge (2010) has the potential to provide a GSK3β mediated second hit in stress models through GSK3β and AMPA receptor interactions with BDNF signaling. As noted above, numerous stress protocols have been shown to decrease BDNF expression. In contrast, antidepressant administration and physical activity have been shown to increase BDNF expression.

BDNF signaling has been shown to increase dendritic complexity (McAllister et al., 1997; Niblock et al., 2000), and recent work with mice that conditionally overexpress GSK3β in the dentate gyrus found that GSK3β overexpression reduced dendritic complexity and synapse number (Llorens-Martin et al., 2013). BDNF signaling activates the upstream GSK3β inhibitor AKT and produces activation of the transcription factor cyclic-AMP response element binding protein (CREB) (Bullock and Habener, 1998; Mai et al., 2002; Xia et al., 2010; Jiang and Salton, 2013). This pathway is implicated in cell survival (Mayr and Montminy, 2001), and interestingly BDNF has been proposed to be a target gene of CREB providing for a positive feedback loop whereby BDNF signaling can result in BDNF transcription (Chen and Russo-Neustadt, 2005; Vogt et al., 2014). Within this pathway, CREB is negatively regulated by active GSK3β (Mai et al., 2002; Ahn et al., 2005; Tang et al., 2014) providing a mechanism through which active GSK3β could negatively regulate BDNF levels. The importance of this GSK3β-BDNF interaction is evident in recent work that suggests that the rapid antidepressant effect of ketamine requires inhibition of GSK3β and BDNF release (Beurel et al., 2011; Liu et al., 2012; Liu et al., 2013). GSK3β-mediated negative regulation of AMPA signaling could also impact BDNF levels. AMPA receptor stimulation results in BDNF release and activation of protein synthesis in dendrites (Jourdi et al., 2009), and AMPA mediated release of BDNF was recently shown to provide a mechanism for the antidepressant effect of ketamine (Lepack et al., 2014). Thus, inhibition of GSK3β would in theory maintain AMPA receptors in synapses thus permitting BDNF release, transcription, and associated maintenance of protein synthesis in dendrites. In contrast, over-active GSK3β could not only inhibit BDNF synthesis, but also its release due to inhibition of AMPA

signalling, resulting in the morphological atrophy predicted in "pathological plasticity", and observed in stress models.

GSK3β regulation is also implicated in neurogenesis. There is abundant evidence that stabilized β-catenin increases proliferation and neurogenesis in the brain providing one avenue by which GSK3β activity could reduce new neuron number (Valvezan and Klein, 2012). Further, growth factor signaling through IGF-1 and BDNF promote neurogenesis and signaling associated with both factors produces inhibition of GSK3β (Mai et al., 2002; Llorens-Martin et al., 2009). The neurogenesis promoting effect of lithium (Chen et al., 2000; Boku et al., 2009; Schaeffer et al., 2014), fluoxetine (Malberg et al., 2000; Santarelli et al., 2003), and leptin (Garza et al., 2012) occur on a background of GSK3β inhibition (Klein and Melton, 1996; Li et al., 2004). In contrast, active GSK3β seems to inhibit neurogenesis. Inflammatory signaling involving the transcription factor Nuclear factor-kB (NF-kB), which is positively regulated by GSK3β, reduces neurogenesis (Koo and Duman, 2008; Koo et al., 2010). Mice with an alanine to serine substitution in both GSK3α and GSK3β that render the proteins incapable of N-terminus inhibition have reduced neurogenesis (Kondratiuk et al., 2013) and are not responsive to the neurogenesis promoting effects of fluoxetine (Eom and Jope, 2009). Inhibition of GSK3β by S^{389} phosphorylation may also play a role in neurogenesis. In vitro analysis demonstrated that hippocampal stem cell proliferation was augmented by GSK3β S^{389} phosphorylation that resulted in β-catenin stabilization (Zhang et al., 2011). Collectively, the research points to GSK3β inhibition by S^{9} or S^{389} phosphorylation as promoting

neurogenesis likely through stabilization of β-catenin as well as through growth factor pathways.

Numerous genetic manipulations have been employed to gain insight into the function GSK3β activity on behavior. Complete knock-out of GSK3β results in inviable offspring (Hoeflich et al., 2000). However, heterozygous mice (GSK3β HET) containing a single copy of GSK3β, and thus reduced GSK3β expression, have been generated. O'Brien et al. (2004) report that GSK3β HET mice and WT controls display similar activity, acoustic startle response, and zero maze behavior. However, GSK3β HET mice show reduced immobility in the forced swim test suggesting an anti-depressive phenotype (O'Brien et al., 2004), though subsequent work failed to replicate this phenotype (Bersudsky et al., 2008). Beaulieu (2008) found similar immobility times in the tail suspension test when comparing GSK3β HET mice to WT controls, and reduced latency to enter the light compartment of a light dark box, a phenotype that suggests reduced anxiety. Taken together these results suggest that downregulating GSK3β expression without modulating phosphorylation state produces and inconsistent phenotype, though suggestions of reduced anxiety and depression-like behavior are present.

Models that result in increased GSK3β levels or decreased phosphorylation produce similarly complex results. O'Brien et. al (2011) reported no change in open field behavior, zero maze open arm time, or forced swim immobility time in mice that overexpress GSK3β, though lithium treatment failed to produce an antidepressant effect in mice that overexpressors GSK3β . In mice that conditionally overexpress GSK3β in

the hippocampus and frontal cortex, no change in anxiety behaviors was observed, though object recognition impairment was evident along with dorsal hippocampal cell death (Fuster-Matanzo et al., 2011). Mutant animals with GSK3β rendered constitutively active through alanine substitution at the S^9 site are more active in an open field, have elevated acoustic startle amplitude, and reduced forced swim immobility time (Prickaerts et al., 2006). Notably, these mice were observed to have upregulated AKT and BDNF, suggesting a compensatory response to increased active GSK3β. Mice with alanine substitutions rendering GSK3β and the GSK3α isoform constitutively active are reported to be more active in an open field, spend more time immobile in forced swim and tail suspension tests, spend reduced time in the open arms of an elevated plus maze, display increased contextual fear conditioning, and be more susceptible to foot shock induced learned helplessness, though it is unclear what the contributions of the individual isoforms are to this phenotype (Polter et al., 2010). Notably, these animals also fail to produce LTD, a finding that would not be predicted by work that suggested active GSK3β is critical to LTD induction (Peineau et al., 2009). Isoform specific constitutive activation was reported by the same group to have no effect on contextual or tone fear conditioning (Polter et al., 2012), and others have reported increased activity, reduced anxiety, decreased susceptibility to stress effects, and reduced forced swim immobility time in the combined GSK3β/GSK3α-active mutants (Ackermann et al., 2010).

While informative, it is important to note that as a molecule with a complex role in development (Kim and Snider, 2011) genetic manipulations of GSK3β that globally affect its regulation may not produce animals that reflect the complexity of GSK3

function in a mature WT animal. Together, results from gain of function, and reduced function, mutations suggest that GSK3β regulation may impact behaviors affected by stress. To date, no studies have been conducted on animals with alternative regulation of the inhibitory GSK3β S^{389} phosphorylation site. Inclusion of GSK3β S^{389} manipulations into the above results may provide a clearer picture of how GSK3β affects behavior as GSK3β S^{389} phosphorylation could also be involved in changes observed in GSK3β HET animals, and GSK3β overexpressors.

Given the role GSK3β plays in cellular plasticity, it is reasonable to predict that stress would alter GSK3β activity. There have been few studies assessing the effects of stress or exercise on GSK3β, and none that include GSK3β S^{389} phosphorylation. No change in GSK3β levels were observed in frontal cortex tissue lysates after 1, 6, or 14 days of one hour cold restraint stress (Kozlovsky et al., 2002). Ten days of 4 hour restraint that produced a depression-like behavioral phenotype did not change GSK3β protein levels or GSK3β S^{9} phosphorylation in the hippocampus (Luo et al., 2015). However, a separate group demonstrated that three weeks of six hour per day restraint reduced GSK3β S^{9} phosphorylation and BNDF levels in the hippocampus, and treatments associated with trophic support, olanzapine and aripiprizole, ameliorated the effects of stress (Park et al., 2011). In a prenatal restraint stress model, in which pregnant dams are stressed from day 14 of pregnancy to parturition, offspring had increased GSK3β in the frontal cortex while S^{9} phosphorylation was reduced, effects not observed in hippocampal lysates (Szymanska et al., 2009). Interestingly, the authors found no change in total GSK3β or S^{9} phosphorylation in WT rats following three weeks of anti-depressant

treatment, a manipulation previously shown to acutely regulate S^9 phosphorylation (Li et al., 2004). However, anti-depressant treatment was shown to normalize frontal cortex GSK3β levels and forced swim immobility time in stressed rats, again without affecting hippocampal protein levels (Szymanska et al., 2009). Forced swim exposure did not change GSK3β protein or S^9 phosphorylation levels in the frontal cortex sampled 24 hours after swim. However, 14 days of repeated swim stress increased GSK3β protein and reduced S^9 phosphorylation concurrent with increased depression-like behavior (Chen et al., 2012). Others have shown that a single forced swim exposure increased GSK3β inhibition, though it is unclear at what time after stress the samples were obtained (Courousse et al., 2014). In a learned helplessness paradigm mice given 180 mild (0.3mA) footshocks and tested in a shuttle box escape procedure 24 hours later displayed increased escape latency and reduced GSK3β S^9 phosphorylation (Polter et al., 2010). A single 1.5mA footshock produced contextual fear 28 days after administration, and was associated with elevated GSK3β S^9 phosphorylation in the amygdala 42 days after its administration, hippocampal phosphorylation levels were unchanged (Dahlhoff et al., 2010). Social defeat stress negatively regulated GSK3β S^9 phosphorylation, however this was only observed in animals that were susceptible to the defeat paradigm (Wilkinson et al., 2011). Studies employing variate stress procedures are fewer than those utilizing repeated or acute stress. A 14 day variate stress paradigm was shown to increase GSK3β mRNA in the hippocampus and reduce markers of neurogenesis, along with increasing depression-like behavior, and these effects were reduced by treatment with lithium or a GSK3β inhibitor during the stress paradigm (Silva et al., 2008). However, a second group demonstrated that hippocampal GSK3β phosphorylation was unchanged after 8

weeks of variate stress that produced a depression-like phenotype, though a robust change in S^9 phosphorylation was observed after a single stressor (Courousse et al., 2014).

Though not entirely consistent, the results from acute and repeated stressor studies described above suggest that stress may reduce GSK S^9 phosphorylation, or increase protein levels, or both, in a brain region specific fashion. Such regulation would permit greater GSK3β activity. Consistent with this, manipulations that would be expected to limit GSK3β activity during stress exposure seem to protect against the effects of stress. For example, conditional manipulations that increased GSK3β inhibition (Silva et al., 2008; Wilkinson et al., 2011) or reduced protein levels (Omata et al., 2011) prior to stress protected against the behavioral and physiological effects of stress exposure. Also, variate stress in animals conditionally overexpressing GSK3β in the dentate gyrus was associated with increased depression-like behaviors and markers of neuronal degradation (Zhang et al., 2013). It is noteworthy that unlike inhibition during stress exposure, post stress GSK3β inhibition did not alter stress-associated behavioral changes (Wilkinson et al., 2011; Ma et al., 2013). This finding is consistent with the multiple roles of GSK3β as a second messenger regulating the cellular response to diverse signals. Altering GSK3β tone during stress exposure might be expected to change the outcomes of stress, as the GSK3β manipulation would be augmenting or inhibiting the stress associated signals. However, GSK3β manipulation would have no effect on those outcomes if the manipulation was employed after stress exposure, as the change in activty would not coincide with stress associated signalling. For example, the rapid antidepressant effects

of ketamine require GSK3β inhibition (Beurel et al., 2011; Liu et al., 2013) and remedy behavioral and physiological effects of stress exposure (Li et al., 2010; Ma et al., 2013), but GSK3β inhibition alone does not have rapid beneficial effects. In this case, GSK3β inhibition alone does not interact with a signal that produces beneficial effects, but when second messenger systems are activated by ketamine GSK3β can impact the outcome of those signals.

Research into the effects of exercise on GSK3β regulation is more limited than that on stress. The sole studies involving voluntary exercise demonstrated that the molecular pathway involved in BDNF signaling was activated following two weeks of exercise. However, a non-significant increase in GSK3β phosphorylation was observed (Chen and Russo-Neustadt, 2005). Hippocampal lysates from rats forced to run on a treadmill for 15 minutes on five days had increased BDNF and GSK3β S^9 phosphorylation levels, and 5 days of running during a 7 day restraint stress paradigm ameliorated restraint induced decreases in BDNF and GSK3β S^9 phosphorylation (Fang et al., 2013). Rats trained to run on a treadmill for 3 hours per day over 36 weeks starting at 5 weeks of age, as well as those that were simply handled during that time, had increased IGF-1 levels and reduced GSK3β tyrosine 216 phosphorylation in the hippocampus, interpreted as reduced active GSK3β (Bayod et al., 2011). Using the same procedure, the authors replicated their GSK3β activation finding in exercised and handled rats but did not observed the reduction in GSK3β tyrosine 216 phosphorylation to be associated with increased β-catenin accumulation in the hippocampus (Bayod et al., 2014). Results from treadmill and voluntary exercise should be compared cautiously as

the manipulations may not produce comparable results (Burghardt et al., 2004). However, given the sparse research on the regulation of GSK3β by exercise, integration of these findings with those suggesting exercise increases growth factor signaling suggests that exercise may be expected to increase inhibition of GSK3β in brain regions such as the hippocampus where increases in BDNF (Neeper et al., 1996; Russo-Neustadt et al., 2000; Cotman and Engesser-Cesar, 2002; Adlard and Cotman, 2004) and IGF-1 (Trejo et al., 2008; Llorens-Martin et al., 2010; Cetinkaya et al., 2013) are observed following exercise. If, as described above, active GSK3β during stress augments stress signaling, increased inhibitory tone as a result of exercise may be expected to limit stress signaling.

Aims of the Following Studies

As a multifunctional protein kinase GSK3β has the ability to act upon many cellular signals and regulate cellular activity in ways that promote or degrade cell health. Stress studies suggest that GSK3β may be negatively impacting cell health during stress exposure, while studies in exercising animals suggest that exercise may produce a context in which greater inhibitory tone over GSK3β activity may be present. Given the opposing effects of stress and exercise on cellular plasticity in brain regions that regulate the response to stress, studies that expand our understanding of the regulation of GSK3β by stress or exercise are warranted. Additionally, there have been no studies examining the role of GSK3β S^{389} phosphorylation in the brain. As GSK3β S^{389} phosphorylation has the ability to regulate β-catenin, and is itself regulated by p38 MAPK, a kinase associated with stress signaling, understanding the effects of S^{389} phosphorylation may open new avenues in treatment of stress-associated pathologies.

The first aim of the research was to expand the understanding of GSK3β S^{389} phosphorylation by examining its cellular localization, regulation of GSK3β expression, and impacts on behavior. This foundational work demonstrates that, as opposed to the cell wide distribution of GSK3β S^{9} phosphorylation, S^{389} phosphorylation is primarily limited to the nucleus of the cell, where it may limit the activity of a nuclear pool of GSK3β that has previously been associated with the pro-apoptotic effects of DNA damaging agents (Watcharasit et al., 2002). Consistent with a protective role of GSK3β S^{389} phosphorylation, restricted neurodegeneration was observed in the hippocampi and cortex of animal generated to produce deficient S^{389} phosphorylation. Behavioral testing in animals with deficient GSK3β S^{389} phosphorylation produced a phenotype that was largely similar to WT animals. However, a fear conditioning phenotype that included exaggerated and overgeneralized fear upon testing suggests that the degeneration observed may be impacting a neuronal population that regulates this phenotype that is commonly observed in stress associated disorders, and rodents subjected to stress exposure.

The second aim of the research was to further our understanding of the regulation of GSK3β by stress and voluntary exercise exposure. Inhibitory phosphorylation of GSK3β was examined in stress associated brain regions from mice subjected to variate stress or voluntary exercise. Stress and exercise were found to similarly regulate GSK3β S^{9} phosphorylation, both positively and negatively, in a regionally specific fashion. However, GSK3β S^{389} phosphorylation was observed to increase in brain regions where changes were evident. Importantly, levels of p38 MAPK phosphorylation, which would be expected to increase GSK3β S^{389} phosphorylation, were increased in brain regions demonstrating increased GSK3β S^{389} phosphorylation. Consistent with the protective role of GSK3β S^{389} phosphorylation that was

postulated in the first study, increased GSK3β S^{389} inhibitory tone was associated with increased levels of H2A-variant-X (ϒH2A.X) phosphorylation, a marker of DNA damage.

Together these studies demonstrate that GSK3β S^{389} phosphorylation is likely playing a role in cellular function that is separable from S^{9} phosphorylation. Thus, regulation of GSK3β activity by S^{389} phosphorylation may be an important component of cellular plasticity observed following stress and exercise. Future studies are necessary to understand the signals driving changes in GSK3β signaling following stress and exercise, and cell types negatively impacted by deficient GSK3β S^{389} phosphorylation. Although foundational in nature, these studies provide an exciting avenue for future research. One of the main difficulties of targeting kinases such as GSK3β for therapeutic effect is the propensity to impact multiple pathways through overgeneralized inhibition or activation. Given the differences observed between GSK3β S^{389} and S^{9} regulation, and distinct cellular distributions of the phosphorylation sites, targeting GSK3β S^{389} phosphorylation may provide an avenue to limit stress signaling.

CHAPTER 2: DEFICIENCY IN GSK3β SERINE 389 PHOSPHORYLATION IS ASSOCIATED WITH NEURODEGENERATION AND PROLONGED FEAR RESPONSE

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Abstract

Glycogen synthase kinase-3β (GSK3β) plays an essential role in mediating cell death and is emerging as a potential target for neurological diseases. Previous work has identified a nuclear accumulation and activation of GSK3β in neurons in response to apoptotic stimuli. The traditional means of inhibiting GSK3β through serine-9 (S9) phosphorylation was not regulated in these models suggesting a separate means of inhibiting nuclear GSK3β may promote survival after cellular stress. Here, we identify that inhibitory regulation of GSK3β by the recently identified serine-389 (S^{389}) phosphorylation site is phosphorylated independently of S^{9} phosphorylation. GSK3β S^{389} is primarily expressed in the nucleus, and the two phosphorylation sites limit the activity of distinct pools of GSK3β. Mutation of S^{389} to Ala prevents inactivation of this nuclear pool of GSK3β. Consistent with a role in cell survival, we show that GSK3β Ser^{389} Ala knockin (KI) mice undergo focal neurodegeneration in limbic brain regions. A fear conditioning phenotype that included overgeneralization of contextual fear and increased tone fear, was observed in GSK3β KI mice in spite of the observed neurodegeneration. This phenotype is similar to that observed in stress pathology. Thus, regulation of GSK3β through S^{389} phosphorylation may provide a unique avenue in the treatment of neurodegenerative and stress associated disease.

Introduction

Glycogen synthase kinase (GSK)-3 is a constitutively active serine threonine protein kinase that has emerged as a critical mediator of the balance between cell survival and death (Beurel and Jope, 2006). GSK3 is present in two constitutively active non-reduntant isoforms, GSK3α and GSK3β (Hoeflich et al., 2000), that are inhibited by phosphorylation (Stambolic and Woodgett, 1994). GSK3 is widely expressed, but levels are greatest in the central nervous system (Woodgett, 1990). Consistent with its role in cell survival and expression, GSK3, particularly GSK3β, has been implicated in numerous disorders that are associated with neurodegeneration including Alzhiemer's disease (Cai et al., 2012) and depression (Jope and Roh, 2006). A better understanding of GSK3 regulation will enhance efforts towards therapeutic interventions involving GSK3 inhibition.

GSK3 inhibition is achieved through N-terminus serine 9 (S^9), and serine 21 (S^{21}) phosphorylation of GSK3β and GSK3α respectively, primarily by AKT (Cross et al., 1995). Multiple second messenger cascades associated with cell survival and proliferation are promoted by GSK3 inhibition (Jope and Johnson, 2004). Recently, a novel mechanism for GSK3β inhibition by p38 mitogen activated protein kinase (MAPK) was demonstrated to be independent of AKT and GSK3α (Thornton et al., 2008). p38 MAPK inhibits GSK3β through phosphorylation of serine 389 (S^{389}) in the C-terminus (threonine 390 (T^{390}) in humans). The function of GSK3β S^{389} phosphorylation remains unclear, though a robust S^{389} phosphorylation signal in brain, as opposed to S^9

phosphorylation which is ubiquitously expressed, suggests that S^{389} phosphorylation may serve a specialized function in the brain.

In neuronal cultures GSK3β levels and activity have been observed to increase in the nucleus in response to diverse cellular stressors including DNA damage (Bijur and Jope, 2001; Watcharasit et al., 2002; Bijur and Jope, 2003). Additionally, hyperactivation of GSK3β in neuronal cells increases cell death in response to pro-apoptotic stimuli (Bijur et al., 2000). Because nuclear changes in GSK3β have been observed to be independent of S^{9} regulation these data suggest an alternate mechanism for nuclear inhibition of GSK3β (Bijur and Jope, 2001; Watcharasit et al., 2002). p38 MAPK levels have also been observed to increase in the nucleus following signals that induce DNA damage (Wood et al., 2009). Together these findings suggest that p38 MAPK mediated inhibition of GSK3β by S^{389} phosphorylation, particularly in the nucleus, may be an unexplored mechanism mediating cell survival following stress.

Here we demonstrate that GSK3β S^{389} is present throughout the brain, and has a nuclear expression pattern that is distinct from S^{9} phosphorylation. Additionally, we show that GSK3β S^{9} phosphorylation is absent when S^{389} phosphorylation is present, and that both sites negatively regulate an independent component of GSK3β kinase activity. Further, we demonstrate that hippocampal and cortical degradation is present in a novel mutant mouse in which the GSK3β S^{389} site was mutated to an alanine, thus blocking inhibition of GSK3β through the C terminus (GSK3β KI). Finally, we assess anxiety and depression-like behavior, spatial learning, acoustic startle, and fear conditioning in GSK3β KI mice and demonstrate a behavioral phenotype that includes augmented

auditory fear conditioning despite the degradation observed in brain areas critical for the task.

Materials and Methods

Subjects

For the generation of GSK3β KI mice, a targeting vector constructed (InGenious Targeting Laboratory, Inc.) from a 9.27kb region was subcloned from the C57BL/B6 BAC clone (RPCI-23: 454D5) into the vector backbone (2.4kb pSP7 (Promega)). The long homology arm extended 6.36kb 5' to the T-G point mutation in exon 11 and the LoxP/FRT flanked Neo cassette was inserted 511bp 3' to the T-G point mutation. The short homology arm extended 2.40 kb 3' to the loxP flanked Neo cassette. The targeting vector was constructed using Red/ET recombineering technology and the T-G point mutation was created by overlap PCR. The linearized targeting vector electroporated into BA1(C57BL/6 x 129/SvEv) hybrid embryonic stem cells. Following G418 selection, surviving clones were screened by PCR to identify homologous recombinant clones for injection into blastocysts. The neomycin cassette was excised by breeding heterozygous GSK3β KI mice with EIIa-cre mice (Lakso et al., 1996) (Jackson Laboratories). GSK3β KI mice were backcrossed to C57BL/6 at least seven generations. Wildtype mice (WT) were purchased from Jackson Laboratories (Bar Harbor, ME).

Prior to experimental manipulation, mice were housed in groups of 4 in acrylic, wire top cages in an Association for Assessment and Accreditation of Lab Animal Care (AAALAC) approved animal facility and provided a 7 day acclimation period. A 12

hour light/dark cycle (0700h-1900h) and ad libitum access to food and water were maintained throughout experimental procedures. All procedures were approved by the University of Vermont Animal Care and Use Committee.

Behavioral Tasks

A cohort of male and female WT and GSK3β KI mice (n=5 females, 5 males per genotype) were examined in the behavioral tasks described below. The order of testing was acoustic startle, open field, zero maze, auditory fear conditioning, terminating with forced swim. A separate cohort was used for the water maze experiment (n=8 females, 7 males per genotype).

Open Field and Zero Maze

To examine locomotion and anxiety like behavior the open field and zero maze were utilized. In the open field, anxiety is measured as avoidance of the center portion of the arena. Individual mice were placed in the open field apparatus (44cm X 39cm opaque acrylic container) located in a room that was dimly lit so as not to amplify anxiety-like behavior. Mice were placed into a corner of the open field and their behavior recorded on video over 10 minutes. Total distance traveled, frequency of crosses into the center of the open field and time in the center of the open field were analyzed with EthoVision software (Noldus Information Technology Inc, Leesburg, VA).

The zero maze is a circular track with opposing walled quadrants. Anxiety-like behavior is represented by time spent in the walled quadrants of the maze. Open quadrant time and entries were quantified over a 5 minute period on the zero maze (51cm

diameter, 7cm track width, elevated 36cm, track width). Mice were placed into a closed quadrant of the zero maze to begin the test. Due to issues with video contrast, zero maze analysis was hand scored. An open quadrant entry was counted only when all four paws of the animal entered the quadrant.

Water Maze

The water maze task is a spatial learning task that requires the hippocampus (Morris et al., 1982). The water maze was a circular galvanized steel tank measuring 1-m in diameter and 61-cm in depth and filled with clear water (22^0 C) to a level of 44 cm (17 cm below the tank rim). The clear acrylic platform (18 cm square) was 0.25 cm below the surface of the water and was located in a fixed position throughout maze training. Mice (n=8 females, 7 males per genotype) were given 4 training trials each day for 8 days. For each training trial, the mouse was placed into the water in one of the three quadrants not containing the platform. A trial ended when the mouse successfully climbed on the platform or after 30 seconds of swimming, following which the mouse was guided to the platform. The interval between trials was 3 minutes. Probe trials, in which the platform was removed from the pool, were carried out after training days 3 and 8. For probe trials, the mouse was placed into the quadrant opposite to the one that formerly contained the platform and allowed to swim for a total of 30 seconds. For maze acquisition and probe trials, swimming was video recorded for offline analysis using EthoVision software.

Forced Swim

The forced swim procedure is a regularly used test of depression-like behavior. Mice were placed into room temperature (~20°C) containers (30cm diameter) for 6 minutes and assessed for immobility. Containers were filled to a depth that would not allow the tail of the mouse to touch the bottom of the container. Immobility was defined as the absence of motion, except that necessary to stay afloat. Immobility was scored using a time sampling procedure in which a mobility assessment was made every 6 seconds during the 6 minute test. Scoring was conducted by a pair of observers and averaged to derive an immobility count for each animal, with greater immobility counts indicative of increased depressive behavior.

Acoustic startle

Acoustic startle was used to assess anxiety following stress and exercise. In acoustic startle testing a brief noise burst is presented and the animal's response is measured. The magnitude of the startle response is interpreted as an index of the animal's anxiety with greater startle responses indicating a more anxious phenotype. Startle was assessed as described previously (Salam et al., 2009). Briefly, mice were placed individually in an acoustic startle chamber (Med-Associates, St. Albans, VT) located within a sound attenuating cubicle and administered 30, 20msec duration, startle-eliciting noise bursts (10 each at 95, 100 and 105 dB) in a pseudo-random order at a 1-minute inter stimulus interval. The average startle amplitude over the 30 trial test is reported.

Meta-chlorophenylpiperazine (mCPP) challenge (0.3mg/kg i.p., Sigma Aldrich, St. Louis, MO) was conducted in a counterbalanced fashion over two days as described previously (Hare et al., 2012). The change in startle amplitude following mCPP administration is reported with positive values representing an increase in acoustic startle.

Auditory Fear Conditioning

Auditory fear conditioning utilizes a tone conditioned stimulus paired with a shock unconditioned stimulus to condition fear, measured by the freezing response, to both the context paired with shock as well as the conditioned stimulus. These components of the fear response are then disambiguated by testing in the training context without tone, and a novel context where the tone is presented. Auditory fear conditioning was carried out in an acrylic conditioning chamber with a metal grid rod floor located in a darkened sound-attenuating cubicle (Video Fear Conditioning System MED-VFC-NIR-M, Med-Associates, St. Albans, Vermont) scented with the odor of Vicks Vaporub. Conditioned freezing was measured in both the conditioning chamber as well as a novel chamber. The novel chamber had walls of a different color than the training chamber, was illuminated, was scented with anise extract, and had a flat plastic floor. Time spent freezing was measured with Video Freeze software (Med-Associates, St. Albans, Vermont) following the recommendations of Anagnostaras et. al. (2010). For fear conditioning, an individual mouse was placed in the conditioning chamber and given a two minute baseline period during which no stimuli were presented. Following baseline, mice were given five tone presentations (75dB, 4500Hz) separated by 30 seconds. Each tone co-terminated with a 1 second 0.3mA foot shock delivered through the grid floor.

Conditioned freezing was measured in two, counterbalanced, six minute tests begun 24 hours after fear conditioning and separated by 24 hours. For measurement of contextual fear conditioning, mice were placed into the training context and no stimuli were presented. Assessment of conditioned fear to the tone was conducted in a novel context. The novel context test consisted of a two minute baseline followed by a three minute tone presentation. A one minute period followed the tone presentation to complete the six minute novel context test.

Auditory Fear Conditioning p38 MAPK inhibitor experiments

To assess the role of p38 MAPK signaling in auditory fear conditioning, the p38 MAPK inhibitor SB203580 (Tocris Bioscience, Bristol, UK) was centrally administered prior to, and following, auditory fear conditioning. WT mice were implanted with intracerebroventricular (ICV, 1mm lateral, 0.4mm posterior, 3mm depth, 26 gauge) cannula under isoflurane anesthesia and allowed seven to ten days to recover prior to fear conditioning. Mice were injected with 2.5µL of vehicle (0.9% saline) or 125 µM SB203580 over 3 minutes, 30 minutes prior to (n=10 males/group), or immediately following (vehicle n=10 males, SB203580, n=8 males) fear conditioning. The auditory fear conditioning protocol followed the same method as above except that the shock intensity was increased to 0.5mA as minimal conditioning was observed in pilot experiments conducted with cannulated mice using a 0.3mA shock intensity. Following conditioning, cannula placements were visually confirmed with ICV cresyl violet injection delivered under anesthesia. In order to assess inhibition of p38 MAPK tissue from a subset of animals was harvested 30 minutes after a second injection of SB203580

injection (n=5 per group) administered 3 days after the final fear conditioning test. p38 MAPK inhibition was measured by analyzing the phosphorylation state of GSK3β S^{389}. p38 MAPK phosphorylates GSK3β S^{389} (Thornton et al., 2008). As such, reduced S^{389} phosphorylation would indicate that p38 MAPK was inhibited. GSK3β is rapidly dephosphorylated by anesthetics and long pre-cooling post mortem intervals (Li et al., 2005). As such, mice were rapidly decapitated and tissue was rapidly harvested (< 3 minutes) and immediately transferred to centrifuge tubes cooled on dry ice prior to storage at -80°C. Coronal slices were obtained using a 1mm brain matrix (Stoelting, Wood Dale, IL). The amgydala (approximate bregma coordinates: anteroposterior -1.06; mediolateral 2.75; dorsoventral -4.5), and whole hippocampus (approximate bregma coordinates: anteroposterior -2.06; mediolateral 1.0; dorsoventral -2.0) were extracted with a 1mm tissue punch (Stoelting, Wood Dale, IL).

Western blot analysis

Whole cell extracts were prepared in Triton lysis buffer and used for Western blot analysis as we previously described (Derijard et al., 1994; Rincon et al., 1997). Nuclear and cytosolic extracts were prepared as previously described (Schreiber et al., 1989; Tugores et al., 1992). For co-immunoprecipitation, nuclear extracts were prepared as previously described (Jamil et al., 2010). ExactaCruz F immunopreciptiation kit (Santa Cruz Biotechnology) was used for immunoprecipatiation/Western blot analysis with rabbit antibodies. Anti-actin, anti-GAPDH, and anti-GSK3β were purchased from Santa Cruz Biotechnology. Anti-phospho-S^{9} GSK3β was purchased from Cell Signaling (Danvers, MA). Anti-phospho-S^{389} GSK3β, and anti-TUJ1 was purchased from

Millipore (Billerica, MA). Anti-phospho-Thr^{390} GSK3β rabbit polyclonal antibody was generated (Proteintech, Inc.) using N-C-ARIQAAASphospho-TPTNATA for immunization. Anti-rabbit HRP and anti-mouse HRP (Jackson ImmunoResearch Laboratories) and anti-goat-HRP (Santa Cruz Biotechnology) were used as secondary antibodies.

GSK3β Kinase Activity Assays

To determine whether S^{389} phosphorylation regulated a distinct component of GSK3β activity, kinase assays were conducted. GSK3β was immunopreciptitated from cerebral cortex protein lysates and kinase activity was determined as previously published (Thornton et al., 2008).

Immunolabeling

The primary antibodies used included rabbit anti-phospho-S^{389} or anti-phospho-T^{390} GSK3β polyclonal antibodies described above. The secondary antibody used was highly adsorbed anti-rabbit Alexa Fluor 568 (Invitrogen). Topro-3 (Invitrogen) was used as a nuclear stain. Images of cells were acquired on a Zeiss LSM-510. Staining of human tissue was performed as previously described (Long et al., 2011). Paraffin-embedded sections of mouse brain were similarly stained except that Sudan Black blocking was excluded. Anti-NeuN (Millipore), anti-TUJ1, and anti-phospho-Ser^{389}/Thr^{390} GSK3β antibodies were used in conjunction with Cy3-conjugated secondary antibodies (Jackson ImmunoResearch). Images were acquired with identical exposure settings using a SPOT

RT digital camera. Fluro-Jade C (Millipore) staining was performed according to manufacturer's instructions.

Statistical Analysis

Data are expressed as means ± standard error of the mean. Brain regions were run on separate blots and analyses were restricted to within brain regions. Western blot analysis following SB203580 administration was evaluated by independent samples t-tests. Behavioral analysis utilized mixed model ANOVAs followed by LSD protected t-tests. Initial analysis included sex. However, only in the mCPP challenge was a sex difference evident and here the sex difference was not dependent on genotype and therefore sex was left out of the analysis that follows. Results were analyzed by SPSS software version 22 (IBM; Armonk, NY). $p < 0.05$ was considered statistically significant.

Results

GSK3β phosphorylation at S^{389} is primarily nuclear and present in all brain regions examined.

Previous research suggests that, unlike the broad tissue distribution of S^{9} phosphorylation of GSK3β, S^{389} phosphorylation of GSK3β is restricted primarily to the brain with a lower levels detected in the thymus and spleen (Thornton et al., 2008). To determine the distribution of phospho-S^{389} within the brain, western blot analysis was performed using lysates from a number of brain regions. Both phospho-S^{389} GSK3β and phospho-S^{9} GSK3β were detected at similar levels in all regions examined (Fig. 1A).

Immunofluorescent staining of the cerebral cortex revealed a distinct subcellular localization of phospho-S^{389} compared to phospho-S^{9} GSK3β. Phospho-S^{389} GSK3β exhibited a punctate nuclear staining pattern while phospho-S^{9} GSK3β was more diffuse and appeared to be more abundant in the cytosol than the nucleus (Fig. 1B). Immunoflorescent staining of postmortem human brain tissue revealed a primarily nuclear staining for phospho-T^{390} GSK3β (human equivalent of mouse S^{389}, Fig. 1C). To further explore the cellular localization of phospho-S^{389} GSK3β, we examined the subcellular distribution of phospho-S^{389} GSK3β in brain. Nuclear and cytosolic extracts from cerebral cortex were examined by Western blot analysis. Phospho-S^{389} GSK3β was also predominantly found in the nucleus of murine cerebral cortex samples whereas phospho-S^{9} staining was found in nuclear and cytosolic compartments (Fig. 1D).

GSK3β S^{389} phosphorylation is not detectable when S^{9} phosphorylation is present, and restrains a distinct pool of GSK3β activity.

To address whether phosphorylation on GSK3β S^{389} was just an additional pathway to further inactivate the same pool of GSK3β, or whether these two mechanisms were targeting different GSK3β pools, we performed co-immunoprecipitation analysis. Whole cell extracts from cerebral cortex were used to immunoprecipiate phospho-S^{9} GSK3β and both the immunoprecipitate and flow-through were examined by Western blot analysis. As expected, phospho-S^{9} was abundantly present in the immunoprecipitate but not in the flow-through (Fig. 1E). In contrast, phospho-S^{389} was not detected in the immunoprecipitate, but was abundantly present in the flow-through (Fig. 1E). Analysis of total GSK3β revealed that only a fraction of GSK3β was actually phosphorylated on S^{9}

(Fig. 1E). Thus, phosphorylation of GSK3β on S^{389} targets an independent pool of GSK3β in the brain that is not phosphorylated on S^{9}. Together, these results indicate that phosphorylation of GSK3β at Ser^{389} (human Thr^{390}) occurs independently of phosphorylation on S^{9} to reduce nuclear GSK3β activity in the brain.

To investigate the role of this alternative pathway for GSK3β inactivation in the brain, we examined GSK3β KI mice where S^{389} of GSK3β was replaced with Ala to prevent phosphorylation of GSK3β at S^{389} (Fig. 2A). Western blot analysis confirmed the absence of phospho-S^{389} GSK3β in whole brain extracts from homozygous GSK3β KI mice while normal levels were present in heterozygous GSK3β KI mice (Fig. 2B). S^{9} phosphorylation of GSK3β was not affected by the Ser to Ala mutation, further demonstrating that these two alternative pathways are independent of each other. To show that phosphorylation on S^{389} contributes to restrain the kinase activity of GSK3β in brain, GSK3β kinase activity in cerebral cortex from WT and GSK3β KI mice was measured. Despite the already high kinase activity, a significant increase in kinase activity in the cortex of GSK3β KI mice was detected ($t_{(9)}$=2.46, p<.05; Fig. 2C). As control, we corroborated that the levels of phospho-S^{9} in the cerebral cortex of GSK3β KI mice were not decreased (Fig. 2C). Thus, phosphorylation of GSK3β on S^{389} contributes to inactivation of GSK3β kinase activity in brain and it is not compensated by phosphorylation on S^{9}.

Restricted cellular degradation is evident in mice that cannot inhibit GSK3β through S^{389} phosphorylation

Previous research has demonstrated nuclear accumulation of activated p38 MAPK in response to DNA damage (Wood et al., 2009). Following DNA damage, active GSK3β forms a complex with the pro-apoptotic factor p53 that may promote inhibition of pro-survival transcription factors (Watcharasit et al., 2002). Recent work has shown the presence of spontaneously generated DNA damage in some neurons diffusely distributed in the cerebral cortex and hippocampus as a result of normal brain activity (Suberbielle et al., 2013). Additionally, failure to repair DNA damage is thought to play a key role in neuronal degradation (Brasnjevic et al., 2008). Thus, preventing inactivation of nuclear GSK3β through serine to alanine substitution at GSK3β S^{389} in KI mice could impact the viability of the neuronal subset with spontaneous DNA damage.

No discernible differences in gross brain anatomy were observed between GSK3β KI mice and WT mice. Immunostaining analysis with NeuN and class III β-tubulin/Tuj1, two standard markers for neurons, did not show drastic differences in the number of neurons within the cerebral cortex and hippocampus of WT and GSK3β KI mice (Fig. 3A). However, histological examination of hematoxylin & eosin (H & E) stained sections revealed the presence of highly eosinophilic (darker) cells with pyknotic nuclei in some regions of the cerebral cortex and hippocampus of GSK3β KI mice (Fig. 3B). The morphology of these cells is characteristic of what in human brain pathology is defined as "dark neurons" and likely represent neurodegenerative cells (Garman, 2011). The presence of these dark cells in GSK3β KI mice was evident in the hippocampal CA1 subfield and the hippocampal CA3 subfield as well as cerebellar cortex (Fig. 3B) and dentate gyrus (not shown). A number of studies have reported that hippocampal CA1 is

particularly vulnerable to DNA damage (Adamec et al., 1999; Jin et al., 1999; Crowe et al., 2011), although the mechanism remains unknown.

Next, we examined whether phospho-S^{389} GSK3β immunostaining in WT mice was evident in the subregions where the dark cells preferentially accumulate in GSK3β KI mice. The specificity for the phospho-Ser^{389} GSK3β staining was demonstrated by its absence in the brain of GSK3β KI mice (Fig. 3C). The highest levels of phospho-S^{389} GSK3β immunostaining were found in the hippocampal regions of WT mice that corresponded with the regions in GSK3β KI mice with higher frequency of dark cells (Fig. 3C). Similarly, the regions of the cerebral cortex with abundant phospho-S^{389} GSK3β staining in WT mice also corresponded with the cortical regions with higher frequency of dark cells in GSK3β KI mice (Fig. 3C).

To demonstrate that the dark cells present in GSK3β KI brains represent degenerative neurons, brains sections from WT and GSK3β KI mice were stained with Fluoro-Jade C, the gold-standard dye used to visualize neurodegeneration (Schmued et al., 2005). Fluoro-Jade staining was nearly undetectable in brains from WT mice (Fig. 3D). In contrast, clear Fluoro-Jade staining was present in regions of the cerebral cortex and hippocampus in GSK3β KI brain (Fig. 3D) and corresponded with the areas where the dark cells were abundant. Thus, these results show that failure to inhibit GSK3β through S^{389} phosphorylation results in neurodegeneration suggesting that inhibition of GSK3β through S^{389} phosphorylation may play a role in neuronal survival.

Hippocampal degeneration in GSK3β KI mice is not associated with deficits in a spatial learning task.

The presence of pyknotic cells by H&E staining, and fluoro-jade labeling, in the hippocampus and cortex of GSK3β KI mice is consistent with neuronal degradation. Given the hippocampal degradation, we chose to assess performance in the water maze, a task that requires the hippocampus (Morris et al., 1982). Animals were trained to find a submerged platform over 8 days, with four trials per day. Performance improved over sessions (Day $f_{(2,56)}$=80.75, p<.0001) though there was no effect of genotype (Genotype $f_{(1,28)}$=1.338, p>.05) or interaction (Day x Genotype $f_{(2,56)}$=1.37, p>.05) indicating that hippocampal spatial learning was similar in GSK3β KI mice and WTs (Fig. 4A). Spatial memory in the water maze can also be measured by utilizing a probe trial in which the platform is removed and the time spent in the general area of the platform is quantified. GSK3β KI and WT mice spent similar time searching in the platform quadrant ($t_{(28)}$=.45, p>.05). Additionally, the time spent searching in the appropriate quadrant was above chance in each group (WT, ($t_{(14)}$=3.61, p<.01; KI, $t_{(28)}$=3.36, p<.01; Fig.4B). Further, when the platform was moved to a new location animals again performed similarly. Over four trials, distance travelled was reduced ($f_{(3,84)}$=9.97, p<.0001), again without a genotype main effect ($f_{(1,28)}$=.44, p>.05) or interaction ($f_{(3,84)}$=.63, p>.05; Fig. 4C). Together, these findings suggest that GSK3β KI mice are capable of performing a hippocampus based task despite apparent neurodegeneration.

Auditory fear conditioning results in increased contextual and tone conditioning in GSK3β KI mice.

As we observed no effect on a hippocampus based task, we sought to understand whether the neurodegenerative phenotype in GSK3β KI mice might alter behavior in a task that involves connections between the hippocampus and other limbic brain regions. Auditory fear conditioning is dependent on amygdala function (LeDoux, 2003; Davis, 2006; Kim and Jung, 2006; Duvarci and Pare, 2014), but is influenced by connections between the hippocampus and amygdala (Maren, 2001). For auditory fear conditioning, mice were given pairings of a tone followed by a footshock in a distinctive context. Beginning twenty-four hours after fear conditioning, mice were tested for freezing to the conditioning context alone and to the tone in a novel context in a counterbalanced fashion. Acquisition of tone fear proceeded equivalently in GSK3β KI and WT animals (Fig. 5A). Each group displayed an increasing fear response to tone presentations subsequent to the first ($f_{(5,90)}$=27.99, p<.0001) and there was no genotype effect ($f_{(1,18)}$=1.06, p>.05) or interaction ($f_{(5,90)}$=1.70, p>.05; Fig. 5A). GSK3β KI mice froze more than WT mice when returned to the conditioning context ($t_{(18)}$=3.18, p<.01; Fig. 5B) and also demonstrated greater conditioned fear to the tone in a novel context ($t_{(18)}$=2.68, p<.05; Fig. 5C). To further examine this exaggerated conditioned fear, we analyzed freezing on test days during the first two minutes after placement into the training and novel contexts. A context effect ($f_{(1,18)}$=32.20, p<.0001) without an accompanying interaction ($f_{(1,18)}$=2.02, p>.05) demonstrates that both groups froze more during the first two minutes after placement in the training context than in the same time period in the novel context (GSK3β KI, $t_{(9)}$=4.25, p<.01; WT, $t_{(14)}$=3.86, p<.01). However, a genotype effect ($f_{(1,18)}$=17.19, p<.01) suggests that GSK3β KI mice overgeneralized fear across contexts. Not only did GSK3β KI mice freeze more than WTs in the training context

($t_{(18)}$=2.87, p<.01), they also froze more immediately after introduction to the novel context ($t_{(9)}$=2.53, p<.05; Fig. 5D). A similar analysis of conditioning to the tone was conducted. During testing, the tone was presented in a single three minute block. Analysis of freezing to each minute of the tone, as well as the final minute of the test when the tone had ended, revealed that each group reduced freezing over the tone exposure (Minute $f_{(3,54)}$=28.07, p<.0001; minute X genotype $f_{(3.54)}$=1.79, p>.05). Similar to the above results, a genotype effect ($f_{(1,18)}$=8.19, p<.01) suggests increased fear in GSK3β KI mice. GSK3β KI and WT mice froze similarly during the first two minutes of tone presentation. However during the final minute of tone presentation ($t_{(18)}$=3.62, p<.01), as well as during the minute after tone termination ($t_{(18)}$=2.22, p<.05), freezing was elevated in GSK3β KI mice (Fig. 5E). Together these results demonstrate exaggerated and overgeneralized freezing in GSK3β KI mice.

Locomotor activity and measures of anxiety and depression are largely similar in GSK3β KI and WT mice.

It is possible that the exaggerated freezing in GSK3β KI mice is evidence of a locomotor deficit. However, when subjected to a 10 minute open field test, GSK3β KI and WT mice travelled similar distances ($t_{(18)}$=.61, p>.05; Fig. 6A) suggesting no change in locomotion. Further, each group entered the center of the arena equivalently ($t_{(18)}$=.71, p>.05), and spent equal time exploring the center of the open field ($t_{(18)}$=1.70, p>.05; Fig. 6B) indicating similar anxiety levels. Zero maze results were similar to those in the open field. Here mice explore a raised circular track with enclosed portions in opposing quadrants over 5 minutes. Anxiety is defined as reduced open quadrant time or reduced

open quadrant entries. Open quadrant entries ($t_{(18)}$=.63, p=.54; Fig. 6C) and open quadrant time ($t_{(18)}$=.85, p=.41; Fig. 6D) were similar between GSK3β KI and WT mice suggesting that GSK3β KI mice are not more anxious than WT mice.

The forced swim test is a commonly used model of depression that measures behavioral despair through immobility, with increasing immobility time suggestive of increased depression (Bogdanova et al., 2013). An analysis of forced swim behavior by minute demonstrated that immobility increased in the later portions of the 6 minute test (Minute $f_{(5,90)}$=24.54, p<.0001; Fig.6E) though no genotype effect (Genotype $f_{(1,18)}$=.23, p>.05) or interaction (Minute X Genotype $f_{(5,90)}$=1.11, p>.05) was observed.

A notable phenotype observed in GSK3β KI mice was lower acoustic startle amplitude (t(18)=3.75, p<.01; Fig. 7A). Acoustic startle is modulated by emotional state with increased startle amplitude being taken as evidence for increased anxiety (Walker et al., 2003). Lower startle amplitude was present across the startle test when stimuli were examined in 6 trial blocks (group $f_{(1,18)}$=14.087, p<.001; trial $f_{(1,18)}$=.02, p>.05; groupXtrial $f_{(1,18)}$=.293, p>.05; Fig. 7B) demonstrating that the startle reduction observed in GSK3β KI animals was not a result of more rapid habituation. In order to assess whether the lower startle amplitude reflected a decrease in emotion-based startle modulation, GSK3β KI and WT mice were tested for acoustic startle following systemic injection of the serotonin agonist mCPP. mCPP dose dependently potentiates startle amplitude and has been used to probe for changes in anxiety responsiveness (Fox et al., 2008; Hare et al., 2012). The change in acoustic startle amplitude following mCPP injection was calculated in comparison to vehicle injection in a counterbalanced, within

subject fashion, separated by 24 hours. Startle potentiation by mCPP was similar in both GSK3β KI and WT mice ($t(18)=.63$, $p>.05$; Fig. 7C) suggesting no difference in serotonergic startle modulation between genotypes.

p38 MAPK inhibition by SB203580 does not alter auditory fear conditioning.

Activation of p38 MAPK is the sole described mechanism for inhibition of GSK3β by S^{389} phosphorylation (Thornton et al., 2008). As such, inhibition of p38 MAPK through SB203580 administration could be expected to reduce GSK3β S^{389} phosphorylation and result an auditory fear conditioning effect that mimics that of the GSK3β KI mice. ICV administration of 125μM SB203580 (2.5μL) 30 minutes prior to fear conditioning did not alter acquisition of tone fear in WT mice (Tone $f_{(5,90)}=103.49$, $p<.0001$, Treatment $f_{(1,18)}=0.00$, $p>.05$, Tone x Treatment $f_{(5,90)}=0.50$, $p>.05$; Fig. 8A). Similarly, context ($t_{(18)}=-0.57$, $p>.05$; Fig. 8B) and tone fear ($t_{(18)}=-0.99$, $p>.05$; Fig. 8C) were similar in SB203580 and vehicle treated mice as was context discrimination (Context $f_{(1,18)}= 25.72$, $p<.0001$, Treatment $f_{(1,18)}=1.43$, $p>.05$, Context x Treatment $f_{(1,18)}=0.09$, $p>.05$; Fig. 8D). In a separate cohort of animals, SB203580 was administered immediately following fear conditioning. Acquisition proceeded equivalently in treated and untreated mice (Tone $f_{(5,80)}=26.69$, $p<.0001$, Treatment $f_{(1,16)}=0.05$, $p>.05$, Tone x Treatment $f_{(5,80)}=0.32$, $p>.05$). Again, context ($t_{(16)}=0.83$, $p>.05$) and tone fear ($t_{(16)}=0.21$, $p>.05$) were similar in SB203580 and vehicle treated mice, as was context discrimination (Context $f_{(1,16)}= 30.51$, $p<.0001$, Treatment $f_{(1,16)}=1.39$, $p>.05$, Context x Treatment $f_{(1,16)}=0.26$, $p>.05$).

While this dose of SB203580 has previously been shown to be effective in altering fear conditioning after immune challenge (Gonzalez et al., 2013), and a much lower dose was effective in regulating swim stress behavior (Bruchas et al., 2007), we sought to understand the effects of SB203580 manipulation through analysis of downstream targets of active p38 MAPK. p38 MAPK inhibits GSK3β through S^{389} phosphorylation (Thornton et al., 2008). Therefore, inhibiting p38 MAPK should elevate levels of GSK3β S^{389} phosphorylation. However, by western blot analysis no change in GSK3β S^{389} phosphorylation was observed in the amygdala ($t_{(5)}$=0.57, p>.05) or hippocampus ($t_{(7)}$=0.01, p>.05) 30 minutes after SB203580 administration (data not shown).

Discussion

Previous investigations into the inhibition of GSK3 have largely focused on the well characterized inhibition of GSK3β by S^9 phosphorylation and GSK3α by S^{21} phosphorylation (Stambolic and Woodgett, 1994; Cross et al., 1995). The recently discovered inhibitory GSK3β phosphorylation site at S^{389} was shown to be independent of GSK3α and mediated by p38 MAPK rather than AKT (Thornton et al., 2008). Here we have extended those results showing that GSK3β S^{389} phosphorylation has a cellular localization pattern that is distinct from S^9 phosphorylation. Further, we have demonstrated that GSK3β S^{389} phosphorylation occurs on GSK3β only when S^9 phosphorylation is absent and represents a means of inhibiting an independent nuclear pool of GSK3β activity. Additionally, we describe a pattern of neuronal degradation and behavioral phenotype that includes exaggerated fear conditioning in GSK3β KI mice that

cannot inhibit GSK3β through S^{389} phosphorylation. Together, these results demonstrate that GSK3β S^{389} phosphorylation may play a unique role in the cellular response to stress by limiting the activity of a nuclear pool of GSK3β, that if unrestrained, promotes neuronal degradation.

As a multifunctional kinase, GSK3β must be precisely regulated. Consistent with this, dysregulation of GSK3β has been implicated in a variety of diseases including those associated with neuronal degeneration such as Alzheimer's disease and depression. GSK3β can promote or inhibit apoptosis making it a critical regulator of cell death (Beurel and Jope, 2006). Of primary importance here is the consistent finding that GSK3β activity promotes apoptosis following insults that activate the intrinsic apoptotic pathway such as DNA damage, oxidative stress, or excitotoxic challenges. For instance, an interaction between active GSK3β and p53, a pro-apoptotic signaling molecule, forms in the nucleus after DNA damage and is associated with increased caspase-3, a key driver of the execution phase of apoptosis (Watcharasit et al., 2002). Notably, observed changes in GSK3β levels in the nucleus after cellular insults including DNA damage (Watcharasit et al., 2002) and excitotoxic challenge (Elyaman et al., 2002) have not been associated with changes in nuclear inhibitory regulation of GSK3β S^{9} phosphorylation. Differentiated neurons cannot be replaced. Thus, these findings suggest that a means of reducing nuclear GSK3β activity that does not involve S^{9} phosphorylation must exist to protect differentiated neurons from the apoptotic implications of GSK3β activation by cellular stress.

Our data suggest that nuclear GSK3β S^{389} phosphorylation may play a role in restraining GSK3β activity and maintaining neuronal viability that is independent of S^{9} phosphorylation. GSK3β S^{389} phosphorylation was shown to be primarily nuclear as opposed to the S^{9} which was distributed in the cytoplasmic and nuclear compartments. The pool of GSK3β phosphorylated at S^{9} was shown to lack S^{389} phosphorylation. Kinase assay results demonstrated increased GSK3β activity when S^{389} phosphorylation was absent. Finally, the fluoro jade labelling in GSK3β KI mice suggests that a deficit in GSK3β S^{389} inhibition promotes neurodegeneration, a finding attributable to that component of GSK3β activity regulated by S^{389} phosphorylation. The nuclear association between GSK3β and p53 was proposed to mediate negative regulation of neuroprotective transcription factors (Watcharasit et al., 2002). One such neuroprotective transcription factor, β-catenin, was shown to be reduced in the nucleus of mice that conditionally overexpress GSK3β and the reduction was associated with increased markers of apoptosis (Lucas et al., 2001). β-catenin was shown to accumulate through inhibition of GSK3β by S^{389} phosphorylation (Thornton et al., 2008) suggesting that regulation of S^{389} phosphorylation may play a role in the neuronal phenotype observed by Lucas et al. (2001). Future work is necessary to determine the components of neuroprotective signaling preserved by GSK3β S^{389} phosphorylation, as well as how such GSK3β inhibition may be protective after cellular insult.

The fear phenotype observed in the GSK3β KI animals suggests that there is an as yet undetermined specificity to the degenerating neurons observed in GSK3β KI mice. We observed that GSK3β KI mice acquired tone fear at a similar rate as WT mice.

However, contextual and tone associated freezing was increased in GSK3β KI mice. The increase was evident as a prolongation of the fear response, as well as an increase in generalized fear between the training context and a novel context. Overgeneralization of fear, and failure to extinguish responses to fear associated cues, are found in anxiety disorders (Lissek et al., 2014), post-traumatic stress disorder (PTSD) (Wessa and Flor, 2007), and are hallmarks of stress exposure models used in rodents to model these disorders (Izquierdo et al., 2006; Baran et al., 2009; Hoffman et al., 2014). A well delineated brain circuitry involving the amygdala, hippocampus, and frontal cortex is involved in fear learning and extinction. Within this circuitry stress may have opposite effects, degrading dendritic arborization in the hippocampus (Watanabe et al., 1992) and frontal cortex (Radley et al., 2008), while driving dendritic hypertrophy in subregions of the amygdala (Vyas et al., 2004).

The hippocampal and frontal cortex degradation observed in rodent stress models is proposed to be a driving factor in the reduced hippocampal and frontal cortex volumes observed in stress associated disorders such as PTSD (Gilbertson et al., 2002; Shin et al., 2006). Within stress associated disorders, amygdala hyperactivation is also commonly observed (Shin et al., 2004; Savitz and Drevets, 2009). Ghosh and colleagues (Ghosh et al., 2013) observed increased interactions between amygdala and hippocampal CA1 following stress concurrent with reduced interaction between hippocampal CA3 and CA1. Another recent study observed hyperactivation of both amygdala and hippocampal CA1 during fear retrieval after stress exposure (Hoffman et al., 2014) suggesting that stress increases amygdalar control over behavior. We observed neuronal degradation in

the CA1 and CA3 fields of the hippocampus. Fluoro Jade labeling limits our interpretation of this finding to a neuronal phenotype. However, determining whether a specific neuronal subtype is disproportionately affected by loss of GSK3β S^{389} inhibition, and how such loss might result in the observed fear conditioning phenotype could aid in understanding the relationship between the complex changes in neuronal morphology and activation observed in models of stress associated disorders.

The behavioral phenotype observed in GSK3β KI animals further indicates a distinct role for S^{389} regulation of GSK3β as opposed to S^{9}. Mice with alanine substitutions at S^{9} and S^{21}, rendering GSK3β and GSK3α constitutively active, are more active in an open field, spend more time immobile in forced swim and tail suspension tests, and reduced time in the open arms of an elevated plus maze, suggestive of an increased anxiety and depression-like phenotype, though it is unclear what the contributions of the individual isoforms are to this phenotype (Polter et al., 2010). These mice also display a complex fear phenotype that is evident as increased contextual fear, better context discrimination, and similar tone fear. In contrast, we observed elevated contextual fear, context generalization, and increased tone fear. Mice with GSK3β S^{9} alone rendered constitutively active are more active in an open field, have elevated acoustic startle amplitude, and reduced forced swim immobility time (Prickaerts et al., 2006). Consistent with the locomotor hyperactivity observed in GSK3β S^{9} mutants, reduced GSK3β S^{9} phosphorylation has previously been associated with locomotor sensitization produced by amphetamine (Enman and Unterwald, 2012). In contrast, we observed no change in locomotor behavior in the GSK3β KI mice described here. Thus,

increased locomotor activity may be unique to manipulations that render the S^9 site on GSK3β constitutively active. The increased acoustic startle observed following S^9 manipulation (Prickaerts et al., 2006) is in contrast to the reduction we observed in GSK3β KI mice. The acoustic startle response is driven by a well-defined circuit (Yeomans and Frankland, 1995) and modulated by limbic brain regions (Davis et al., 1997). It is unclear whether the neurodegenerative phenotype observed here extends to the primary acoustic startle circuit and might therefore drive the reduction in startle amplitude. Thus, at this time we would not suggest that the reduction in startle amplitude is evidence of reduced anxiety in GSK3β KI mice. However, our observation that the anxiogenic 5HT-2c agonist mCPP modulates startle to a similar extent in WT and GSK3β KI mice suggests that the ability to modulate the emotional response is preserved in GSK3β KI mice.

Inhibition of p38 MAPK was not found to replicate the phenotype observed in GSK3β S^{389} mice. Previous research has utilized an ICV injection of SB203580 at an order of magnitude lower concentration to regulate the dysphoric effects of stressor exposure (Bruchas et al., 2007) suggesting that the dose utilized in the current experiment was sufficient to inhibit some portion of p38 MAPK activity. Others have utilized a concentration similar to that reported here, injected directly into the hippocampus, to protect against the impairing effects of inflammatory cytokine injection on fear conditioning (Gonzalez et al., 2013). Further research is necessary to understand the requirements for p38 MAPK mediated inhibition of GSK3β, as well as the developmental

effects of deficient GSK3β S^{389} phosphorylation that may underlie the observed fear phenotype.

The data reported here demonstrate a distinct nuclear localization of GSK3β S^{389} phosphorylation, as well as an independent role of S^{389} phosphorylation in limiting GSK3β activity. Further, the novel GSK3β KI animals reported here demonstrate a fear conditioning phenotype that mimics that observed in models of stress associated disorders. Based on the fear conditioning findings, and neurodegenerative phenotype observed in GSK3β KI animals, the relationship between GSK3β S389 phosphorylation and stress deserves further investigation.

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Figures

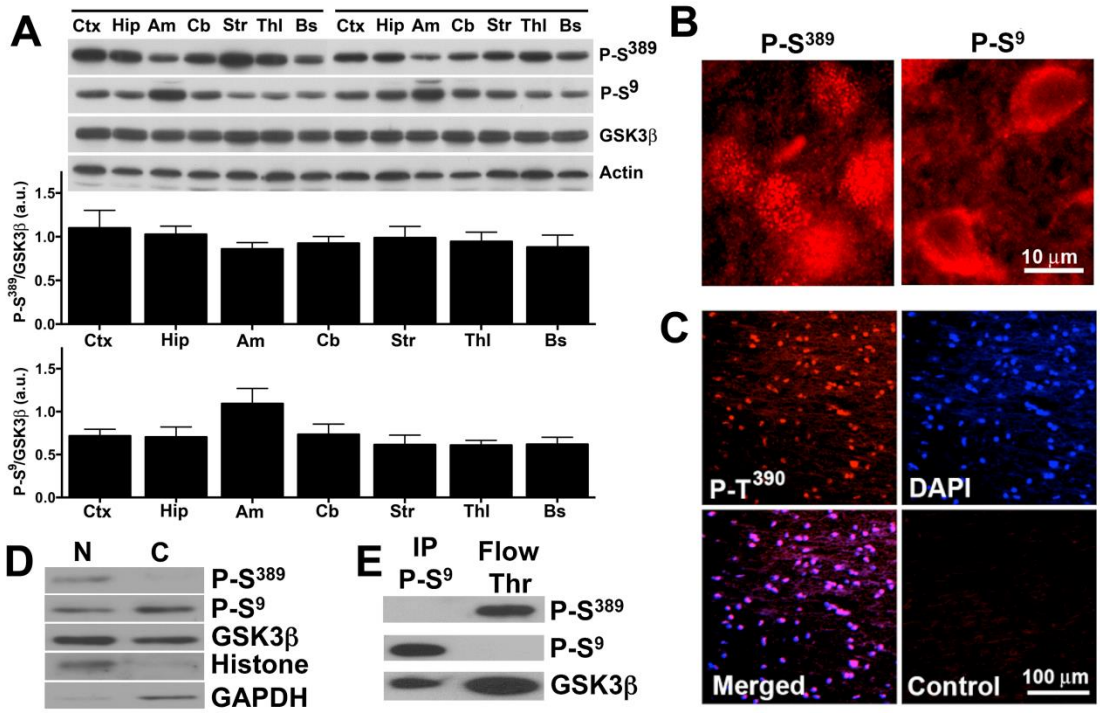

Figure 1. GSK3β serine 389 phosphorylation is expressed in a primarily nuclear fashion and present in all brain areas examined. **A.** Western blot demonstrating phosphorylation of serine 9 (P-S^{9}) and serine 389 (P-S^{389}) in all examined brain areas. Immunolabeling demonstrates a unique cellular distribution of GSK3β P-S^{389} and P-S^{9} in **B.** murine, and **C.** human (P-T^{390} is human S^{389} equivalent) tissue. **D.** Western blot data confirms a nuclear (N) localization of the P-S^{389} signal, P-S^{9} is evident in nuclear and cytosolic (C) compartments. **E.** Immunoprecipitation of P-S^{9} demonstrates that GSK3β phosphorylated at S^{9} was not simultaneously phosphorylated at S^{389}.

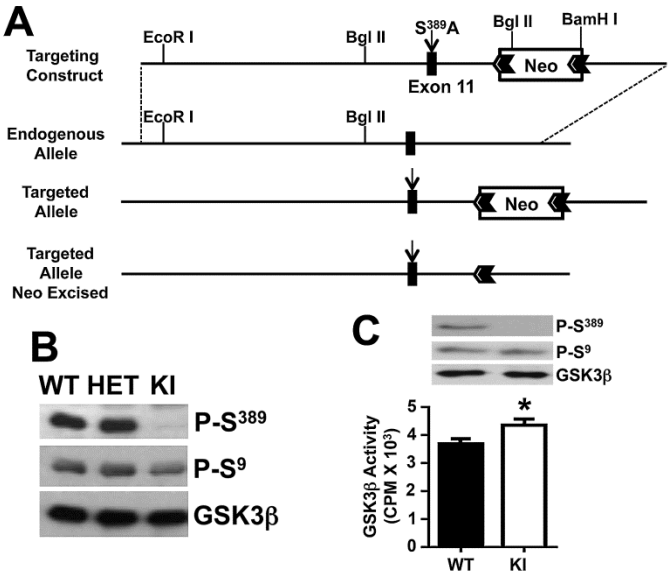

Figure 2. Characterization of the GSK3β serine 389 to alanine mutant mouse. **A.** Diagram depicting the GSK3β Ser^{389}Ala knockin (KI) construct, the endogenous GSK3β allele containing exon 11 (black box), the targeted allele with the neomycin selection cassette still present and the targeted allele with the neomycin cassette removed by Cre recombinase. White notched arrow heads represent LoxP sites. **B.** Western blot image of whole cell lysates from brains of wildtype (WT), heterozygous (Het) and homozygous GSK3β KI mice demonstrate lack of serine 389 phosphorylation (p-S^{389}) in GSK3β KI mice. **C.** GSK3β kinase activity and western blot analysis for P-S^{389} and P-S^{9} demonstrating increased GSK3β activity in cerebral cortex of KI mice.

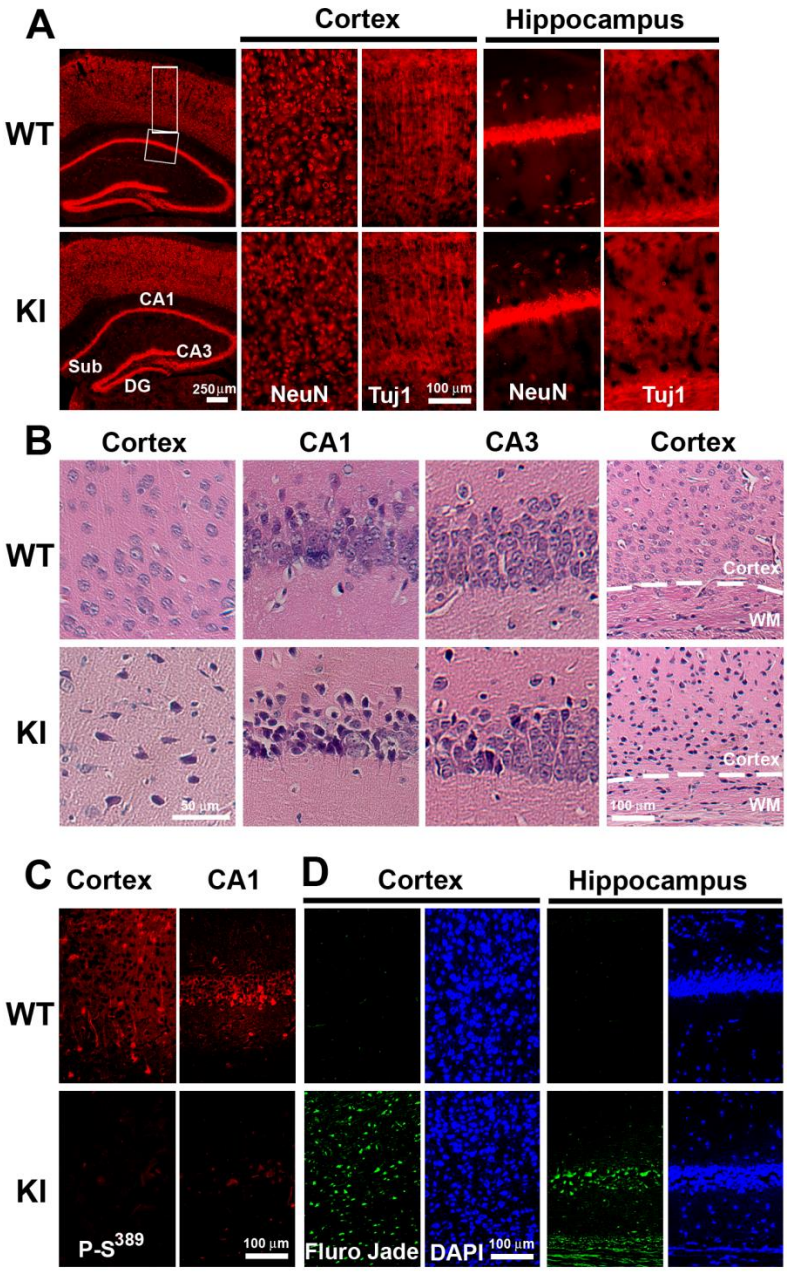

Figure 3. Limited neuronal degradation, without apparent neuronal loss, is observed in GSK3β mutant mice. **A.** Immunolabelling of NeuN and TUJ-1 demonstrates no overt neuronal loss in GSK3β KI mice. **B.** Dark neurons indicative of neurodegeneration are evident in haematoxylin and eosin stained cortical and hippocampal tissue of GSK3β KI mice. **C.** GSK3β serine 389 phosphorylation (P-S^{389}) is evident in brain regions where dark neurons are present. **D.** Fluoro jade labeling in cortex and hippocampus further demonstrates neuronal degradation in GSK3β KI mice.

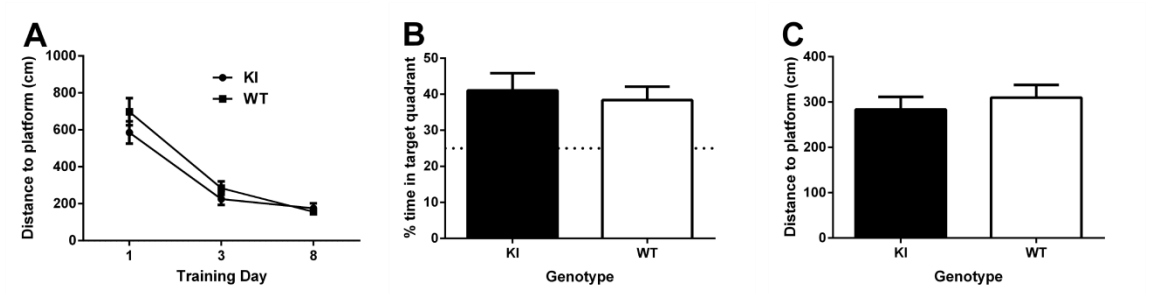

Figure 4. Assessment of spatial learning by Morris water maze in wild type (WT) and GSK3β S^{389} knock-in (KI) mice. **A.** Water maze acquisition proceeded similarly. **B.** Time spent searching for the platform in the target quadrant during a probe trial was similar in WT and KI mice. Both groups demonstrated greater than chance performance (dotted line) **C.** Reversal test performance was similar in WT and KI mice. Data are Mean±SEM.

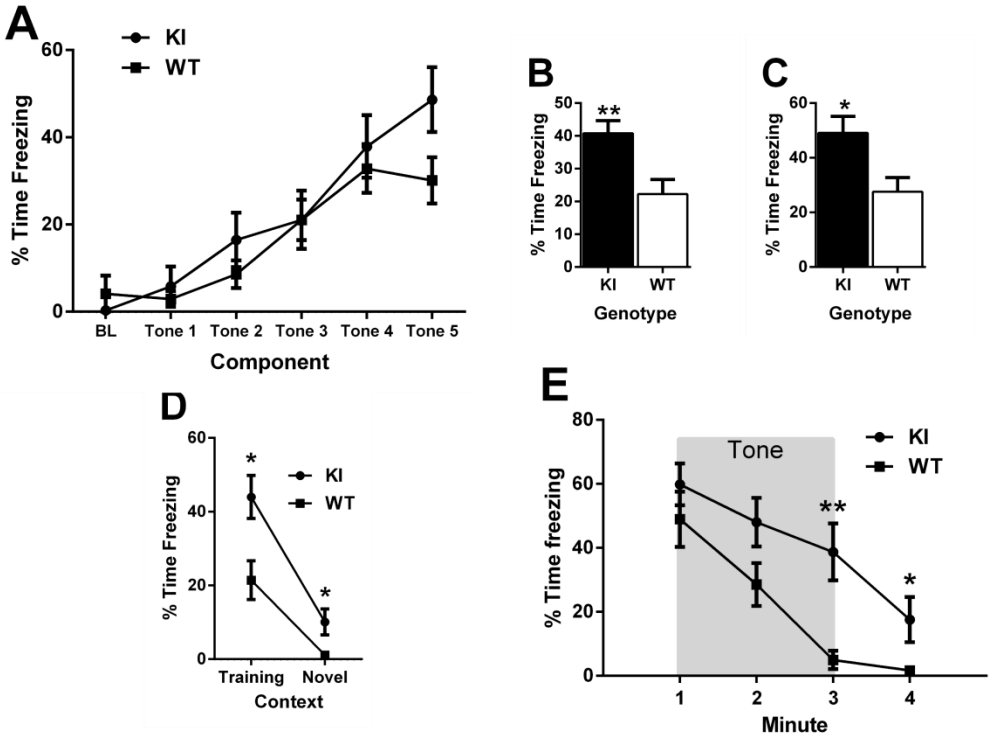

Figure 5. Auditory fear conditioning in wild type (WT) and GSK3β S389 knock-in (KI) mice. **A.** Acquisition of fear conditioning proceeded similarly in WT and KI mice. KI mice demonstrated greater **B.** contextual, and **C.** tone fear. **D.** Context discrimination was evident in KI and WT mice, though KI mice froze more than WT mice in the first two minutes of exposure to the training and novel context after conditioning. **E.** Decay of tone fear was more rapid in WT than KI mice. Data are Mean±SEM, *p<.05, **p<.01

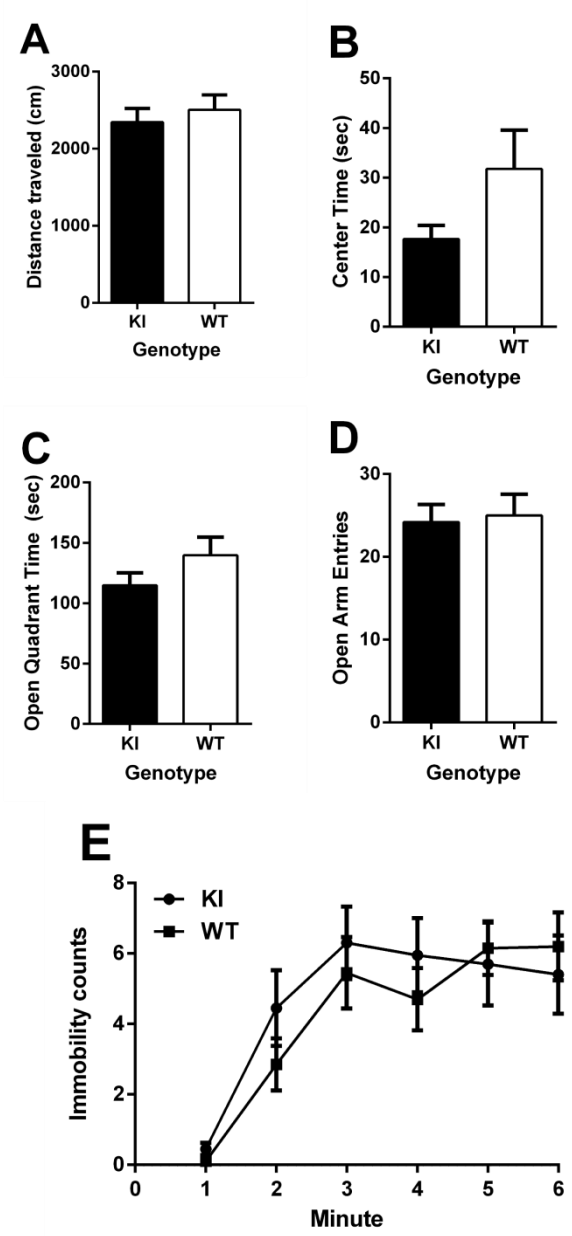

Figure 6. Assessment of anxiety and depression-like behavior in wild type (WT) and GSK3β S389 knock-in (KI) mice. **A.** Open field distance and **B.** center time were similar in WT and KI mice. **C.** Zero maze open quadrant time **D.** and open quadrant entries were also similar. **E.** Forced swim immobility counts were similar in WT and KI mice across the 6 minute test. Data are Mean±SEM.

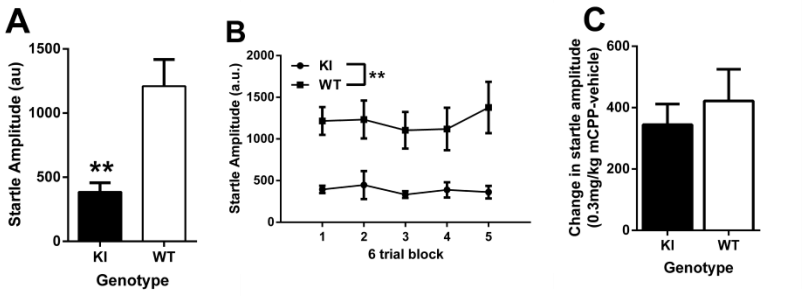

Figure 7. Acoustic startle assessment in wild type (WT) and GSK3β S389 knock-in (KI) mice. **A.** Acoustic startle was reduced in KI mice. **B.** The acoustic startle response was lower in KI mice across the entire startle session. **C.** mCPP administration produced a similar elevation in startle amplitude in KI and WT mice. Data are Mean±SEM, **p<.01

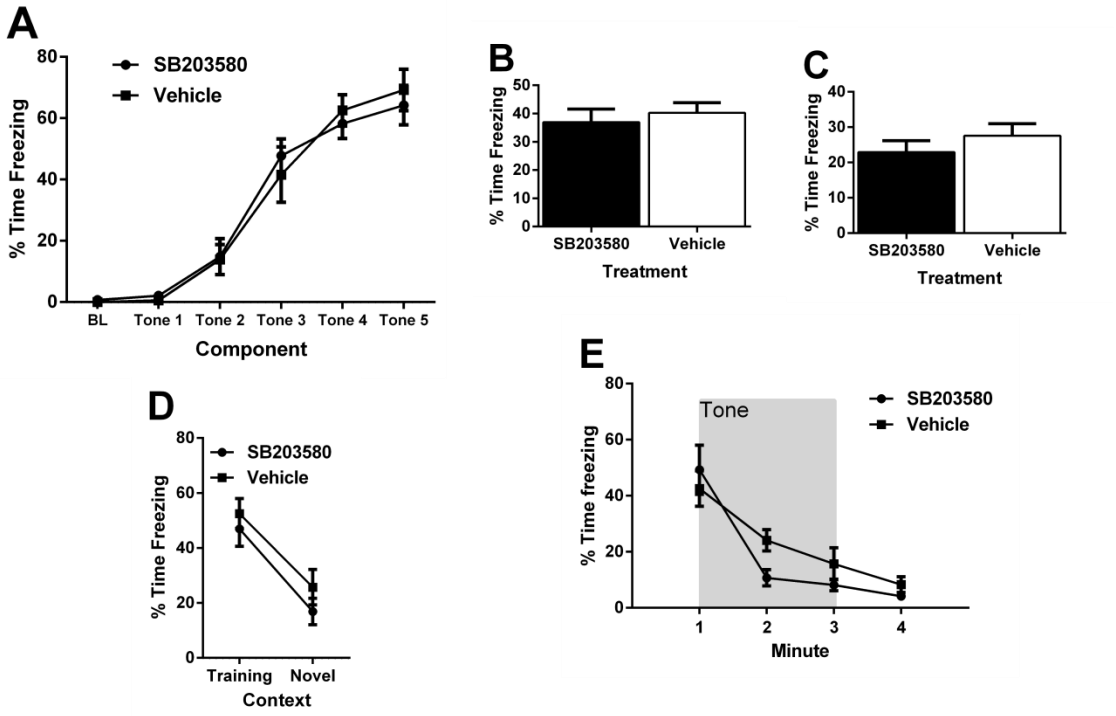

Figure 8. Pre-training intracerebroventricular injection of SB203580 did not alter auditory fear conditioning in wild type mice. **A.** Training **B.** Contextual and **C.** Tone fear were unchanged after drug administration. **D.** Context discrimination was evident and similar in drug and vehicle treated mice. **E.** Decay of tone fear was similar in drug and vehicle treated mice.

CHAPTER 3: VARIATE STRESS, VOLUNTARY EXERCISE, AND DNA DAMAGE ARE ASSOCIATED WITH REGULATION OF P38 MAPK AND GSK3β

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Abstract

Stress associated psychiatric disorders affect millions worldwide. In rodents, variate stress exposure models the negative effects of stress, while voluntary exercise typically confers stress-resistance. p38 mitogen associated protein kinase (p38 MAPK) is implicated in the cellular response to stress, and has recently been implicated in the behavioral response to stressors in rodents, though its regulation by exercise is unknown. Downstream targets of p38 MAPK following stress are only beginning to be elucidated. A novel inhibitory phosphorylation site on glycogen synthase kinase 3β (GSK3β) was recently shown to be sensitive to p38 MAPK activity. Here we examine the phosphorylation state of p38 MAPK and GSK3β in stress-sensitive brain regions following variate stress and voluntary exercise. Variate stress increased p38 MAPK levels in the hippocampus, bed nucleus of the stria terminalis, amygdala, and frontal cortex. In contrast, voluntary exercise increased p38 MAPK levels only in the amygdala. GSKβ regulation largely mirrored that of p38 MAPK in a phosphorylation site specific fashion suggesting a novel association between the kinases following stress. Variate stress and exercise were also associated with increased histone H2A-variant-X (ϒH2A.X) indicative of DNA damage. Notably, acute stress did not increase ϒH2A.X indicating that the increase observed following variate stress may represent accumulation of DNA damage. Our findings extend the association between behavioral challenges and DNA damage, and suggest that activation of the p38MAPK/GSK3β pathway may be protective, engaged in response to DNA damage to prevent central nervous system cell loss.

Introduction

Psychiatric disorders precipitated by stress, such as anxiety and depression, affect approximately 25% of the population and are often found to be comorbid (Kessler et al., 2005). In animals, variate stress exposure, in which the stressor is varied over multiple days, leads to increased anxiety and depression like behaviors (Hammack et al., 2009; Li et al., 2011), while voluntary exercise mitigates the negative consequences of stress exposure (Greenwood et al., 2003; Greenwood et al., 2005a). Recent work suggests that activation of p38 mitogen associated protein kinase (p38 MAPK) may mediate the aversive effects of stress exposure (Bruchas et al., 2007; Bruchas et al., 2011).

Though p38 MAPK has been implicated in stress effects, examination of its downstream targets is limited, and its regulation by exercise is unknown. Recent work identified glycogen synthase kinase 3β (GSK3β) as a substrate of p38 MAPK (Thornton et al., 2008). GSKβ regulates cell properties including structure, gene expression, and apoptosis (Kaidanovich-Beilin and Woodgett, 2011). Inhibitory phosphorylation of GSK3β at the serine 9 (S^9) residue has been implicated in the therapeutic effects of lithium (Klein and Melton, 1996), selective serotonin reuptake inhibitors (Li et al., 2004), and ketamine (Beurel et al., 2011; Liu et al., 2013). p38 MAPK inhibition of GSK3β is independent of the traditionally studied S^9 residue, and rather, exclusively achieved by phosphorylation of the recently discovered serine 389 (S^{389}) residue (Thornton et al., 2008). p38 MAPK accumulates in the nucleus, and nuclear GSKβ activity is increased in response to DNA damage (Watcharasit et al., 2002; Wood et al., 2009). Cell survival following such damage may be enhanced by p38 MAPK activation and GSK3β inhibition

(Mikhailov et al., 2004; Phong et al., 2010; Yang et al., 2011; Tan et al., 2014; Wang et al., 2014). GSK3β S^{9} phosphorylation is unchanged following DNA damage, suggesting that GSK3β inhibition through p38 MAPK mediated S^{389} phosphorylation may function to limit GSK3β activity and facilitate the DNA damage response.

Accumulation of DNA damage may lead to neurodegeneration (Brasnjevic et al., 2008), which could result in the neuronal atrophy observed in variate stress models (Li et al., 2011). However, recent reports suggest that neuronal activity is sufficient to produce DNA damage in activated circuits (Suberbielle et al., 2013), suggesting that any challenge sufficient to result in neuronal activation may produce DNA damage. Thus, stress, as well as voluntary exercise, challenges that produce neuronal activation and engage numerous brain regions, may be expected to result in DNA damage and regulation of p38 MAPK and GSK3β S^{389} phosphorylation. However, to date such associations have not been made. Here we show that variate stress and exercise regulate p38 MAPK levels in stress-sensitive brain regions including the hippocampus, amygdala, bed nucleus of the stria terminalis (BNST) and frontal cortex concurrent with increased inhibitory GSK3β S^{389} phosphorylation . In addition, we demonstrate that each challenge is associated with increased H2A-variant-X (ϒH2A.X) phosphorylation, a marker of DNA damage (Rogakou et al., 1998).

Materials and Methods

Subjects

Male C57BL6/J mice (Jackson Laboratories, Bar Harbor, ME) were obtained at six weeks of age and provided a 7-day acclimation period prior to experimental manipulation. Prior to experimental manipulation, mice were housed in groups of 4 in acrylic wire top cages in an Association for Assessment and Accreditation of Lab Animal Care (AAALAC) approved animal facility. Following group assignment, mice undergoing variate stress were single housed. A 12 hour light/dark cycle (0700h-1900h) and ad libitum access to food and water were maintained throughout experimental procedures. All procedures were approved by the University of Vermont Animal Care and Use Committee.

Acoustic startle

Acoustic startle was used to assess anxiety following stress and exercise. Startle was assessed as described previously (Salam et al., 2009). Briefly, mice were placed individually in an acoustic startle chamber (Med-Associates, St. Albans, VT) located within a sound attenuating cubicle and administered 30, 20msec duration, startle-eliciting noise bursts (10 each at 90, 95 and 105 dB) at a 1-minute inter stimulus interval. The average startle amplitude over the 30 trial test is reported.

Variate stress and voluntary exercise

Following the 7-day acclimation period, acoustic startle was assessed in all mice as described above. The average startle amplitude for each mouse was used to assign mice to control, exercise, and variate stress groups. Mice assigned to the control (n=10) and exercise (n=10) conditions were group housed whereas mice assigned to the variate stress condition (n=10) were individually housed immediately after the baseline startle test. The variate stress procedure was adapted from previous studies (Hammack et al., 2009; Hare et al., 2012) and was 14 days in duration. Stressors used were exposure to a 5 minute forced swim followed by 30 minutes in a bedding free cage, exposure to two 0.5mA 5 second foot shocks, 30 minutes of 1Hz oscillation, 60 minutes of restraint in an overturned 50ml beaker, and 30 minutest on an elevated pedestal (20 cm W X 20 cm D X 60 cm H). Stressors were varied across the stress days to prevent response habituation using a pseudorandom sequence in which all stressors were performed before a stressor was repeated. Swim stress was conducted only on days 1 and 14. Mice in the control and variate stress groups were weighed daily to assess stress-associated attenuation of weight gain (Hare et al., 2012). The exercise group was provided ad libitum access to a running wheel (Superpet, 11.4cm diameter) in the home cage for 14 days. Distance run was measured daily using bicycle pedometers (Cateye Enduro 8, Osaka, Japan). Acoustic startle was measured on day 15 in all groups and followed by tissue harvest.

Tissue Harvest

GSK3β is rapidly dephosphorylated by anesthetics and long pre-cooling post mortem intervals (Li et al., 2005). As such, mice were rapidly decapitated and tissue was rapidly harvested (< 3 minutes) and immediately transferred to centrifuge tubes cooled on

dry ice prior to storage at -80F. Coronal slices were obtained using a 1mm brain matrix (Stoelting, Wood Dale, IL). The amgydala (approximate bregma coordinates: anteroposterior -1.06; mediolateral 2.75; dorsoventral -4.5), bed nucleus of the stria terminalis (BNST) (approximate bregma coordinates: anteroposterior 0.26; mediolateral 1.0; dorsoventral -4.25), dentate gyrus of the hippocampus (approximate bregma coordinates: anteroposterior -2.06; mediolateral 1.0; dorsoventral -2.0; punched in a posterior to anterior fashion), and frontal cortex (approximate bregma coordinates: anteroposterior 1.34; mediolateral 0.25; dorsoventral -3.0; punched in a posterior to anterior fashion) were localized extracted with a 1mm tissue punch (Stoelting, Wood Dale, IL). Tissue was harvested 24 hours after the single forced swim exposure, and 24 hours after the final stressor in control, variate stress, and exercised mice. All tissue harvest was performed between 1200h and 1300h, and all animals were age matched at the time of harvest.

Western blot analysis

Western blot analysis was performed using crude whole cell lysates as described previously (Lluri et al., 2008), except the tissue was homogenized in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). Densitometry was performed as described previously (Long et al., 2013).

Immunohistochemistry

Brains were rapidly dissected, blocks with exposed BNST and amygdala generated, and submersion fixed in 10% buffered formalin (Fisher Scientific) for 4 hrs prior to paraffin embedding. Sections (8 μm) were mounted onto gelatin-coated slides and processed for immunohistochemistry (IHC) as described previously (Long et al., 2011), except the use of Sudan Black was excluded.

Antibodies

Rabbit anti-human phospho-GSK3β (ser9) (7,500X for blots, 800X for IHC; 9323), rabbit anti-human phospho p38 MAPK (Thr180/Tyr182) (1,000X, 9215), rabbit anti-human p38 MAPK (1,000X, 9212), and rabbit anti-human phospho-histone H2A.X (Ser139) (1,000x for blots, 2577) were obtained from Cell Signaling Technology (Danvers, MA). Rabbit anti-mouse phospho-GSK3β (Ser389) (3,000 for blots, 400X for IHC; 07-2275) and mouse anti-human phospho-histone H2A.X (Ser139) (500X for IHC, 05-636) were obtained from Millipore (Billerica, MA), while mouse anti-chicken α-tubulin (50,000X, T6199) was obtained from Sigma (St. Louis, MO). Species-specific horseradish peroxidase-conjugated (3,000X), Cy3-conjugated (500X) and Cy2-conjugated (100X) secondary antibodies were from Jackson ImmunoResearch (West Grove, PA).

Statistical Analysis

Data are expressed as means ± standard error of the mean. Due to lane limits (n=4-6/group) in the western blot apparatus, brain regions were run on separate blots and analyses were restricted to within brain regions. Western blot analysis following variate

stress or exercise was evaluated with ANOVA followed by LSD protected independent samples t-tests exclusively between experimental groups (variate stress or exercise) and the control group. Western blot analyses of acute challenges were by independent sample t-tests. Behavioral analysis utilized mixed model ANOVAs with day as the within subject factor and group as the between subject factor. Mixed model ANOVAs were followed by lower order ANOVAs, independent samples t-tests, and paired sample t-tests. A single animal in the voluntary exercise group was removed from startle analysis due to results greater 2.3 standard deviations from the group mean. Results were analyzed by SPSS software version 22 (IBM; Armonk, NY). $p < 0.05$ was considered statistically significant.

Results

Variate stress reduces weight gain and increases acoustic startle amplitude.

We have shown previously that variate stress reduces weight gain in mice (Hare et al., 2012). Body weight was similar in control and stressed mice on the first day of stress exposure ($t_{(18)}$=0.24, p=.82). A group by day repeated measures ANOVA produced significant main effects for day ($F_{(13,234)}$=8.42, p<.0001) and group ($F_{(1,18)}$=14.29, p=.001) along with a significant interaction ($F_{(13,234)}$=5.94, p<.0001) demonstrating that mice in the control condition gained weight over the fourteen day stress period at a greater rate than those in the stress condition (Fig.1A). Control (day 1 v day 14, $t_{(9)}$=5.53, p<.0001), but not stressed mice, showed an increase in normalized body weight over the 14 days (day 1 v day 14, $t_{(9)}$=1.17, p=.26). At day 14, control mice weighed more as a percentage of their body weight than stressed mice ($t_{(18)}$=3.08, p=.006).

Acoustic startle was assessed prior to the initiation of variate stress and following termination of stress (Fig. 1B). Stressed (day1 v day 14, $t_{(9)}$=2.68, p=.013), but not control mice (day 1 v day 14, $t_{(9)}$=.29, p=.78) showed an increase in acoustic startle over these two tests. At 14 days, acoustic startle amplitude was greater in stressed than in control mice ($t_{(18)}$=2.79, p=.01) suggesting an anxiogenic effect of stress.

Voluntary exercise increases weight gain and decreases acoustic startle amplitude.

Mice, housed in groups of four, averaged 14.55±.78 km of wheel running per day (data not shown). As is typical, mice increased wheel activity over days (F(13,26) = 9.79, p<.001), with no significant differences in running observed after the seventh day

of wheel access. A post hoc comparison indicated that mice in the voluntary exercise condition gained more weight than those in the control condition during the fourteen days of wheel access (exercise v control, day 14, $t_{(18)}$=2.73, p=.01).

To assess the anxiety reducing effects of voluntary exercise (Fox et al., 2008; Salam et al., 2009; Hare et al., 2013), mice were tested for acoustic startle before and 14 days after the introduction of the running wheels (Fig.1B). In contrast to the increase in acoustic startle amplitude observed following stress, exercising mice showed lower startle amplitude than control mice at 14 days ($t_{(17)}$=2.51, p=.02), and reduced startle amplitude when compared to their own pre-exercise baseline (day 1 v day 14, $t_{(8)}$=3.37, p=.002).

Stress and exercise regulate p38 MAPK in stress responsive brain areas

To examine the regulation of p38 MAPK by variate stress or voluntary exercise, tissue homogenates were analyzed by western blot. We chose to examine p38 MAPK in stress-sensitive brain regions where p38 immunoreactivity has been previously observed (Lee et al., 2000; Beardmore et al., 2005; Reus et al., 2014; Robles et al., 2014). Analysis of total (t-p38MAPK) and phosphorylated p38 MAPK (p-p38 MAPK) suggests regulation by stress across all sampled regions, while exercise resulted in a more restricted activation of p38 MAPK (Fig.2). Stress resulted in increased p-p38 MAPK (Fig.2A) and t-p38 MAPK (Fig.2B) levels in the amygdala (stress v control, t-p38 MAPK/tubulin, $t_{(6)}$=2.59, p=.03; p-p38 MAPK/tubulin, $t_{(6)}$=2.80, p=.021). Similarly, stress resulted in an increase in t-p38 MAPK and p-p38 MAPK in the BNST (stress v

control, t-p38 MAPK/tubulin, $t_{(6)}$=5.85, p<.001; p-p38 MAPK/tubulin, $t_{(6)}$=4.85, p=.001). Previous research demonstrated no change in p38 MAPK in whole hippocampal homogenates following stress (Li et al., 2009). However, dentate gyrus homogenates in the current study produced significantly increased t-p38 MAPK and p-p38 MAPK levels following stress (stress v control, t-p38 MAPK/tubulin, $t_{(6)}$=4.62, p=.001; p-p38 MAPK/tubulin, $t_{(6)}$=2.91, p=.02). Stress also increased t-p38 MAPK and p-p38 MAPK levels in the frontal cortex (stress v control, t-p38 MAPK/tubulin, $t_{(6)}$=3.04, p=.01; p-p38 MAPK/tubulin, $t_{(6)}$=2.43, p=.04).

In contrast to stress, a limited regulation of p38 MAPK was observed following exercise (Fig.2A,B). Exercise increased levels of t-p38 MAPK as well as p-p38 MAPK in the amygdala (exercise v control, t-p38 MAPK/tubulin, $t_{(6)}$=4.66, p=.001; p-p38 MAPK/tubulin, $t_{(6)}$=3.92, p=.004). Exercise produced a trend toward increased t-p38 MAPK and p-p38 in the BNST (exercise v control, t-p38 MAPK/tubulin, $t_{(6)}$=2.16, p=.06; p-p38 MAPK/tubulin, $t_{(6)}$=1.94, p=.08). In the hippocampus exercise failed to regulate t-p38 MAPK or p-p38 MAPK (exercise v control, t-p38 MAPK/tubulin, $t_{(6)}$=85, p=.42, p-p38 MAPK/tubulin, $t_{(6)}$=.71, p=.50). In the frontal cortex exercise increased t-p38 MAPK though p-p38 MAPK was unchanged (exercise v control, t-p38 MAPK/tubulin, $t_{(6)}$=2.8, p=.02; p-p38 MAPK/tubulin, $t_{(6)}$=1.40, p=.20)

Changes in GSK3β Serine 389 phosphorylation mirror observed increases in p38 MAPK activity following stress and exercise

Catalytic activity of GSK3β is inhibited by phosphorylation. The majority of GSK3β research has focused on inhibition of its activity by N-terminus S^9 phosphorylation. GSK3β C-terminus serine S^{389} phosphorylation was recently demonstrated to be target p38 MAPK (Thornton et al., 2008). Phosphorylation of S^{389} by p38 MAPK was demonstrated to be site specific as pharmacological inhibition of p38 MAPK reduced S^{389} phosphorylation while leaving S^9 phosphorylation unchanged. Here we sought to identify GSK3β S^{389} phosphorylation in brain regions where p38 MAPK regulation was evident following stress or exercise. GSK3β S^{389} phosphorylation has been demonstrated in whole brain homogenate but has yet to be examined in a region specific fashion. GSK3β S^{389} phosphorylation, as well as GSK3β S^9 phosphorylation, was evident by western blot (Fig. 3B). To further confirm our western blot analysis brain areas were examined by immunofluorescence. Fluorescence signal for GSK3β S^{389} and S^9 phosphorylation was evident in all brain areas examined (Fig. 3C).

Following demonstration of GSK3β S^{389} and S^9 phosphorylation in our regions of interest, we aimed to associate S^{389} phosphorylation with stress and exercise p38 MAPK regulation. Increased GSK3β S^{389} phosphorylation was identified in brain regions that demonstrated increased p38 MAPK levels following stress (Fig. 4A). Specifically, increased GSK3β S^{389} phosphorylation was observed in the amygdala (stress v control, $t_{(6)}$=5.06, p=.001), BNST (stress v control, $t_{(6)}$=3.09, p=.01), and hippocampus (stress v control, $t_{(6)}$=3.55, p=.006) following stress. Only in the frontal cortex was an observed increase in p-p38 MAPK not associated with increased GSK3β S^{389} phosphorylation (stress v control, $t_{(6)}$=.99, p=.35) following stress. GSK3β S^{389} regulation by voluntary

exercise was similarly examined (Fig. 4A). Following exercise GSK3β S^{389} phosphorylation was increased only in the amygdala (exercise v control, $t_{(6)}$=3.93, p=.003), though consistent with stress results this was the only brain region in which an increase in p-p38 MAPK was observed following exercise. Notably, a trend towards increased S^{389} phosphorylation was observed in the BNST (exercise v control, $t_{(6)}$=1.86, p=.10). A similar trend towards increased t-p38 MAPK and p-p38 MAPK were observed in the same region.

Regulation S^{9} phosphorylation was observed following both stress and exercise (Fig. 4B). Consistent with previous reports demonstrating the independence of GSK3β S^{9} and S^{389} phosphorylation (Thornton et al., 2008), the pattern of regulation was different when compared to S^{389} phosphorylation and p-p38 MAPK regulation. Reduced GSK3β S^{9} phosphorylation was observed in the amygdala following both stress (stress v control, $t_{(6)}$=-4.13, p=.003) and exercise (exercise v control, $t_{(6)}$=-3.06, p=.014). In contrast, GSK3β S^{9} phosphorylation in the BNST was increased following both stress (stress v control, $t_{(6)}$=4.14, p=.003) and exercise (exercise v control, $t_{(6)}$=4.27, p=.002). Exercise increased GSK3β S^{9} phosphorylation in the frontal cortex ($t_{(6)}$=2.79, p=.02).

Previous work has shown that acute stress (Meller et al., 2003; Shen et al., 2004) does not regulate p38 MAPK, and that increased p-p38 MAPK produced by a pair of forced swim stressors separated by 24 hours is transient, decaying within 6 hours (Bruchas et al., 2007). Similarly, studies have shown that acute restraint stress does not regulate GSK3β (Kozlovsky et al., 2002; Batandier et al., 2014) though these studies were focused on S^{9} phosphorylation. To determine whether the observed increase in

GSK3β S^{389} phosphorylation after stress was due to the repeated stress exposure of the variate stress paradigm or was regulated by the final stressor administered, a cohort of animals was subjected to a single forced swim stressor and tissue was collected 24 hours later. GSK3β S^{389} phosphorylation was unchanged in the amygdala, BNST, hippocampus, or frontal cortex 24 hr following acute swim stress ($t_{(10)}$<1.03, p.34 for all comparisons, data not shown). This finding suggests that the increase in S^{389} phosphorylation observed following multiple stressors is indeed due to variate stress exposure rather than regulation by the final stressor in the paradigm.

Region specific increases in ϒH2A.X are evident following exercise and variate stress

ϒH2A.X is rapidly induced following DNA damage inducing stimuli (Rogakou et al., 1998). p38 MAPK levels are increased by stimuli that produce DNA damage (Mikhailov et al., 2004; Wood et al., 2009; Phong et al., 2010). Active GSK3β has been shown to slow DNA repair (Yang et al., 2011), though GSK3β accumulation in the nucleus following DNA damage inducing stimuli is not associated with increased inhibitory S^{9} phosphorylation (Watcharasit et al., 2002). Thus, the p38 MAPK regulation and increased inhibitory GSK3β S^{389} phosphorylation observed here may be evidence of a response to DNA damage following variate stress or exercise initiated to facilitate DNA repair. Though increased ϒH2A.X was observed in all brain regions following variate stress or exercise, only in the BNST did results achieve significance (Fig. 5A). In comparison to controls, both stress (stress v control, $t_{(6)}$=2.46, p=.04) and exercise (exercise v control, $t_{(6)}$=2.29, p=.05) increased ϒH2A.X in the BNST. To further

understand this observed increase in ϒH2A.X following stress BNST samples from animals subjected to acute forced swim were analyzed for ϒH2A.X and compared to a second cohort of stress animals (Fig. 5B). Consistent with results demonstrating no change in GSK3β S^{389} phosphorylation after acute forced swim, no changes in ϒH2A.X were observed in the BNST 24 hours after acute forced swim (acute stress v control, $t_{(6)}$=.04, p=.97). Increased ϒH2A.X was again observed following the variate stress paradigm (stress v control, $t_{(9)}$=3.16, p=.01), suggesting that DNA damage is accumulating over the variate stress period. Immunoblotting is the least sensitive means of assessing ϒH2A.X accumulation in response to DNA damage (Sharma et al., 2012). ϒH2A.X forms dense foci around DNA damage sites that can be observed by immunofluorescence. Fluorescence images obtained from BNST tissue are consistent with the western blot analysis observation of increased ϒH2A.X signal following stress or exercise (Fig.5C).

Discussion

Cellular stress promotes activity of p38 MAPK though to date the literature on physical and psychological stress regulation of p38 MAPK is limited. The current study demonstrates increased p38 MAPK activity in brain regions associated with stress driven plasticity. Further, we demonstrate that increased GSK3β S^{389} phosphorylation, a recently identified substrate of p38 MAPK, is evident following stress and exercise. Finally, our data are the first to demonstrate that ϒH2A.X an indicator of DNA damage is increased following stress and exercise. These observations are consistent with in-vitro, and peripheral in-vivo work demonstrating a relationship between p38 MAPK and

GSK3β S^{389} phosphorylation (Thornton et al., 2008) but are the first to demonstrate such an association in limbic brain regions in response to challenge.

Consistent with previous reports (Hare et al., 2012; Roman et al., 2012) stress resulted in reduced weight gain and increased acoustic startle amplitude, suggestive of an increased depressive and anxiety-like phenotype following stress. In contrast, voluntary exercise reduced acoustic startle amplitude suggestive of reduced anxiety. We observed increased t-p38 MAPK and p-p38 MAPK largely in stressed mice. Stress associated increases in GSK3β S^{389} phosphorylation were observed in the amygdala, BNST, and hippocampus of stressed mice, brain regions that also demonstrated increased p38 MAPK activity. In contrast, acute stress produced no change in GSK3β S^{389} phosphorylation in the brain regions examined. While increased ϒH2A.X was observed in multiple brain regions only in the BNST was ϒH2A.X significantly increased. Surprisingly, this increase was evident following stress and exercise. Consistent changes in GSK3β S^{9} phosphorylation were observed following stress and exercise in the BNST, and amygdala though regulation was directionally different with increased GSK3β S^{9} evident in the BNST and decreased S^{9} evident in the amygdala. The differences observed in GSK3β S^{9} and S^{389} regulation provide in-vivo support for previous work suggesting the independence of activity across the pair of phosphorylation sites (Thornton et al., 2008).

p38 MAPK activity plays a role in a number of cellular responses associated with stress pathology including synaptic destabilization (Collingridge et al., 2010) and inflammation (Correa and Eales, 2012). Stress has previously been demonstrated to increase phosphorylated p38 MAPK immediately following a series of forced swim

exposures over 48 hours (Bruchas et al., 2007). Here we observed increased p38 MAPK activity following stress in the amygdala, BNST, and hippocampus. As opposed to the previous studies where phosphorylated p38 MAPK immunoreactivity was elevated for approximately 6 hours, the increased immunoreactivity present here was observed 24 hours after the final stress exposure demonstrating sustained activation of p38 MAPK following chronic challenge. Inhibition of p38 MAPK has been demonstrated to reduce the negative behavioral effects of acute stress (Bruchas et al., 2007; Sharma et al., 2011) and serotonin specific knock out of p38 MAPK is protective against depression like behavior in a social stress model (Bruchas et al., 2011) suggesting that inhibition of p38 MAPK may have therapeutic benefit in stress associated disorders. However, numerous reports suggest that p38 MAPK activity is increased in response to stimuli that induce DNA damage, and that such activity may be protective (Phong et al., 2010; Tan et al., 2014; Wang et al., 2014) potentially through GSK3β inhibition (Yang et al., 2011). The findings reported here, and previous work demonstrating that GSK3β inhibitory S^{389} phosphorylation (Thornton et al., 2008), and basal p38 MAPK activity (Lee et al., 2000), are elevated in brain compared to peripheral cells, suggests that the association between p38 MAPK and GSK3β S^{389} phosphorylation may be protective in central nervous system cells that are not subject to replacement if lost. Further work is clearly necessary to understand this novel association between these multifunctional kinases, as well as how extracellular and intracellular stress signals may differentially modulate their individual and associated activity.

That both stress and exercise were associated with DNA damage as indicated by ϒH2A.X expression is novel and deserves further investigation. Experience associated transcription and translation in neurons results in high rates of metabolism, mitochondrial activity, and associated reactive oxygen species that may damage mitochondrial and neuronal DNA (Barzilai et al., 2008). Unrepaired DNA damage may result in cell death, and accumulated DNA damage is hypothesized to underlie numerous pathologies (Brasnjevic et al., 2008). However, a recent report of DNA damage in activated neuronal populations postulated that the repair process may play a necessary role in chromatin remodeling and resulting experience associated modification of gene expression (Suberbielle et al., 2013). Such epigenetic modulation is likely to play an important role in stress outcomes (Covington et al., 2009; Vialou et al., 2013) and is also evident following voluntary exercise (Gomez-Pinilla et al., 2011; Intlekofer et al., 2013). We observed the strongest increase in ϒH2A.X within the BNST, a site of robust plasticity during stress exposure (Vyas et al., 2003), and voluntary exercise (Greenwood et al., 2005a), and therefore a region that may be particularly susceptible to neuronal activity associated DNA damage. Directly tying p38 MAPK inhibition of GSK3β to the DNA damage response, as well as determining whether such damage is playing a role in stress or exercise associated behavioral outcomes are compelling targets for further investigation.

Our results suggest that DNA damage was accumulating in stressed animals as chronic but not acute stress exposure increased ϒH2A.X signal. It should be noted that exercise was not terminated 24 hours prior to tissue harvest as stress exposure was, and

damage after an acute bout of exercise was not measured. Therefore, it cannot be suggested that DNA damage is accumulating in exercising animals, only that increased signal associated with damage was present. Voluntary exercise in rodents takes place during the dark phase of the circadian cycle and therefore would be expected to terminate approximately five to seven hours prior to tissue harvest. Thus, in animals that exercised it is possible that elevated ϒH2A.X signal represents a response to the prior period of running rather than an accumulation of DNA damage. There is evidence that locking a wheel 24 hours prior to tissue harvest may itself be stressful (Greenwood et al., 2012a) therefore time-locking of the manipulations utilized here may itself confound results. To our knowledge this is the first demonstration of increased DNA damage following exercise, though it is notable that recent work suggests that the commonly observed increase in brain derived neurotrophic factor following exercise was recently associated with increased transcription of AP endonuclease 1, a protein associated with DNA repair, in exercising animals (Yang et al., 2014). Thus, while exercise may produce DNA damage it is conceivable that the same activity may result in more efficient repair. Indeed stress hormones are associated with slowed DNA repair in-vitro (Flint et al., 2007) and thus the time course of repair may be expected to be different following stress or exercise. Work is currently underway in our laboratory to assess changes in the time course of DNA repair following stress or exercise.

Here we have demonstrated an anxiogenic effect of stress and anxiolytic effect of exercise though protein regulation was similar in many cases. It is important to consider that while stress and exercise produce divergent behavioral outcomes, as demonstrated

here, each is a metabolic challenge that activates the hypothalamic pituitary adrenal (HPA) axis though activation by each challenge is associated with opposite hedonic states (Wosiski-Kuhn and Stranahan, 2012). Exercise is associated with reduced anxiety and depression (Binder et al., 2004; Salam et al., 2009; Sciolino et al., 2012), while activation of the HPA axis in a negative context is associated with increased anxiety and depression (Li et al., 2011). These findings suggest experiences, both positive and negative, may act through similar systems to produce divergent behavioral outcomes based on the contextual milieu associated with the experience. Each of the kinases examined here is known to provide a regulatory hub for convergent signaling pathways upstream of transcription factor activation. Plasticity following transcription factor activation brought about by stress or exercise could be gated in a fashion that is dictated by the respective experience. For example, anxiogenic and anxiolytic behavioral effects in the BNST have been observed through optical stimulation of glutamatergic and GABAergic projection neurons respectively (Jennings et al., 2013). Identifying the neuronal populations affected by exercise and stress manipulations would provide clarification of these effects that tissue punches and western blot analysis fail to capture.

The research presented here demonstrates stress regulation of p38 MAPK activity across stress-sensitive brain regions. Further, we demonstrate that the understudied GSK3β S^{389} phosphorylation site is regulated in a fashion consistent with p38 MAPK activity. Finally, our results are the first to demonstrate DNA damage, by stress and exercise, in a brain region that is known to be involved in stress and exercise associated plasticity. Understanding the role the described pathway plays in behavioral outcomes

following stress or exercise, as well as the DNA damage response will require a better understanding of kinases involved. Further work is necessary to delineate the causes of DNA damage in stress and exercise, and demonstrate that inhibition of p38 MAPK activity or GSK3β S^{389} phosphorylation exacerbates challenge associated DNA damage. Additionally an understanding of whether a specific p38 MAPK isoform, for example p38β which is preferentially expressed in the nucleus (Lee et al., 2000), is driving the increase in p38 MAPK activity observed here requires additional work.

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Figures

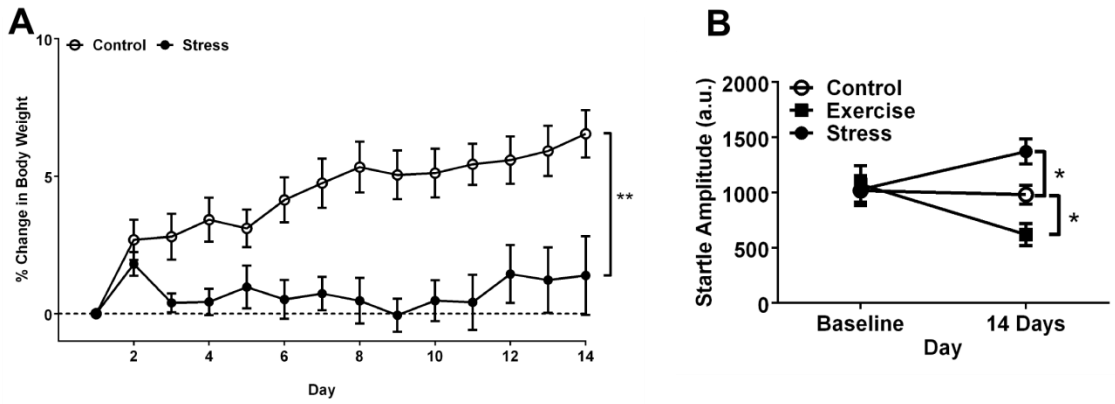

Figure 1. Stress reduces weight gain, exercise and stress differentially affect startle amplitude. **A,** Effect of variate stress (stress) on body weight over the 14 day variate stress period. **B,** Opposing effect of stress or voluntary exercise on acoustic startle amplitude. Data are means ± SEM, **p<.01 v control, *p<.05 v control

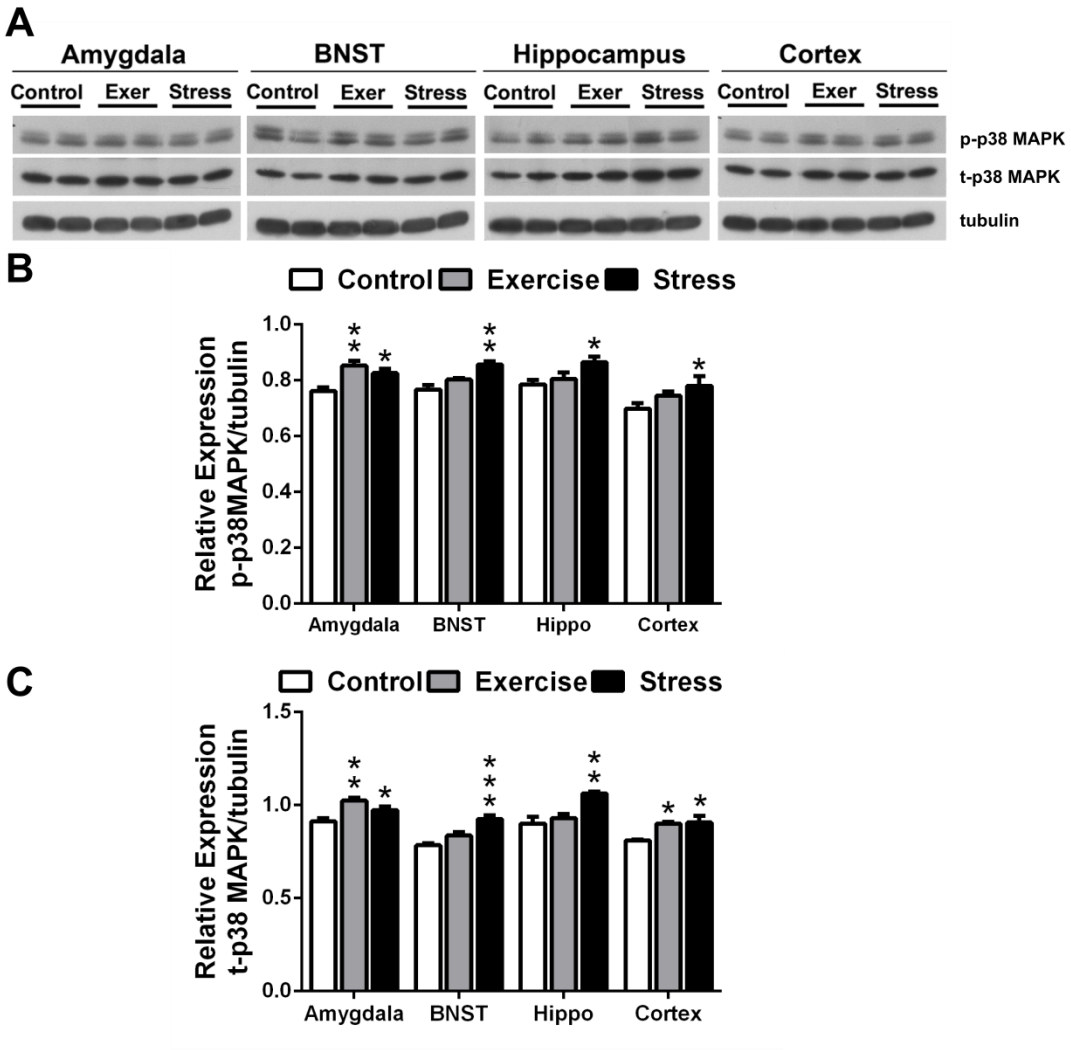

Figure 2. Regulation of p38 MAPK by stress and exercise. **A.** Representative western blots from analysis of phosphorylated p38 MAPK (p-p38 MAPK), total p38 MAPK (t-p38 MAPK), and α-tubulin (tubulin) in control, voluntary exercise (exer), and variate stress (stress) animals. **B.** Comparison of relative p-p38 MAPK immunoreactivity in regional lysates following exercise and variate stress. **C.** Comparison of relative t-p38 MAPK immunoreacitivty in regional lysates following exercise and variate stress. All data are Mean ± SEM, ***p<.001 v control, **p<.01 v control, *p<.05 v control.

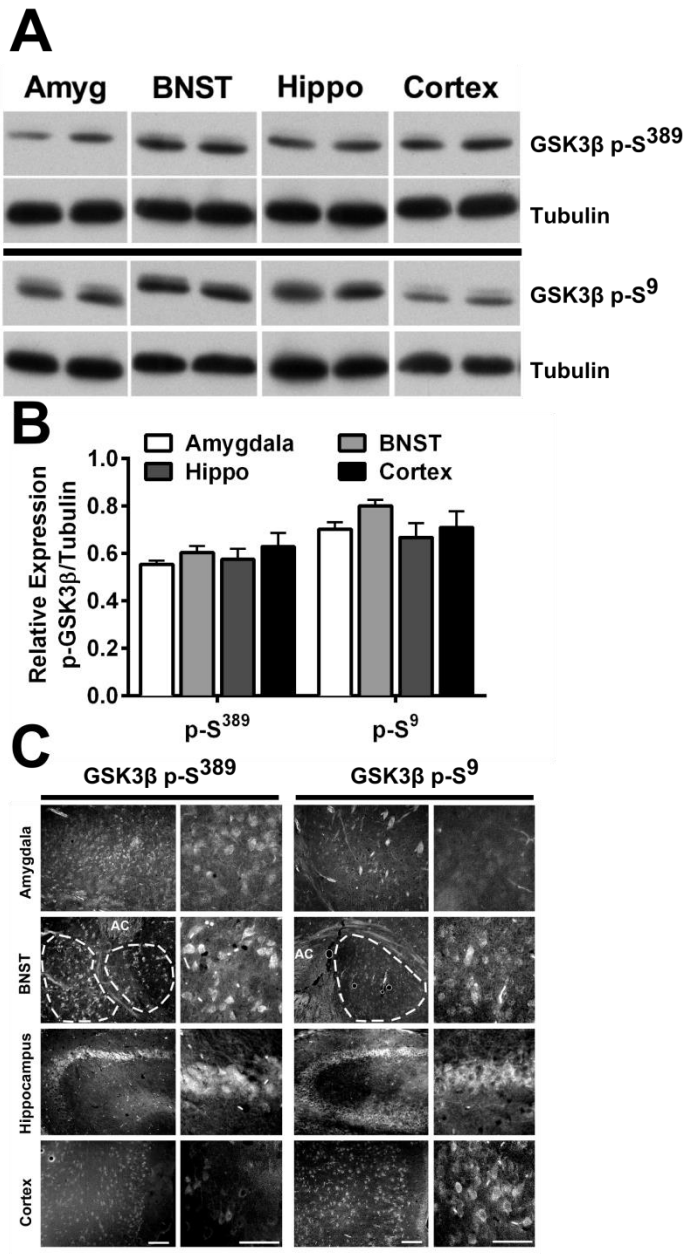

Figure 3. Demonstration of GSK3β phosphorylation in amygdala, BNST, hippocampus, and frontal cortex. **A.** Representative western blot samples from analysis of GSK3β S^{389} phosphorylation (p-S^{389}), and S^{9} phosphorylation (p-S^{9}), α-tubulin loading control (tubulin). **B.** Relative GSK3β p-S^{389} and S^{9} expression across sampled brain regions. **C.** Immunofluorescence images confirming GSK3β p-S^{389} and S^{9} expression in sampled brain regions. Left panel for each phosphorylation site is representative 20x magnification image, right panel is 40x magnification of same image. Scale bar = 50μM.

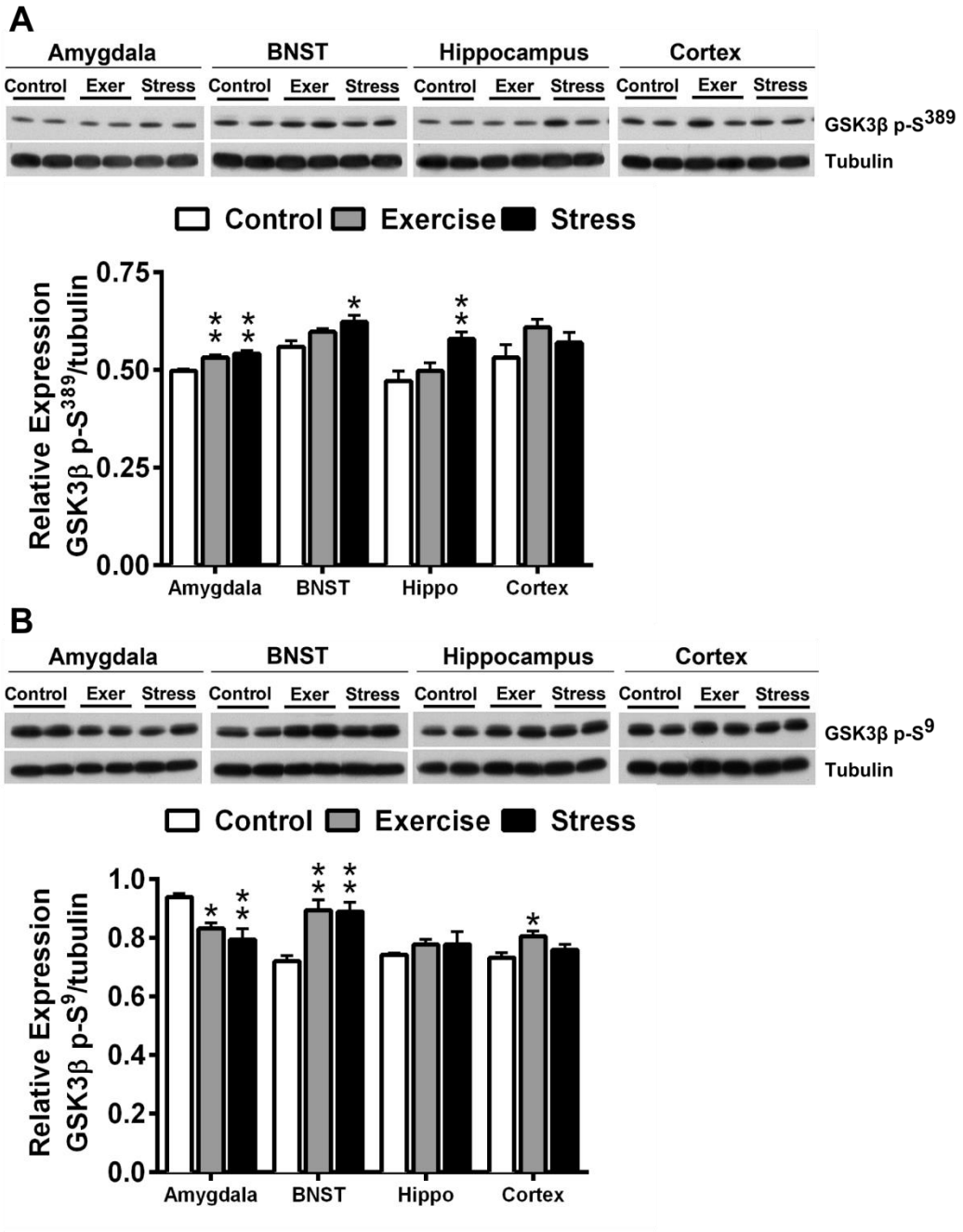

Figure 4. GSK3β phosphorylation state analysis by western blot following stress or exercise. **A.** Representative western blot samples relative expression from regional analysis of GSK3β S^{389} phosphorylation (p-S^{389}). **B.** Representative western blot samples and relative expression from regional analysis of GSK3β S^{9} phosphorylation (p-S^{9}). All samples normalized to α-tubulin (tubulin) for analysis. Data are Mean ± SEM, **p<.01 v control, *p<.05 v control.

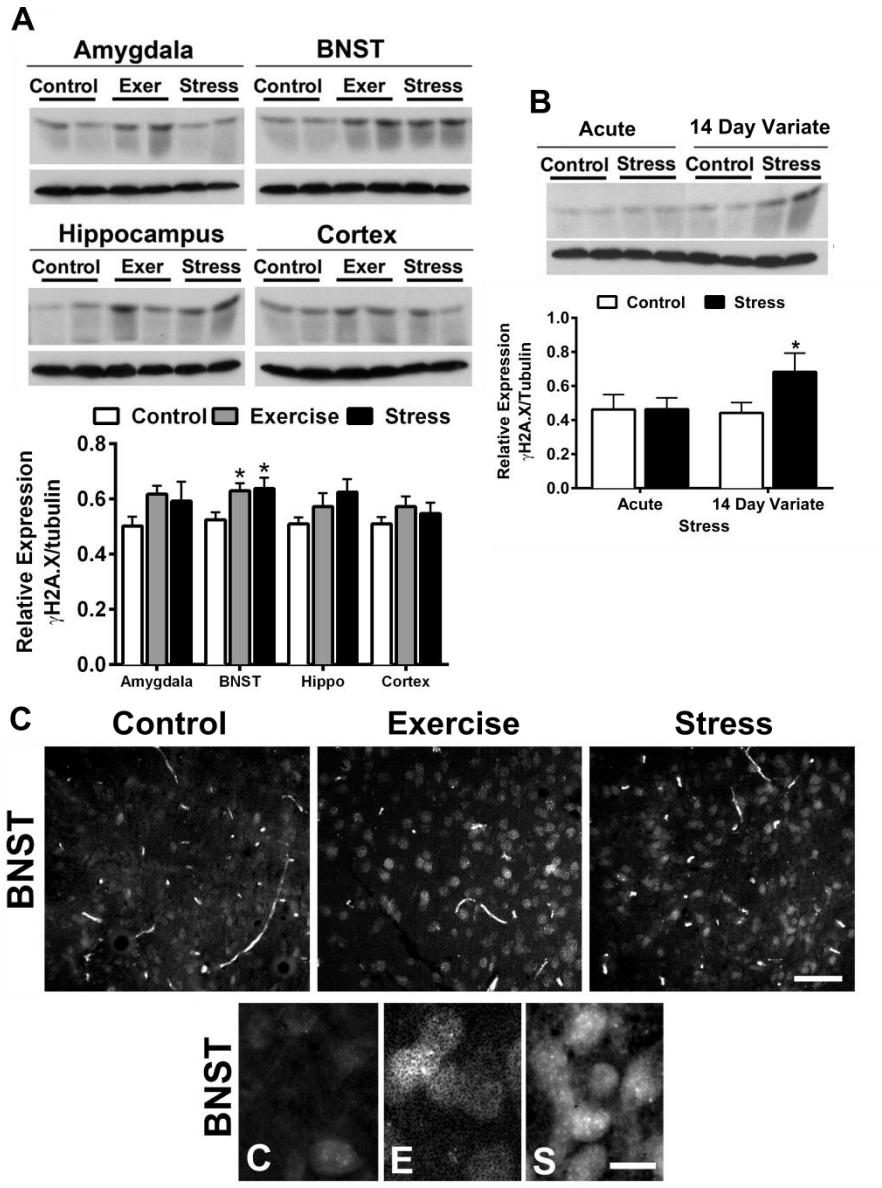

Figure 5. Evidence for DNA damage following stress and exercise. **A.** ϒH2A.X analysis by western blot following variate stress (stress) or exercise. A. ϒH2A.X analysis by western blot following variate stress (stress) or exercise. **B.** A comparison of ϒH2A.X 24 hours after an acute forced swim experience or 14 days of variate stress terminating with forced swim. **C.** ϒH2A.X foci that reliably mark DNA damage can be observed in representative BNST immunofluorescence images following variate stress (stress) or exercise confirming western blot analysis. All samples normalized to α-tubulin (tubulin) for analysis. Data expressed as mean ± SEM. *p<.05 v control. Fluorescence images are 20x magnification with expanded sample. Scale bar = 50μM.

CHAPTER 4: GENERAL DISCUSSION

The initial investigation into inhibition of GSK3β by S^{389} phosphorylation produced findings that made understanding regulation of S^{389} phosphorylation a compelling research target (Thornton et al., 2008). First, inhibition of GSK3β through S^{389} phosphorylation was found to be mediated by p38 MAPK rather than AKT, which is known to phosphorylate the S^{9} site (Stambolic and Woodgett, 1994) indicating that the two sites may be regulated by separable signaling pathways activated by similar, or different, stimuli. Second, GSK3β S^{389} phosphorylation was especially apparent in brain tissue suggesting a specialized role in the central nervous system. Finally, inhibition of GSK3β by S^{389} phosphorylation was correlated with accumulation of β-catenin suggesting that S^{389} phosphorylation may play a role in cell survival. The findings reported here further suggest that GSK3β S^{389} phosphorylation may be involved in neuroprotection. GSK3β S^{9} and S^{389} phosphorylation were found to have different cellular distributions. GSK3β S^{389} phosphorylation was found to have a nuclear distribution, while S^{9} phosphorylation was apparent throughout the cell. GSK3β activity was found to be increased in GSK3β KI animals, and this was associated with a pattern of neurodegeneration in the hippocampus and cortex that was not evident in WT mice. These findings suggest a neuroprotective role for S^{389} phosphorylation within the central nervous system. Despite the observed hippocampal degeneration, GSK3β KI mice were found to perform normally in the water maze, a task that requires the hippocampus (Morris et al., 1982). However, auditory fear conditioning was exaggerated and contextual fear was overgeneralized in GSK3β KI mice suggesting that the

neurodegeneration observed may influence fear conditioning circuitry perhaps through hippocampal connections with the amygdala (Maren, 2001).

The auditory fear conditioning phenotype observed in GSK3β KI animals was similar to what might be expected in stressed animals (Izquierdo et al., 2006; Baran et al., 2009; Hoffman et al., 2014). The effect of stress on GSK3β S^{389} phosphorylation had not been examined, nor had regulation of GSK3β by stress been examined outside of the hippocampus or frontal cortex. This paucity of research prompted assessment of GSK3β regulation by stress, and exercise, a behavior with stress protective effects, in brain regions associated with regulation of the stress response. We observed that two weeks of variate stress, and exercise, produced divergent effects on anxiety-like behavior, but regulated GSK3β phosphorylation in a largely similar fashion. Notably, stress and exercise increased GSK3β S^{389} phosphorylation. GSK3β S^{9} phosphorylation was positively, and negatively, regulated in a region-dependent manner further supporting separable roles for the inhibitory phosphorylation sites. Phosphorylated p38 MAPK would be expected to mediate the increase in GSK3β S^{389} phosphorylation and indeed, we observed an increase in p38 MAPK phosphorylation in brain regions where S^{389} phosphorylation was increased. Previous work suggests that DNA damage may produce a cellular context in which p38 MAPK mediated inhibition of GSK3β would promote neuronal survival (Watcharasit et al., 2002; Wood et al., 2009). ϒH2A.X, a marker of DNA damage (Rogakou et al., 1998), was found in both stressed and exercising animals in brain regions where GSK3β S^{389} phosphorylation was elevated, suggesting that the increased inhibition may be a response to DNA damage. We observed no change in

GSK3β S^{389} phosphorylation or ϒH2A.X signal when animals were exposed to a single stressor indicating the increased ϒH2A.X signal observed after two weeks of stress or exercise may represent an accumulation of DNA damage that necessitates increased GSK3β S^{389} phosphorylation to prevent neurodegeneration as observed in GSK3β KI animals.

Incorporation of GSK3β KI neurodegeneration and the observed regulation of GSK3β by stress and exercise

The results reported here suggest that deficient inhibition of GSK3β through S^{389} phosphorylation results in neurodegeneration. Importantly, fluoro jade labelled degeneration occurs prior to cell death, and can represent shrinkage of cell bodies and cellular processes (Ehara and Ueda, 2009). Stress exposure is regularly associated with retractions of the dendritic arbor, spine loss, and labeling of shrunken neurons (Uno et al., 1989; Watanabe et al., 1992; Magarinos and McEwen, 1995), suggesting that deficient GSK3β S^{389} phosphorylation may play a role in stress associated plasticity. This loss of complexity is particularly evident in the hippocampal CA3 field, a region in which we observed evidence of neurodegeneration in GSK3β KI mice. Previous research supports a role for loss of trophic factor support in the morphological changes brought about by stress (Duman and Monteggia, 2006; Magarinos et al., 2011). On the other hand, exercise enhances expression of multiple trophic factors (Neeper et al., 1996; Llorens-Martin et al., 2010), and is associated with structural changes in neurons that oppose those of stress (Yau et al., 2011; Baj et al., 2012). If GSK3β S^{389} phosphorylation plays a role in the dendritic remodeling associated with stress and exercise, one might predict

that exercise would result in decreased GSK3β activity by increasing S^{389} phosphorylation to promote plasticity, while the loss of trophic support in stressed animals would be associated with increased GSK3β activity through reduced S^{389} phosphorylation. However, our results demonstrate that 14 days of stress or exercise are each associated with increased GSK3β S^{389} phosphorylation. Thus it seems that GSK3β inhibition is a neuroprotective response to the increase in DNA damage evident in both conditions (Fig. 1).

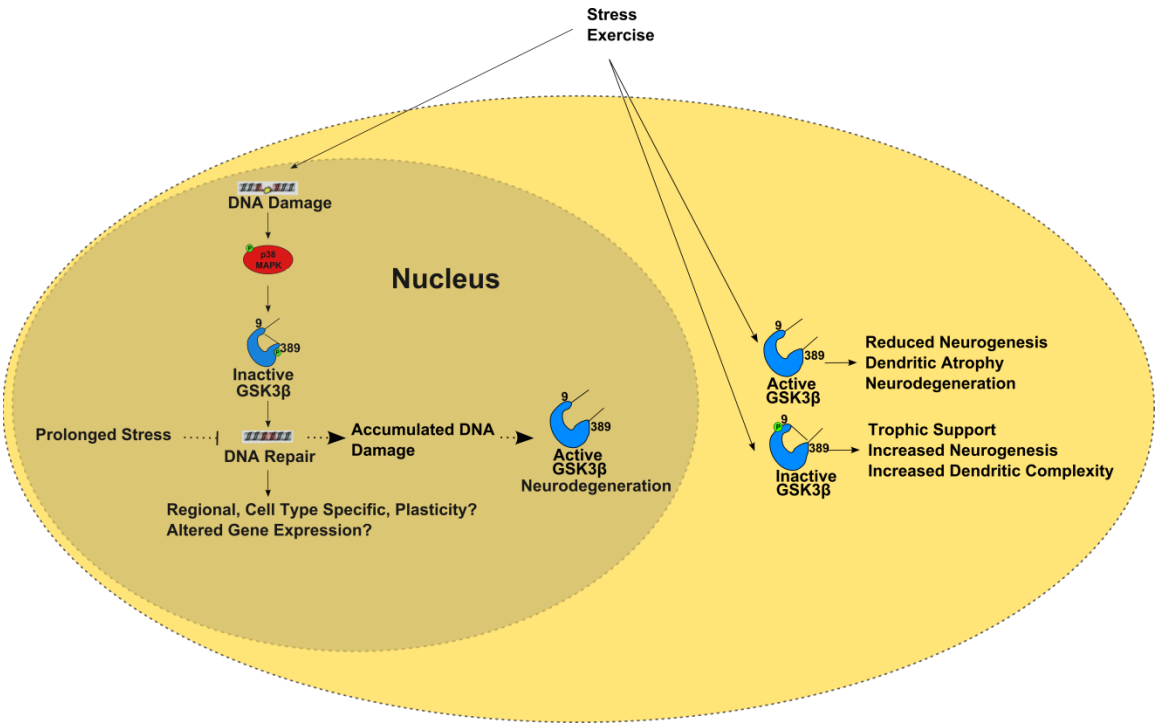

Figure 1. Regulation of GSK3β by stress or exercise. DNA damage signaling further inhibits nuclear GSK3β through p38 MAPK phosphorylation of the S389 site. Under prolonged conditions that inhibit DNA repair DNA damage may accumulate leading to neurodegeneration (dashed arrows). Cytoplasmic GSK3β S9 phosphorylation is regulated in a region specific fashion that may lead to changes in cellular plasticity.

The underlying cause of dendritic remodeling by stress is not known though excitotoxicity, mitochondrial dysfunction, altered gene transcription and exposure to reactive oxygen species have been proposed (Sapolsky, 2000; Duman and Monteggia,

2006; Popoli et al., 2012; Picard et al., 2014). Neuronal activity, high rates of metabolism and associated reactive oxygen species, and experience driven gene transcription have similarly been proposed to produce DNA damage suggesting that such damage might be a central factor in neuronal degradation (Brasnjevic et al., 2008; Barzilai, 2010; Suberbielle et al., 2013; Aymard et al., 2014). Accumulated DNA damage and impaired repair processing are thought to underlie a number of neurodegenerative disorders (Brasnjevic et al., 2008) though an association with stress has not previously been made. Increased ϒH2A.X, a marker of DNA damage, was observed following exercise and stress, though only in the stress condition can we say that DNA damage accumulated as 14 days, but not 1 day, of stress resulted in increased levels of ϒH2A.X. The damage associated with exercise may reflect the previous night's activity rather than an accumulation of damage over the 14 day wheel access period. In either case, the suggestion would be that failure to repair the damage may eventually lead to the neuronal degradation observed in GSK3β KI mice (Fig. 1).

Elevated levels of ϒH2A.X in exercise and stress conditions may be induced by a similar mechanism, experience associated neuronal activation, metabolism, and gene transcription, but the repair outcome may be quite different. The cellular context associated with stress has previously been suggested to impair DNA repair (Jenkins et al., 2014), and in contrast, that associated with exercise may enhance repair. BDNF, which is elevated in exercising mice (Neeper et al., 1996), has been associated with more rapid DNA repair (Yang et al., 2014). Exercising animals terminate stress responses more rapidly (Hare et al., 2013), and habituate to stressors faster (Sasse et al., 2008; Campeau

et al., 2010; Nyhuis et al., 2010), suggesting reduced exposure to stress hormones which have been demonstrated to slow DNA repair (Flint et al., 2007). In contrast, stress reduces BDNF (Nibuya et al., 1995), and impairs the organisms ability to limit the stress response (O'Connor et al., 2003; Mizoguchi et al., 2008) potentially setting up a situation in which DNA repair is less efficient and more likely to lead to degeneration. A purposeful physiological process may represent a second similar mechanism to account for the DNA damage signal observed following stress and exercise. Induction of DNA damage has been proposed to represent a physiological process through which gene transcription may be regulated by experience (Suberbielle et al., 2013; O'Hagan, 2014). Such purposeful damage could lead to opposing patterns of gene expression following stress and exercise (Duman and Monteggia, 2006) while providing a similar ϒH2A.X signal.

The degeneration observed in GSK3β KI animals led to an initial hypothesis that stress would lead to a reduction in GSK3β S^{389} phosphorylation. If deficiency in GSK3β S^{389} phosphorylation does lead to stress associated changes in neuronal morphology in response to DNA damage, it is possible that the 14 day stress protocol employed may not have been sufficient to result in the type of structural changes seen with longer stress protocols. Three, but not two, weeks of stress has been shown to produce dendritic remodeling in the hippocampus (Magarinos and McEwen, 1995). Three week protocols also result in stress associated changes in neuronal morphology in the frontal cortex (Radley et al., 2006; Radley et al., 2008). However, a single stressor has been demonstrated to alter spine morphology in the frontal cortex (Sciolino et al., 2015),

though this may represent a more subtle effect than the changes in dendritic arbor observed after longer stress paradigms. If stress impairs DNA repair processes (Jenkins et al., 2014), and sufficient accumulation of DNA damage leads to neurodegeneration (Brasnjevic et al., 2008), one may expect that stress paradigms of sufficient duration may eventually lead to the increased nuclear GSK3β activity observed in in-vitro neuronal studies of DNA damage associated apoptotic signaling (Watcharasit et al., 2002). Indeed, accumulated DNA damage may very well be a component of the allostatic load that stress places on the nervous system and eventually result in initiation of degenerative signaling cascades of which active GSK3β would likely be a part. These cascades could be apoptotic in nature (Watcharasit et al., 2002), or result in GSK3β activation of proteins such as tau that can function to destabilize structural proteins (Hanger et al., 1992; Lucas et al., 2001). In contrast, in the pro-repair environment of an exercising animal DNA damage may not accumulate sufficiently to lead to GSK3β activation.

It should also be acknowledged that the result of neuronal remodelling need not be negative. Research into the beneficial effects of exercise has focused on increased neuronal complexity and increased trophic factor support, while that into stress has largely focused on the opposite. However, there is evidence that stress associated changes are regionally specific and can produce the type of physiological changes typically associated with exercise. Stress increases BDNF expression and dendritic complexity in the BNST (Vyas et al., 2003; Hammack et al., 2009), while having degenerative effects on frontal cortex projections to the BNST (Radley et al., 2013). Increased amygdala activation is observed in patients with stress associated disorders yet

in many cases decreased volume is observed (Savitz and Drevets, 2009). In these cases it is unclear whether the decreased volume is a developmental outcome that predisposes for the disorder or is caused by the disorder, but the possibility is present that the observed increase in activity is the result of degeneration in an as yet unknown neuronal subtype. A very complex pattern of results can be seen in stress associated amygdala plasticity that seems to be stressor and neuronal subtype specific (Vyas et al., 2002), and can result in increased and decreased complexity. Changes in neuronal complexity associated with exercise have focused on increased complexity in the hippocampus and frontal cortex as mediating the beneficial effects of exercise (Yau et al., 2011; Sciolino et al., 2015). However, posterior hypothalamic, periaqueductal grey, and nucleus tractus solitaris dendritic complexity is observed to be reduced in animals that exercise as adults (Nelson et al., 2010). This reduced dendritic complexity is thought to contribute to the beneficial effect of exercise by reducing sympathetic tone. However, it is notable that the brain regions involved play a role in defensive behaviors, and have projections to areas that control the stress response such as the BNST and amygdala (Bienkowski and Rinaman, 2013). Thus, should DNA damage be associated with neuronal degradation following stress and exercise, the functional outcome of such changes should be examined in a region and cell type specific fashion before the finding is interpreted in a negative or positive light.

Regulation of p38 MAPK by stress

While the finding of increased DNA damage and regulation of associated second messengers suggests a specific, protective role for nuclear p38 MAPK activity in

inhibiting GSK3β by S^{389} phosphorylation, as with GSK3β, consideration of the localization of p38 MAPK's regulation by stress is warranted. We observed a robust increase in both total and phosphorylated p38 MAPK levels across all brain regions examined in the stress group, and a much more limited increase in exercising mice. p38 MAPK has multiple isoforms that have been reported to display different distributions (Lee et al., 2000). p38α MAPK is thought to inhibit GSK3β by S^{389} phosphorylation (Rincon and Thornton unpublished observations). p38α is localized in the nucleus as well as the cytoplasm (Lee et al., 2000). Synaptic p38 MAPK signaling is implicated in the depressive and anxiogenic effects of acute stress exposure. Indeed, deletion of p38α MAPK in the dorsal raphe was shown to reduce social avoidance, a measure of depression, after social defeat (Bruchas et al., 2011). Inhibition of p38 MAPK similarly attenuated the increase in forced swim immobility time observed 24 hours after an initial swim exposure (Bruchas et al., 2007). The effect of p38 MAPK inhibition was likely due to disrupting second messenger signaling occurring after activation of kappa opioid receptors by stress (McLaughlin et al., 2003; Bruchas et al., 2006; McLaughlin et al., 2006a; McLaughlin et al., 2006b; Bruchas et al., 2007). These findings, suggest that understanding the role of p38 MAPK in mediating stress effects, such as the changes in anxiety-like behavior or changes in GSK3β phosphorylation observed here, will require an understanding of how stress signals are interacting with p38 MAPK in a location specific fashion.

We observed a stress-like phenotype in GSK3β KI animals given auditory fear conditioning. As p38 MAPK is the only known regulator of GSK3β S^{389}

phosphorylation, p38 MAPK activity increases GSK3β S389 phosphorylation levels (Thornton et al., 2008), it was expected that inhibition of p38 MAPK by central administration of SB203580 in WT mice would replicate the exaggerated, and overgeneralized fear observed in GSK3β KI mice that cannot phosphorylate S^{389}. We observed no change in fear conditioning when 125uM SB203580 was administered prior to or immediately following fear conditioning. However, we observed no change in GSK3β S389 phosphorylation 30 minutes after SB203580 administration in the hippocampus or amygdala. As such, this result is inconclusive as to the effect of p38 MAPK inhibition on fear conditioning, as well as the role GSK3β S^{389} phosphorylation might play in fear conditioning. Bruchas et. al. (2007) disrupted stress effects with a 25nM ICV injection of SB203580 along a similar time-course and volume, and Gonzalez et. al. (2013) observed a facilitation of fear conditioning after IL-1β challenge when 125uM SB203580 was administered directly into the hippocampus. In contrast, impaired contextual discrimination was observed when ~2mM SB203580 was administered into the hippocampus prior to habituation, conditioning, and testing. These findings demonstrate that the effect of p38 MAPK on behavior is likely specific to injection timing, dose, region, and cellular context. As such, identification of an injection protocol that alters GSK3β S^{389} phosphorylation is valuable as it provides a target p38 MAPK substrate that has been identified to play a role in fear behavior. Disruption of GSK3β phosphorylation by p38 MAPK was observed after a series of injections (Rincon and Thornton personal communications). A new fear conditioning series is underway that will examine whether such a protocol will reduce GSK3β S^{389} phosphorylation and mimic the GSK3β KI phenotype. Alternatively, the fear phenotype observed in GSK3β

KI animals may be due to developmental or compensatory changes that occur with deficient S^{389} phosphorylation. Future work is necessary to determine what those changes might be, but it is known that similar genetic manipulations to block GSK3β S^{9} phosphorylation paradoxically increase BDNF levels (Prickaerts et al., 2006) suggesting that compensatory changes may be similarly taking place in GSK3β KI animals.

Conclusion

The set of data presented here suggests a fundamentally different role for GSK3β S^{389} and S^{9} inhibitory phosphorylation. Analysis of GSK3β KI animals demonstrated a behavioral phenotype that was dissimilar to that reported in animals with deficient S^{9} phosphorylation. Regulation of GSK3β phosphorylation by stress and exercise also demonstrated that S^{9} regulation was more dynamic, showing regional increases and reductions, while S^{389} levels increased, in association with a pathway likely initiated by DNA damage. Given that GSK3β has well over 50 substrates (Kaidanovich-Beilin and Woodgett, 2011), this work provides an exciting avenue to pursue a therapeutic target that may serve to regulate a smaller portion of GSK3β activity.

The necessity of targeting the cellular pathway directly associated with the therapeutic need being addressed is exemplified in findings assessing the effects of the GSK3β ATP competitive inhibitor SB216763 that would limit GSK3β activity indiscriminately. The actions of SB216763 are neuroprotective when co-infused with an amyloid-beta peptide that mimics some neurodegenerative aspects of Alzheimer's disease, but produce a similar phenotype to the amyloid-beta peptide when injected alone

(Hu et al., 2009). Similarly, the rapid-antidepressant actions of ketamine are enhanced by GSK3β inhibition (Beurel et al., 2011; Liu et al., 2013), but GSK3β inhibition alone does not produce beneficial effects when initiated after stress exposure (Wilkinson et al., 2011; Ma et al., 2013). These findings suggest that modulation of GSK3β needs to be taking place on an active pathway at the time of insult, rather than broadly administered after the fact. Lithium, a compound that directly inhibits GSK3β activity only in part due to inhibitory S^9 phosphorylation (De Sarno et al., 2002), is used as a treatment for bipolar depression and has protective effects against development of dementia and Alzheimer's disease in depressed patients when compared to disease rates in depressed patients that did not take lithium (Nunes et al., 2007; Kessing et al., 2010) . Each of these disorders displays neurodegenerative characteristics (Sheline et al., 1999; Vakili et al., 2000; Savitz and Drevets, 2009; Medina et al., 2011), suggesting that similar pathways may be engaged. Given the findings reported here it is intriguing to consider that the direct inhibition of GSK3β by lithium may be in part targeting that portion of GSK3β activity regulated by S^{389} phosphorylation, and that a S^{389} specific inhibitor may provide a better therapeutic target.

The foundational work reported here demonstrates that dysregulated inhibition of GSK S^{389} phosphorylation may play an important role in neurodegenerative disorders. Further studies would benefit from the ability to conditionally manipulate GSK3β S^{389} phosphorylation to ensure similar developmental backgrounds between subjects. Direct comparisons between similar manipulations of the GSK3β S^9 phosphorylation site would help to clarify the individual roles of the inhibitory phosphorylation sites. Though

research into multifunctional protein kinases is complex, utilizing tools that allow a clearer dissection of activity in neuronal subtypes, or cellular compartments, of interest will allow for clearer interpretation of results. Given the number of disorders associated with neuronal degradation continued investigation into the role of GSK3β S^{389} phosphorylation in the brain is warranted.

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