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Elucidating the role of GSK3 in synaptic and cognitive deficits in Fragile X Syndrome

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ELUCIDATING THE ROLE OF GSK3 IN SYNAPTIC AND COGNITIVE DEFICITS IN FRAGILE X SYNDROME

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ABSTRACT

Fragile X Syndrome (FX) is the most common inherited form of mental retardation. Prominent characteristics of FX are mimicked in a mouse model with deleted *fmr1* including hyperactivity, anxiety, developmental delay and social deficits. Additionally, FX mice display deficits in n-methyl-d-aspartate receptor (NMDAR) dependent long-term potentiation (LTP) at medial perforant path synapses onto dentate granule cells (MPP-DGC synapses). Because LTP is a cellular correlate of learning and memory, deficits in LTP at this synapse are thought to underlie impairments in pattern separation, a form of learning and memory dependent on proper DG function. Identifying the pathological mechanisms that cause impaired synaptic plasticity will aid in the development of novel therapeutic targets and treatments in FX.

The activity of glycogen synthase kinase-3 (GSK3) is enhanced in hippocampus of FX mice. Accordingly, treatment with the GSK3 inhibitor lithium corrects several behavioral phenotypes in FX mice. In order to investigate whether hyperactive GSK3 is causally related to the deficit in LTP at MPP-DGC synapses, we utilized FX mice and assessed the magnitude of LTP in the presence of lithium and CT99021, GSK3 inhibitors. We find that acute inhibition of GSK3 reverses deficits in LTP and steady-state depolarization (SSD) during high frequency stimulation but fails to rescue NMDAR hypofunction during SSD. Importantly, blockade of mGluRs, which rescues many of the phenotypes in FX mice, fails to rescue LTP at MPP-DGC. In order to identify whether HDAC3, a

downstream target of GSK3, contributes to LTP deficits in FX mice we assessed the magnitude of LTP in the presence of a selective HDAC3 inhibitor. We find that HDAC3 inhibition fails to rescue LTP deficits in FX mice. However, non-selective HDAC inhibition and selective HDAC3 inhibition enhance LTP at MPP-DGC synapses in WT mice. These results suggest that FMRP is required for enhanced LTP following HDAC inhibition. Overall, these findings establish that synaptic deficits in FX can be reversed by interventions with GSK3 inhibitors, which may prove beneficial in the treatment of cognitive symptoms in FX. Furthermore, these findings identify a novel role for FMRP which may lead to a better understanding of disease pathology in FX.

DEDICATION

I dedicate my dissertation to my parents, Rick and Angelia Vinson. The two most patient, kind, selfless, humble, forgiving, sincere, graceful, enduring, loyal human beings I know. Your unconditional love for me has, without a doubt, greatly contributed to any and every success I have achieved in life.

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LIST OF ABBREVIATIONS

AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CREB	cAMP response element-binding protein
DG	dentate gyrus
DGC	dentate granule cell
DHPH	(*S*)-3,5-Dihydroxyphenylglycine
DMSO	dimethyl sulfoxide
ERK	extracellular regulated kinase
fEPSP	extracellular field dendritic potential
FMRP	Fragile X Mental Retardation Protein
FX	Fragile X Syndrome
GSK3	glycogen synthase kinase-3
HDAC	histone deacetylase
HDAC3	histone deacetylases 3
HFS	high frequency stimulation
LTD	long-term depression
LTP	long-term potentiation
mGluR	metabotropic glutamate receptor
mGluR-LTD	metabotropic glutamate receptor long-term depression
MPP	medial perforant path
Nrf2	Nuclear factor (erythroid-derived 2)-like 2

NMDAR	*N*-Methyl-D-aspartic acid receptor
PP1	protein phosphatase 1
PP2	protein phosphatase 2
PPR	paired pulse ratio
SSD	steady state depolarization
TA	temporoammonic
TBS	theta burst stimulation
TSA	Trichostatin A
VGCC	voltage gated calcium channel

INTRODUCTION

Fragile X Syndrome

Since the discovery that a mutation in a single gene, *fragile x mental retardation 1(fmr1)*, is responsible for Fragile X Syndrome (FX) (Fryns et al., 1984; Fu et al., 1991; Pieretti et al., 1991), two decades of studies have searched for adequate treatments for impaired cognition to no avail. FX is a neurodevelopmental disorder that is the most frequently inherited form of mental retardation caused by a single gene defect. Because it is an X-linked disorder, FX has a higher occurrence rate in males versus females, affecting approximately 1:3000-5000 males and 1:4000-6000 females (Coffee et al., 2009). Transmission to female offspring can occur when either parent is affected whereas transmission to male offspring occurs when only the female parent is affected. *Fmr1* is located on the long arm of the X chromosome and contains up to 45 CGG repeats with AGG repeats interspersed in the 5'UTR of the promoter in unaffected individuals (Maddalena et al., 2001). Over 98% of reported cases of FX result from an expansion repeat of CGG and concomitant decrease in AAG, which normally provide DNA stability. An expansion with 45-54 repeats is considered a gray zone and individuals are typically unaffected. However 55-200, considered a pre-mutation, alters mRNA translation (Feng et al., 1995). A full mutation results when over 230 repeats accumulate (Maddalena et al., 2001). The presence of excessive repeats leads to a thinning of the X chromosome causing a "fragile" appearance, hence the name "Fragile X Syndrome". The

full mutation results in hypermethylation at the expanded CpG islands that completely prevents gene transcription and ultimately results in loss of the protein product, Fragile X Mental Retardation Protein (FMRP) (Sutcliffe et al., 1992).

Because FMRP is expressed throughout the body (Hinds et al., 1993), lack of FMRP results in diverse physical and behavioral alterations in addition to cognitive impairments. Physical symptoms in FX patients include elongated ears and face, flat feet, low muscle tone and macroorchidism. Behavioral abnormalities can range from mild to severe and include hyperactivity, inattentiveness, seizures, anxiety, sleep disturbances, visuo-spatial processing deficits and autistic-like behaviors (Cordeiro et al., 2011). Additionally, analysis of post-mortem tissue in early studies revealed numerical and morphological abnormalities in dendritic spines in cortex of males afflicted with FX. Studies show that while the density of neurons remains the same, the number of dendritic spines is dramatically increased. Furthermore, the morphology of the spines is long and spiney, which is characteristic of immature spines (Rudelli et al., 1985; Hinton et al., 1991; Irwin et al., 2001).

FMRP is an RNA binding protein that regulates translation of over 800 mRNA (Darnell et al., 2011) targets and binds to approximately 4% of the mRNA in the mammalian brain (Brown et al., 2001). It is located in dendritic shafts and spines, which positions it to target genes involved in synaptic signaling. It contains three RNA-binding domains (Ashley et al., 1993; Siomi et al., 1993) and suppresses translation of mRNA by preventing either initiation or elongation (Bagni and Greenough, 2005; Bhakar et al., 2012; Santoro et al., 2012). Therefore, basal protein synthesis of hundreds of proteins is

unregulated in FX, causing a global increase in translation. Importantly, high throughput cross-linking immunoprecipitation (HITS-CLIP) assays have recently discovered that of the 800 targets, 347 genes are synaptically expressed (Darnell et al., 2011) and include mRNA's that encode for 58 genes in the NMDAR complex, 3 genes in the AMPAR complex, and 32 genes in the mGluR5 complex. NMDA, AMPA, and mGlu5 receptors all play integral roles in the induction and expression of long-term potentiation (LTP) and long term depression (LTD), implicating FMRP as a translational regulator of synaptic plasticity (Darnell et al., 2011; Sidorov et al., 2013).

Synaptic Plasticity

It is generally accepted that memory is processed and stored via alterations in neuronal connections in the nervous system. A unique feature of neurons, the principal cells of the nervous system, is their ability to transmit information to adjacent neurons through electrical or chemical (neurotransmitter release) signals. Electrochemical communication between neurons primarily occurs at synapses which are particularly suited for memory formation and storage due to their ability to modify the strength of connections based on prior experience (or input). Changes in synaptic strength can last for only seconds to minutes (short-term plasticity) or can persist up to the life time of an organism (long-term plasticity) (Kandel, 2001; Dudai, 2004). Long-term potentiation (LTP) is the most widely studied form of long-term synaptic plasticity and most heavily implicated as a cellular mechanism underlying learning and memory.

Long-term potentiation

LTP is persistent increase in synaptic strength (Bliss and Gardner-Medwin, 1973; Bliss and Collingridge, 1993). It is triggered by activation of NMDARs (Collingridge, 1987). NMDARs are ligand (glutamate) gated ion channels that are also voltage dependent. At resting membrane potentials, magnesium (Mg^{2+}) blocks the pore thus preventing ion flow. The Mg^{2+} block is dispelled after post-synaptic depolarization (primarily achieved by influx of sodium (Na^{+}) through AMPA receptors) allowing an influx of calcium (Ca^{2+}) into the post-synaptic cell (Mayer et al., 1984; Nowak et al., 1984). The robust and rapid increase in Ca^{2+} leads to activation s Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), an essential regulator of LTP (Lisman et al., 2002). CamKII activation initiates several signaling cascades that ultimately lead to the expression of LTP. Several mechanisms can contribute to LTP expression, like changes in pre-synaptic release probability (Zakharenko et al., 2003) or structural changes (Lisman, 2003a), however the best understood mechanism is an enhanced post-synaptic response to pre-synaptic glutamate release due to increased AMPA receptors in the PSD (Lisman, 2003b; Nicoll, 2003).

LTP can be experimentally induced using a variety of stimulation patterns. The two most commonly used protocols in experimental set-ups are high frequency tetanus (HFS) and theta burst stimulation (TBS). HFS consists of a single or multiple rounds of 100 Hz stimulation lasting between 0.5 to 1 second, whereas TBS is multiple trains of 10 stimulus bursts at 5 Hz separated by 20 seconds.

It is important to note that non-NMDAR forms of LTP have been observed experimentally. Delivery of a 200 Hz tetanus elicits potentiation that is insensitive to

NMDAR antagonists (Grover and Teyler, 1990). Injection of voltage-gated calcium channel (VGCC) inhibitors or Ca^{2+} chelators into the post-synaptic cell prevents induction of 200 Hz LTP. Therefore the current theory explaining 200 Hz LTP induction is that the strong stimulus intensity provides sufficient post-synaptic depolarization to activate non-NMDA VGCCs (Grover and Teyler, 1990). Application of tertraethylammonium (TEA^{+}) induces a form on NMDA-independent form of potentiation called LTP_k (Aniksztejn and Ben-Ari, 1991; Powell et al., 1994). Similar to 200 Hz LTP, LTP_k is blocked by injection of VGCC inhibitors and Ca^{2+} chelators in the post-synaptic cell. It is likely that enhanced depolarization due to blocked K^{+} flow is sufficient to activate VGCCs thus allowing Ca^{2+} influx into the post-synaptic cell. Ca^{2+} then triggers necessary signaling cascades to express LTP. Finally, LTP at mossy fiber synapses in CA3 of hippocampus is NMDA-independent. There is much debate regarding the precise mechanism however it is generally agreed upon that the locus for induction is in the pre-synaptic terminal (Zalutsky and Nicoll, 1990).

Long-term depression

Long-term depression (LTD) is defined as persistent weakening of synaptic strength. LTD is commonly induced by delivering low frequency stimulation (LFS: 900 pulses, 1Hz). Like LTP, the most widely studied form of LTD is NMDAR-dependent and triggered by influx of Ca^{2+} into the post-synaptic cell. The distinction between LTP and LTD lies in the specific signaling cascade activated by Ca^{2+}. When an LTP inducing stimulus is applied, there is a robust but brief influx of Ca^{2+} through NMDARs which leads to of CamKII (Lisman et al., 2002)(discussed previously). However, pre-synaptic activity that results in modest Ca^{2+} influx over a sustained period of time activates protein

phosphatase-1 (PP1). PP1 activation eventually leads to a removal or internalization of AMPARs, thus resulting in a decrease in post-synaptic response during synaptic transmission (Malenka and Bear, 2004).

Several forms of NMDA-independent forms of LTD have been observed experimentally. These are mainly mediated by G protein-coupled receptors. Activation of Group 1 metabotropic glutamate receptors (mGluRs) leads to extracellular regulated kinase (ERK) activation and AMPAR internalization (Snyder et al., 2001; Volk et al., 2007), termed mGluR-LTD. Similar to mGluR-LTD, activation of muscarinic acetylcholine receptors (mAchRs) induces mLTD which is also coupled to ERK activation and requires AMPA internalization (Volk et al., 2007; Scheiderer et al., 2008). Finally, activation of α-1 adrenergic receptors activates ERK and induces LTD termed NE-LTD (Scheiderer et al., 2008).

Hippocampus

While synaptic plasticity is observed at synapses in many brain regions, it has been most extensively studied in the hippocampus due to the integral role of hippocampal function in cognitive processing and memory consolidation (Scoville and Milner, 1957; Milner, 1972). The earliest indications of the relationship between the hippocampus and cognition came from the study of patient H.M who suffered from temporal lobe epilepsy. To alleviate seizures, parts of hippocampus and amygdala were surgically resected, and as a result, H.M. suffered from anterograde amnesia of episodic and semantic memory, while his short-term and procedural memory remained intact (Corkin, 2002), demonstrating that hippocampus was required for these cognitive functions. Additional

studies from patients and animal models over several decades have confirmed these findings and solidified the role of hippocampus in declarative memory formation.

The hippocampal formation is divided into distinct subregions: dentate gyrus (DG), CA1, CA2, CA3, and CA4 with each subregion containing specific synaptic connections and contributing uniquely to memory formation. Sensory information to be processed enters the hippocampus from the entorhinal cortex via the perforant path. Perforant path projections synapse primarily in two locations, dentate granule cells or distal dendrites of CA1 pyramidal neurons (forming temporoammonic-CA1 synapses; TA-CA1). Information propagated to DG is processed through the hippocampus via the trisynaptic circuit which consists of synaptic connections onto three major principal neurons: dentate granule cells, CA3 pyramidal neurons, and CA1 pyramidal neurons. Briefly, medial and lateral perforant path projections from EC synapse onto dentate granule cells (forming the medial/lateral perforant path dentate granule cell synapses: MPP-DGC or LPP-DGC) which send their projections via mossy fibers to CA3 pyramidal neurons. CA3 pyramidal cells synapse onto CA1 pyramidal neurons along the Schaffer collateral pathway (CA3-CA1 synapses). Information processed through the trisynaptic circuit is then projected back to EC directly or indirectly via subiculum (Figure 1) (Deng et al., 2010).

Initial studies investigating the role of hippocampus in learning and memory processing often relied on complete hippopcampal lesions or pharmacological inactivation. While these studies confirmed the role of hippocampus in processing space, time, and sensory perception, they did not account for the possibility of individual contributions from the specialized subregions (Kesner et al., 2004). While there is now a

general consensus that each subregion performs specific functions it is important to note that there is still much debate about the precise functions of each region. However numerous studies using focal lesions, electrophysiological recordings and computational models have provided insight into individual processing roles. In fact, several studies have demonstrated that DG is responsible for orthogonalization of sensory inputs and spatial pattern separation. Additionally, studies suggest that CA3 also assists in spatial pattern separation in addition to spatial pattern completion, short-term memory, and novelty detection, whereas CA1 is heavily involved in processing temporal information and intermediate-term memory (Marr, 1971; O'Reilly and McClelland, 1994; Rolls, 1996; Eichenbaum et al., 1999; Tanila, 1999; Kesner et al., 2004; Rolls and Kesner, 2006).

Animal Models of FX

Drosophila (Wan et al., 2000; Zhang et al., 2001) and mouse (Bakker, 1994) models have been developed in order to dissect the underlying pathophysiology of FX. Because insertion of CGG repeats does not result in hypermethylation or gene silencing in these models (Brouwer et al., 2007), *Fmr1* (mouse) or *dmf1* (drosophlia) must be deleted or inactivated in order to mimic FX. The *Fmr1* -/- mouse (FX mouse) is the most commonly used model as it recapitulates many of the characteristics of FX including, macroorchidism, increased locomoter hyperactivity, stereotypic movements, seizure susceptibility, and decreased sociability as well as increased spine density and altered morphology (Musumeci et al., 2000; Nielson et al., 2002; Spencer et al., 2005; Brennan et al., 2006; Gibson et al., 2008). Due to the alterations in dendritic spines (Liu et al., 2012) and the intimate relationship between FMRP and several receptors essential for

synaptic plasticity and cognition (Darnell et al., 2011), namely NMDRs, it was expected that FX mice would display robust deficits in NMDAR-dependent plasticity and cognition. However, these predicted characteristics proved difficult to elucidate. Initially, studies focused on area CA1 of hippocampus and reported that NMDAR-dependent LTP (induced using strong HFS or TBS) and LTD (induced using LFS) at CA3-CA1 synapses was normal in adult FX mice (Godfraind et al., 1996). Accordingly, FX mice performed normally or with mild impairments in CA1-dependent cognitive tasks, such as radial arm maze, operant conditioning paradigms, contextual and conditioned fear memory, and Morris water maze (Bakker, 1994; Kooy et al., 1996; D'Hooge et al., 1997; Fisch et al., 1999; Paradee et al., 1999; Dobkin et al., 2000; Peier et al., 2000; Mineur et al., 2002; Yan et al., 2004; Baker et al., 2010). It is important to mention that a few studies have now observed impairments in LTP at CA3-CA1 synapses induced using weak stimulation protocols (Lauterborn et al., 2007; Hu et al., 2008; Shang et al., 2009) in FX mice. However, it is likely that the induction protocols utilized to elicit LTP in these studies induce forms of plasticity that share a similar expression mechanism with traditional NMDAR-dependent LTP but are mediated by different signaling cascades (Shang et al., 2009). While NMDAR-dependent plasticity at CA3-CA1 synapses was unaffected in FX mice, expression of metabotropic glutamate receptor dependent long term depression (mGluR-LTD) was significantly enhanced compared to wild type littermates (Huber et al., 2002). This led investigators to propose the mGluR Theory of FX (Bear et al., 2004) (described in detail below).

Recently, two independent studies reported the much anticipated discovery that both NMDAR-dependent long term potentiation (LTP) and long term depression (LTD)

are deficient at medial perforant path-dentate granule cell (MPP-DGC) synapses in FX mice (Yun and Trommer, 2011; Eadie et al., 2012). Furthermore, these deficits are associated with NMDAR hypofunction and impaired context discrimination, a model of pattern separation dependent on functional NMDARs in DG (McHugh et al., 2007; Eadie et al., 2012). This is the first study to discover hippocampal cognitive deficits coupled with synaptic deficits in a mouse model of FX.

mGluR Theory of Fragile X

Initial studies in FX were aimed at trying to understand the function of FMRP in order to gain insight into why its absence causes such a wide variety of symptoms. These studies revealed that activation of Group 1 mGluRs could robustly induce the synthesis of FMRP in synaptoneurosomes (Weiler et al., 1997). This sparked over a decade's worth of research focused on understanding the role of mGluRs in FX. Because mGluR activation stimulates FMRP, it was initially suggested that mGluR function might be impaired due to the loss of FMRP. Surprisingly, investigations of mGluR-LTD revealed that mGluR signaling was not only intact but significantly enhanced. Studies showed that after application of DHPG, an exogenous agonist of Group I mGluR's, mGluR-LTD was increased at CA3-CA1 synapses in FX mice when compared to WT littermates (Huber et al., 2002). The proposed explanation for this unexpected finding, that still holds merit today, was that under normal conditions, mGluR activation initiates synthesis of many proteins including the translational suppressor FMRP, which acts as regulatory feedback mechanism to constrain protein synthesis. When FMRP is absent, protein synthesis stimulated by mGluR activation is uninhibited, therefore mGluR signaling is enhanced

and can manifest as enhanced, mGluR-LTD (Bear et al., 2004). In support of this idea, the enhanced mGluR-LTD no longer requires synthesis of new proteins, presumably because required proteins are already present due to unrestricted translation (Huber et al., 2002). This led Bear and colleagues to propose the mGluR Theory of FX with the idea that signaling through mGluR5 (a member of the Group 1 family of mGluRs) is pathologically enhanced and dampened mGluR signaling would repair the irregularities in FX. Indeed, genetic reduction of mGluR5 decreases signaling and corrects several behavioral and morphological phenotypes in FX mice including deficits in ocular dominance plasticity, inhibitory avoidance extinction, and reversed enhancement of dendritic spines, basal protein synthesis, seizure susceptibility (Dolen et al., 2007) However, this theory does not entirely explain all of the phenotypes, as mGluR5 reduction or complete removal does not reverse macroordchidism in FX mice (Dolen et al., 2007). Furthermore, there still remains no identified connection between enhanced mGluR-LTD and impaired cognition.

Glycogen Synthase Kinase-3

Glycogen synthase kinase-3, originally named for its role in glycogen metabolism (Embi et al., 1980), is a serine threonine kinase that regulates a myriad of cellular functions including cell adhesion, cell division, gene transcription, cell structure, and cell survival (Frame and Cohen, 2001). GSK3 is ubiquitously expressed in all eukaryotes (Ali et al., 2001) and has two paralogs expressed in mammalian brain, GSK3α and GSK3β (Mukai et al., 2002). While GSK3α and GSK3β share identical kinase domains, they are encoded by different genes and maintain unique genetic sequences outside of the kinase

domain. Like the majority of kinases, GSK3α and GSK3β contain a catalytic site where bound ATP is necessary for enzymatic activity (Woodgett, 1990). Because GSK3 is involved in a wide variety of cellular functions (Frame and Cohen, 2001) and targets over 40 substrates (Jope and Johnson, 2004), regulation of GSK3 activity within cells must be tightly regulated. Activation of GSK3 requires phosphorylation of tyr216 (GSK3β) or tyr279 (GSKα) (Hughes et al., 1993). However, regulation of GSK3 is primarily mediated by inhibitory phosphorylation, subcellular location, and substrate priming. Multiple pathways converge on GSK3 and modulate activity through inhibitory phosphorylation at serine 9 (GSK3β) and serine21 (GSK3α) (Sutherland et al., 1993). Akt, PKA, and PKC are examples of kinases that can phosphorylate GSK3 and turn off enzymatic activity. Conversely, phosphatases, like protein phosphatase-1 (PP1) and protein phosphatase-2 (PP2), dephosphorylate and activate GSK (Frame and Cohen, 2001) (Figure 2). Additionally, P38 mitogen-activated protien kinase (p38MAPK) phosphorylates GSK3β at serine 389 and inhibits GSK3 activity similar to serine 9 phosphorylation (Thornton et al., 2008). GSK3 is located throughout neurons including the nucleus, mitochondria, cytoplasm (Jope and Roh, 2006), and post-synaptic density (Hooper et al., 2007; Peineau et al., 2007). Because of this, GSK3 activity can also be regulated in specific cellular localizations. GSK3 activation can depend on the proximity to upstream enzymes or downstream substrates. Furthermore, many GSK3 substrates require phosphorylation of priming sites (Frame and Cohen, 2001) before GSK3 can phosphorylate and activate/inhibit proteins. Therefore, to be enzymatically active, GSK3 must be localized to the appropriate cellular compartment where active regulators are

located and primed substrates are waiting. Impairments in any of these regulatory mechanisms can lead to altered GSK3 efficiency.

GSK in synaptic plasticity

GSK3 is highly expressed in the brain (Leroy and Brion, 1999) and fractionation studies have identified GSK3β is present in synaptosomes (Hooper et al., 2007; Peineau et al., 2007). The presence of GSK3 in dendrites and spines suggests that GSK3 plays a role in synaptic plasticity. Indeed, induction of LTP increases inhibitory serine-21 phosphorylation of GSK3β (Peineau et al., 2007) and over expression of GSK3β impairs LTP at CA3-CA1 and MPP-DGC synapses (Hooper et al., 2007) indicating that inhibition of GSK3 is required for LTP. Conversely, studies using several structurally different GSK3 inhibitors revealed a dose-dependent attenuation and eventual blockade of LTD at CA3-CA1 synapses (Peineau et al., 2007). Therefore, GSK3 is a bidirectional modulator of NMDAR-dependent plasticity. The PI3 kinase (PI3K) -Akt pathway is one of at least 8 pathways that converge to regulate inhibitory phosphorylation of GSK3 (Hong and Lee, 1997) and was recently identified as the signaling cascade initiated by NMDAR activation that results inhibition of GSK3 in LTP (Figure 3). This was not surprising given the established role of the PI3K-Akt pathway in synaptic plasticity (Mizuno et al., 2003). Inhibition of PI3K using LY294002 or wortmannin inhibits LTP in cultured neurons. Additionally, an increase in phosphorylation at Ser473 of Akt, the location where PI3K phosphorylation occurs, is observed 5 minutes after induction of LTP in area CA1 of hippocampus (Man et al., 2003), further implicating the PI3K-Akt pathway in LTP. While decreased Akt activity due to PP1 dephosophorylation may play an indirect role in modulating GSK3 activity during LTD, direct dephosphorylation of

ser21 via PP1 (Morfini et al., 2004; Lee et al., 2005; Szatmari et al., 2005) is the primary mechanism for activation of GSK3 during LTD induction (Peineau et al., 2007). PP1 is activated by calcineurin (PP2B) once Ca^{2+} enters through NMDARs during the induction of LTD (Mulkey et al., 1993; Mulkey et al., 1994) (Figure 3).

The majority of studies establishing the role GSK3 in LTD and LTP investigated plasticity at CA3-CA1 synapses (Peineau et al., 2007), however it is likely that GSK3 regulation of plasticity occurs at MPP-DGC synapses as well. In fact, inhibition of PI3K using wortmannin prevents LTP at MPP-DGC synapses *in vivo* (Kelly and Lynch, 2000), indirectly implying that GSK3 inhibition is necessary. Furthermore, overexpression of GSK3 in the dentate gyrus prevents LTP at MPP-DGC synapses (Hooper et al., 2007). Therefore, GSK3 likely plays the same role in NMDAR-dependent plasticity at all glutamatergic synapses. It is also important to note that studies investigating GSK3 in synaptic plasticity have primarily focused on GSK3β, however a role for GSK3α cannot be discounted as future studies are necessary to determine whether GSK3α similarly regulates plasticity.

The precise mechanism for GSK3 modulation of LTP and LTD is currently under investigation. Several possibilities exist, however GSK3β mediated AMPAR trafficking has gained the most attention. Briefly, GSK3β forms a complex with AMPARs which is regulated by LTP (Peineau et al., 2007) and inhibition of GSK3 prevents AMPAR endocytosis during chemical LTD (Du et al., 2010). Furthermore, kinesin light chain 2 (KLC2), a key regulator in the kinesin cargo delivery system, has been identified as a downstream target of GSK3β. Studies have demonstrated that GSK3β mediated phosphorylation of KLC2 causes a dissociation of the KLC2/AMPA complex from the

kinesin cargo chain thus impeding the movement of AMPARs. Additionally, treatment with TAT-KLCpCDK which prevents GSK3β phosphorylation of KLC2, blocks AMPAR internalization and LTD expression at CA3-CA1 synapses (Du et al., 2010). In addition to AMPAR endocytosis, proposed mechanisms for GSK3 mediation of synaptic plasticity include trafficking of NMDARs (Chen et al., 2007) and regulation of transcription factors (Manahan-Vaughan et al., 2000). However the recent identification of a novel downstream target, histone deacetlyase 3 (HDAC3) (Bardai and D'Mello, 2011), raises an interesting possible mechanism (discussed below).

GSK3 inhibition

GSK3 is a target of lithium, an FDA approved drug that has been used clinically for decades in the treatment of bipolar disorder (Klein and Melton, 1996; Stambolic et al., 1996). Lithium directly competes with Mg^{2+} for its binding site, which is required for enzymatic activity of GSK3 (Ryves and Harwood, 2001). Lithium treatment also inhibits GSK3 indirectly by increasing inhibitory serine 9/21 phosphorylation (Chalecka-Franaszek and Chuang, 1999; De Sarno et al., 2002; Li et al., 2007) . It is important to note that in addition to targeting GSK3, lithium is also known to inhibit phosphoglucomutase (Ray et al., 1978) inositol-1,4 bisphosphate 1-phosphatase, insoitol-1 (or-4)-monophosphatase (Harwood, 2005), and other inositol polyphosphatases (Inhorn and Majerus, 1987). Therefore, there has been an emergence of novel GSK3 inhibitors in order to confirm the beneficial effects of selective GSK3 inhibition. The majority of inhibitors that have been developed compete with the ATP binding site on the catalytic domain of GSK3. These inhibitors include indirubin derivatives (Leclerc et al., 2001), paullone derivities (Leost et al., 2000) SB216763 (Coghlan et al., 2000) and

CT99021 (Bain et al., 2007). Of importance, kinase specificity studies have identified CT99021 as the most selective GSK3 inhibitor (Wagman et al., 2004). Several non-ATP competitive inhibitors have recently been developed. These include substrate-competitive inhibitors (Plotkin et al., 2003; Kaidanovich-Beilin et al., 2004; Licht-Murava et al., 2011), allosteric inhibitors (VP0.7) (Palomo et al., 2011), and TDZD-8, which is hypothesized to bind and inhibit the catalytic domain (Martinez et al., 2002).

Disease pathology and cognitive impairments

Due to the diversity of GSK3 substrates and their involvement in various cellular functions, it is not surprising that GSK3 has been implicated in a number of disease pathologies. GSK3 dysfunction has been connected to peripheral diseases like cardiac hypertrophy (Hardt and Sadoshima, 2002; Haq et al., 2003), cancer (Hill and Hemmings, 2002; Manoukian and Woodgett, 2002), and diabetes (Frame and Cohen, 2001). Additionally, hyperactive GSK3 has been identified as playing a pathological role in several neurobiological disorders including bipolar mood disorder (Klein and Melton, 1996; Grimes and Jope, 2001) schizophrenia (Beasley et al., 2001; Eldar-Finkelman, 2002) Alzheimer's disease (Hanger et al., 1992; Kirschenbaum et al., 2001; Avila, 2004), and most recently Fragile X Syndrome (McBride et al., 2005; Min et al., 2009; Yuskaitis et al., 2010b; Choi et al., 2011; Liu et al., 2011; Liu et al., 2012) (discussed in further detail below), making GSK3 a prime target for therapeutic interventions. In fact, numerous studies have demonstrated that GSK3 contributes to cognitive impairment. Pharmacological or genetic inhibition of GSK3 reverses cognitive deficits in many disorders including Alzheimers's disease (De Ferrari et al., 2003; Liu et al., 2003; Engel et al., 2006; Rockenstein et al., 2007; Terwel et al., 2008; Hu et al., 2009; Sereno et al.,

2009; Toledo and Inestrosa, 2010; Avrahami and Eldar-Finkelman, 2013), Parkinson's disease (Castro et al., 2012) and diabetes (Ponce-Lopez et al., 2011; King et al., 2013) among others. In the majority of these studies, inhibition of GSK3 did not alter learning and memory in control animals. Importantly, this suggests that GSK3 does not normally mediate cognition however is readily recruited in disease pathologies.

GSK3 in FX

Studies conducted in Drosophila first identified the therapeutic potential of lithium in FX. Lithium treatment reverses impaired courtship behavior in *dmf1*-/- mutant flies (McBride et al., 2005). Furthermore, lithium repairs age-dependent cognitive decline in adult *dfmr1* mutants (Choi et al., 2010). Subsequent studies extended these findings and demonstrated that treatment with lithium reduces seizure susceptibility (Min et al., 2009), reverses hyperactivity (Min et al., 2009; Yuskaitis et al., 2010b; Liu et al., 2011), increases sociability (Mines et al., 2010), corrects macroorchidism (Yuskaitis et al., 2010a), reverses the enhancement in dendritic spines (Liu et al., 2011), and the increase in mGluR-LTD (Choi et al., 2011) in FX mouse models. Because lithium is not a selective inhibitor of GSK3, additional studies were necessary to firmly establish a role for GSK3 in the pathology of FX. Studies from FX mice on two different genetic backgrounds demonstrated that inhibitory serine 9/21 phosphorylation of GSK3α and GSK3β are decreased in FX mice compared to WT littermates. There were no changes in total protein (Min et al., 2009; Yuskaitis et al., 2010b) suggesting impaired regulatory phosphorylation and increased activity. Importantly, treatment with lithium normalized serine phosphorylation of both GSK3α and GSK3β (Yuskaitis et al., 2010b) implying that lithium's beneficial effects are likely as a result of GSK3 inhibition. Additionally,

inhibition of GSK3 using selective inhibitors reversed hyperactivity and normalized seizure susceptibility (Min et al., 2009). Taken together, these results firmly established a pathological role of GSK3 in FX.

Histone Deacetylases

Long-term memory formation requires changes in gene transcription. Therefore, processes that influence transcription also modulate cognition. Accordingly, histone deacetylases (HDACs), enzymes that encourage chromatin condensation through removal of acetyl groups from histone core proteins, decrease transcription and negatively regulate memory formation in hippocampus as well as other brain regions (Levenson et al., 2004; Kouzarides, 2007; Vecsey et al., 2007; Stefanko et al., 2009). Conversely, pharmacological inhibition or deletion of HDACs facilitates transcription and enhances learning and memory (Vecsey et al., 2007; Stefanko et al., 2009; Kilgore et al., 2010). Because alterations in cognition are oftentimes associated with parallel changes in LTP, it is not surprising that HDACs also modulate LTP. In fact, a few studies have demonstrated this by showing that HDAC inhibition enhances LTP at CA3-CA1 synapses in hippocampus (Levenson et al., 2004; Vecsey et al., 2007). However because all studies investigating the role of HDACs in hippocampal LTP thus far have focused on CA3-CA1 synapses, it is not known whether HDAC modulation of synaptic plasticity is specific for this synapse or is a general mechanism modulating synaptic efficacy at all excitatory synapses.

To date, the majority of studies investigating how HDACs regulate synaptic efficacy and plasticity have used non-selective pharmacological approaches. However individual HDAC isoforms likely have differential roles in modulating learning and

memory and synaptic plasticity, prompting the use of approaches that specifically target select HDAC subtypes. Consistent with this, recent work showed that increased activity of HDAC2, but not HDAC1, impairs hippocampus-dependent learning and memory and LTP at CA3-CA1 synapses (Guan et al., 2009). The class I HDAC3 isoform has the highest expression in hippocampus (Broide et al., 2007) and its selective inhibition enhances object recognition and location memory, hippocampus-dependent learning and memory tasks (McQuown et al., 2011). However, whether enhanced LTP at relevant synaptic pathways is an underlying mechanism contributing to enhanced cognition has yet to be explored. Given that GSK3 and HDAC3 activity negatively regulate cognition and that GSK3 phosphorylates and activates HDAC3, it is possible that GSK3 constrains LTP through increased HDAC3 activity. Furthermore, HDACs have emerged as potential therapeutic targets for improving memory deficits in cognitive disorders after a study demonstrated beneficial effects in a mouse model of Alzheimer's disease (Kilgore et al., 2010). However, it is not known if these findings can be extended to other models with cognitive impairments like FX

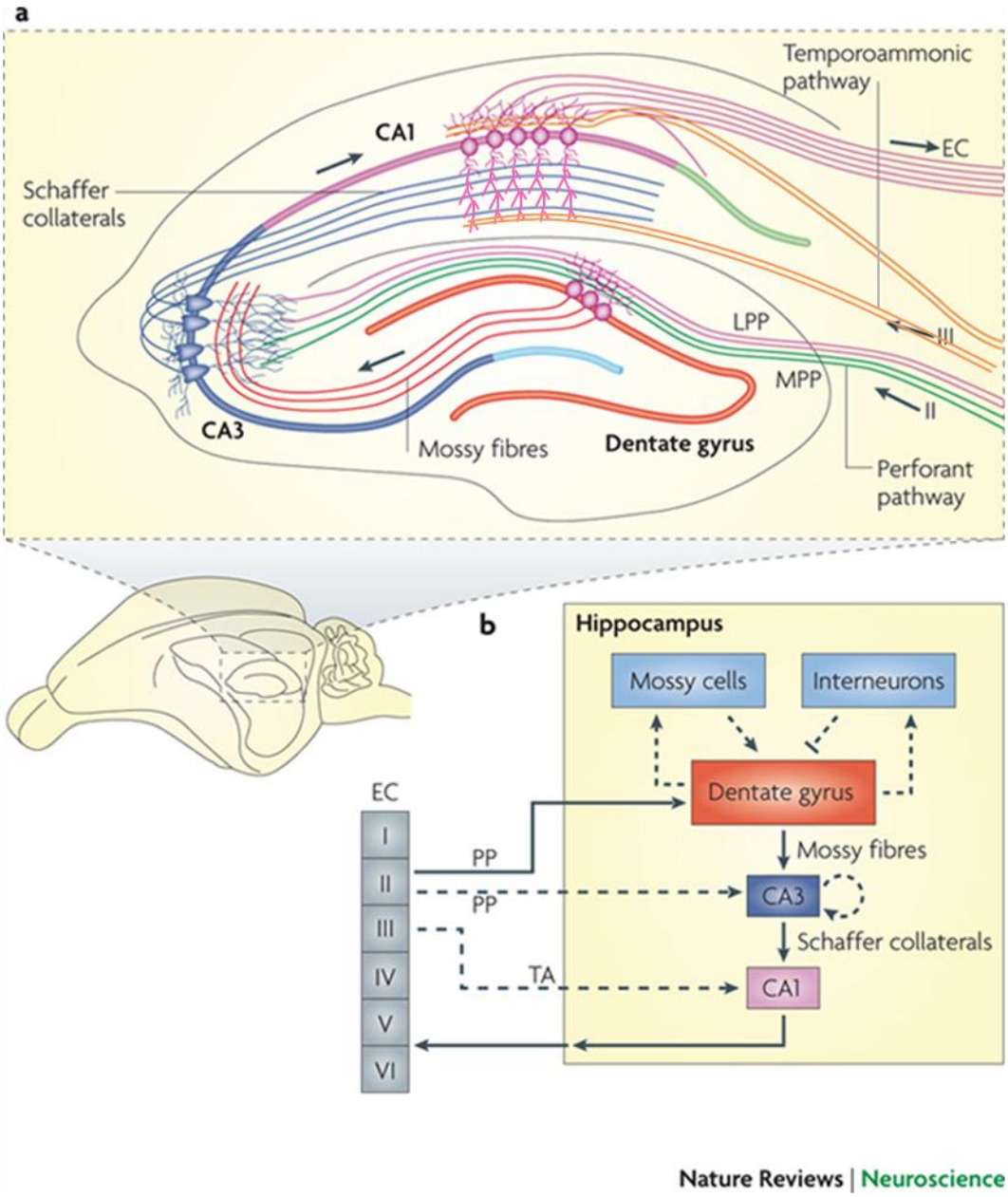
Hypothesis

Loss of FMRP causes a decrease in synaptic plasticity in dentate gyrus due to hyperactive GSK3 and increased HDAC3 activity.

Glycogen synthase kinase-3 (GSK3) is a constitutively active kinase (Hughes et al., 1993) that has increased activity in hippocampus of FX mice. Previous studies have shown that inhibition of GSK3 normalizes several behavioral abnormalities including locomotor hyperactivity, seizure susceptibility, passive avoidance learning, anxiety-related behaviors, and sociability in FX mice, therefore establishing a role for increased GSK3 activity in the pathology of FX (McBride et al., 2005; Min et al., 2009; Mines et al., 2010; Yuskaitis et al., 2010b; Choi et al., 2011; Liu et al., 2011). Importantly, GSK3 bi-directionally modulates NMDAR-dependent synaptic plasticity at hippocampal synapses. Inhibition of GSK3 is required for induction of LTP and conversely, its activation is necessary for induction of LTD (Peineau et al., 2007). Because GSK3 is a key regular of plasticity and its activity is pathologically enhanced in FX mice, it is likely that hyperactive GSK3 contributes to decreased NMDAR dependent LTP.

Furthermore, GSK3 has over 40 putative targets (Jope and Johnson, 2004); therefore, it is important to identify key downstream effector molecules that are mediating the synaptic and learning deficits in FX mice. One candidate may be HDAC3 (Bardai and D'Mello, 2011). HDAC3 negatively regulates learning and memory (McQuown et al., 2011) and HDAC inhibition has proven to be effective in reversing learning deficits in mouse models of neurodegeneration (Kilgore et al., 2010). Therefore,

HDAC3 could similarly contribute to the pathophysiology in learning and synaptic function in FX.


a
Temporoammonic pathway
CA1
EC
Schaffer collaterals
LPP
III
MPP
II
CA3
Mossy fibres
Dentate gyrus
Perforant pathway
b
Hippocampus
Mossy cells
Interneurons
Dentate gyrus
EC
I
II
III
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PP
PP
Mossy fibres
CA3
Schaffer collaterals
TA
CA1
Nature Reviews | Neuroscience

Figure 1. a | An illustration of the hippocampal circuitry. **b** | Diagram of the hippocampal neural network. The traditional excitatory trisynaptic pathway (entorhinal cortex (EC)–dentate gyrus–CA3–CA1–EC) is depicted by solid arrows. The axons of layer II neurons in the entorhinal cortex project to the dentate gyrus through the perforant pathway (PP), including the lateral perforant pathway (LPP) and medial perforant pathway (MPP). The dentate gyrus sends projections to the pyramidal cells in CA3 through mossy fibres. CA3 pyramidal neurons relay the information to CA1 pyramidal neurons through Schaffer collaterals. CA1 pyramidal neurons send back-projections into deep-layer neurons of the EC. CA3 also receives direct projections from EC layer II neurons through the PP. CA1 receives direct input from EC layer III neurons through the temporoammonic pathway (TA). The dentate granule cells also project to the mossy cells in the hilus and hilar interneurons, which send excitatory and inhibitory projections, respectively, back to the granule cells. (Modified and Used with permission from Deng et al 2010)

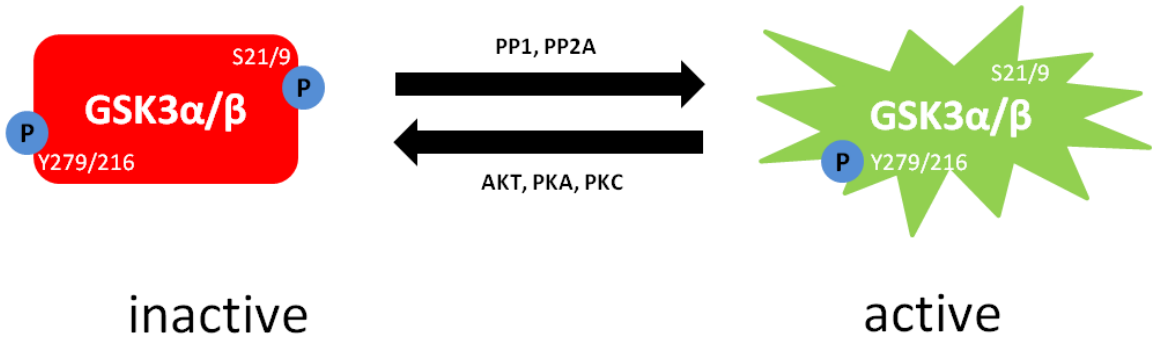

Figure 2. Regulation of GSK3 activity is tightly regulated by phosphorylation. Inhibitory phosphorylation of serine 21 or serine 9 of GSK3α and GSKβ, respectiively, by protein kinases (AKT, PKA and PKC) prevents activity of GSK3. De-phosphorlation of serine 21/9 by protein phosphatases (PP1, PP2A) activates GSK3. Additionally, activation of GSK3 requires tyrosine 279 or 216 phosphorylation of GSK3α and GSKβ, respectiively, for activation, however this site is normally phoshorlylated at basal conditions, possibly through autophosphorylation.

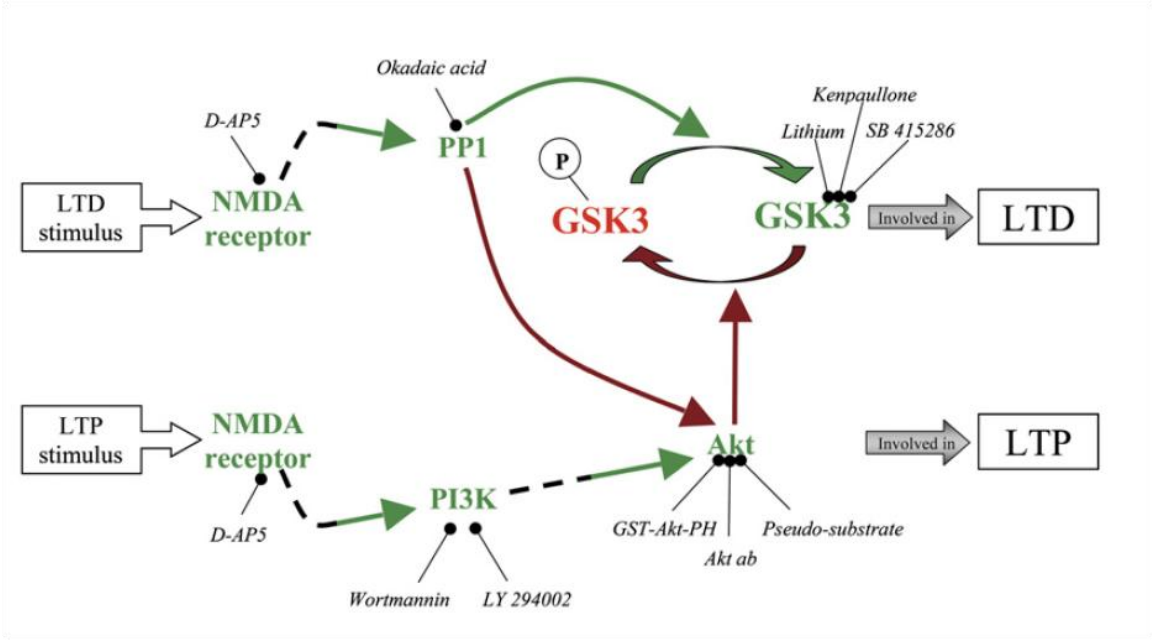

Figure 3. The Regulation of GSK3β in the Control of NMDA Receptor-Dependent Synaptic Plasticity. The activity of GSK3β determines whether NMDA receptor activation induces LTD or inhibits LTD. During LTD, activation of PP1 leads to dephosphorylation of GSK3β at ser9 to further activate GSK3β and enable LTD to occur. PP1 also inhibits Akt. During LTP, activation of NMDA receptors leads to stimulation of the PI3K-Akt pathway, which phosphorylates GSK3β at ser9 to inhibit its activity and prevent the induction of LTD. Thus, GSK3β, under the control of Akt and PP1, is a critical determinant of the direction of NMDA receptor-dependent plasticity. (Used with permission from Peineau et al 2007)

GLYCOGEN SYNTHASE KINASE-3 INHIBITORS REVERSE DEFICITS IN LONG-TERM POTENTIATION IN FRAGILE X MICE

by

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Abstract

Background

Identifying feasible therapeutic interventions is crucial for ameliorating the intellectual disability and other afflictions of Fragile X Syndrome (FX), the most common inherited cause of intellectual disability and autism. Hippocampal glycogen synthase kinase-3 (GSK3) is hyperactive in the mouse model of FX (FX mice), and hyperactive GSK3 promotes locomotor hyperactivity and audiogenic seizure susceptibility in FX mice, raising the possibility that specific GSK3 inhibitors may improve cognitive processes.

Methods

We tested if specific GSK3 inhibitors improve deficits in *N*-methyl-D-aspartate receptor (NMDAR)-dependent long-term potentiation (LTP) at medial perforant path synapses onto dentate granule cells (MPP-DGC).

Results

GSK3 inhibitors completely rescued deficits in LTP at MPP-DGC synapses in FX mice. Furthermore, synaptosomes from the dentate gyrus of FX mice displayed decreased inhibitory serine-phosphorylation of GSK3β compared with wild-type littermates.

Conclusions

These findings establish that synaptic plasticity and cognitive deficits in FX mice can be improved by intervention with inhibitors of GSK3, which may prove therapeutically beneficial in FX.

Introduction

Fragile X Syndrome (FX), an X-linked disorder, is the most frequently inherited form of mental retardation. FX is caused by an expansion repeat of cytosine-guanine-guanine (CGG) in the *fragile x mental retardation 1(fmr1)* gene, which silences it and eliminates production of fragile x mental retardation protein (FMRP), an important regulator in RNA binding and translation regulation (Pieretti et al., 1991; Verkerk et al., 1991; Turner et al., 1996; Bardoni et al., 2000).Because FMRP is expressed throughout the body, lack of FMRP results in diverse physical and behavioral characteristics, in addition to cognitive impairment. Physical symptoms in FX patients include elongated ears and face, flat feet, low muscle tone and macroorchidism. Behavioral abnormalities include hyperactivity, inattentiveness, seizures, sleep disturbances, visuo-spatial processing deficits and autistic-like behaviors. In order to understand the underlying pathophysiology of FX, researchers developed the *fmr1 knock out mouse*, which mimics the loss of FMRP expression in FX patients.(Bakker, 1994) Importantly, loss of FMRP in *Fmr1-/-* mice recapitulates several characteristics of FX, including macroorchidism, increased locomotor hyperactivity, stereotypic movements, seizure susceptibility and dendritic spine density, as well as decreased sociability (Musumeci et al., 2000; Nielson et al., 2002; Spencer et al., 2005; Brennan et al., 2006; Gibson et al., 2008)

Fmr1-/- mice (FX mice) have served as a useful tool in understanding the pathology of the physical and behavioral characteristics of FX. Normally, FMRP is highly expressed in brain, specifically in dentate gyrus (DG) granule neurons (Hinds et al., 1993), and it interacts with many different mRNAs. Of importance to this study, FMRP targets mRNA encoding NMDAR (N-methyl-D-aspartate receptor) subunits

(Brown et al., 2001; Darnell et al., 2004; Zalfa et al., 2007; Schutt et al., 2009). Because dendritic spines are altered and NMDARs are essential to synaptic plasticity and cognition (Herron et al., 1986), it is expected that FX mice would display robust deficits in NMDAR-dependent plasticity and cognition. However, these predicted characteristics proved difficult to elucidate. Initially, studies focused on subfield CA1 of hippocampus, a region necessary for learning and memory, (Zola-Morgan et al., 1986) and reported that NMDAR dependent plasticity in CA1 was normal in adult FX mice (Godfraind et al., 1996), but expression of metabotropic glutamate receptor dependent long term depression (mGluR-LTD) was significantly enhanced compared to wild type littermates (Huber et al., 2002). This led investigators to propose the mGluR Theory of FX with the idea that signaling through mGluR5 is pathologically enhanced and that dampened mGluR signaling would repair the irregularities in FX. Indeed, decreased mGluR5 signaling corrects several behavioral and morphological phenotypes in FX mice (Dolen and Bear, 2008). However, this theory does not entirely explain all of the phenotypes, as there still remains no identified connection between enhanced mGluR-LTD and impaired cognition. In fact, *Fmr1-/-* mice display no deficits in CA1 dependent learning as evident by normal performance in Morris water maze as well as other CA1 dependent learning tasks(D'Hooge et al., 1997; Eadie et al., 2009). Recently, it was reported that NMDAR-dependent long-term potentiation (LTP) and NMDAR transmission are deficient at medial perforant path-dentate granule cell (MPP-DGC) synapses in FX mice (Eadie et al., 2010; Yun and Trommer, 2011). Furthermore, these deficits are associated with impaired context discrimination, a model of pattern separation dependent on functional NMDARs in DG (Eadie et al., 2010). This is the first study to discover cognitive deficits coupled

with synaptic deficits in a mouse model of FX. Understanding the underlying molecular mechanisms synaptic deficits will lead to the identification of therapeutic targets in the treatment of cognitive symptoms in FX.

Glycogen synthase kinase (GSK3) is involved in a wide variety of cellular functions, including cognition (Gomez de Barreda et al., 2010). Regulation of GSK3 is primarily mediated by inhibitory serine-phosphorylation, occurring through multiple signaling pathways. Impairments in these pathways can lead to insufficient phosphorylation (thus inhibition) of GSK3, resulting in hyperactive GSK3 activity. Hyperactive GSK3 has been implicated in a number of diseases including, cancer, diabetes, Alzheimer's disease, and mood disorders (Frame and Cohen, 2001; Zhu et al., 2002; Jope and Johnson, 2004; Hooper et al., 2008; Mines et al., 2011). In its role as a modulator of cognitive function, GSK3 is capable of bi-directionally regulating NMDAR-dependent long-term plasticity. In order for LTP induction and expression to occur, GSK3 activity must decrease. Conversely, GSK3 activation is necessary for LTD induction and expression.(Peineau et al., 2007) The involvement of GSK3 in both NMDAR-dependent LTP and LTD implicate abnormal GSK3 activity as contributing to DG associated deficits in FX mice. In support of this idea, studies have demonstrated that FX mice have lower levels of inhibitory phosphorylation of GSK3 (with no alteration in total protein levels), suggesting that GSK3 activity is pathologically increased in several brain regions, including hippocampus, compared to wild-type (WT) littermates (Min et al., 2009; Yuskaitis et al., 2010b). These findings suggest that GSK3 expression is unaltered in FX mouse brain, but that inhibitory control of GSK3 activity is impaired. Decreased inhibition of GSK3 was observed in FX mice on both the FVB and C57Bl6

backgrounds, indicating this is a robust change not dependent on mouse strain(Liu et al., 2011).Importantly, pharmacological inhibition of GSK3 reduces seizure susceptibility (Min et al., 2009), reverses hyperactivity (Min et al., 2009; Yuskaitis et al., 2010b; Liu et al., 2011), increases sociability , corrects macroorchidism(Yuskaitis et al., 2010a), and reverses the enhancement in dendritic spines (Liu et al., 2011) and of mGluR-LTD (McBride et al., 2005) thereby establishing a role for hyperactive GSK3 in the pathology of FX. Since GSK3 is known to regulate NMDA-dependent plasticity and hyperactive GSK3 contributes to physical and behavioral abnormalities in animal models of FX, it is the goal of this study to examine the role of hyperactive GSK3 in impaired LTP in FX mice. Here we tested the hypothesis that pharmacological inhibition of GSK3 rescues deficits in LTP at MPP-DGC synapses. We report that lithium and selective GSK3 inhibitors, but not mGluR5 inhibition, reverse the LTP deficit in FX mice, establishing GSK3 as an independent target for therapeutic development to treat FX.

Materials and Methods

Reagents

Antibodies were obtained from Cell Signaling Technology (Beverly, MA), LiCl and picrotoxin from Sigma (St. Louis, MO), Chir99021 (CT99021) from Biovision (Milpitas, CA) and 2-methyl-6-(phenylethynyl)pyridine (MPEP) from Tocris Bioscience (Ellisville, MO).

Animals

Mice were housed in light and temperature controlled rooms and treated in accordance with National Institutes of Health and the University of Alabama at Birmingham Institutional Animal Care and Use Committee regulations.

Synaptsomal Preparation and Western blot analysis

Mice were anesthetized then decapitated. Brains were removed and dissected on wet ice. Hippocampal subfields were dissected and rapidly frozen. Samples were homogenized in TEVP buffer (10mM Tris base, 5mM NaF, 1mM Na_3VO_4, 1mM EDTA, 1mM EGTA, 2.5% protease inhibitor cocktail, 1% phosphatase cocktail), pH 7.4 containing 320mM sucrose. Samples were centrifuged (800xg,10 min 4°C). The supernatant was then centrifuged (9200xg, 15 min, 4°C) and pellet discarded. The resultant supernatant (cytosol fraction) was discarded and the pellet (crude synaptosomal membrane) was resuspended in TEVP buffer. Samples were then separated on 7.5% SDS PAGE and transferred for 1 h to polyvinylidene difluoride membrane. Membranes were incubated in primary antibody (anti-p-GSK3b (1:500) or anti-GSK3b (1:5000), overnight at 4°C, then washed with TBST/TBS. After 3 washes, membranes were incubated in secondary antibody (GAR (1:10,000), GAM (1:5000), Cell Signaling) for 1 h at room temperature followed by 3 washes in TBST/TBS. The blots were visualized with enhanced chemiluminescence (Perkinelmer, Waltham, MA).

Hippocampal slice preparation and electrophysiology

Mice were anesthetized, decapitated and brains rapidly removed. Coronal slices (400uM) were prepared from dorsal hippocampus in modified aCSF[in mM: 85 NaCl, 2.5 KCl, 4 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 glucose and 75 sucrose saturated in 95% O_2 and 5% CO_2]. Slices were transferred to a submersion chamber containing standard artificial CSF (aCSF) [in mM; 124 NaCl, 3 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 15 glucose] and stored at room temperature and continuously bubbled with 95% O_2 /5% CO_2 for up to 5 hours before recording. Extracellular dendritic field potential recordings (fEPSPs) at either schaffer collateral or medial perforant pathway-dentate granule cell synapses were carried out in a submersion chamber and slices perfused with standard aCSF or aCSF containing 20 mM LiCl (equivalent NaCl removed) at 25-28 F. Following a 20 minute stable baseline (0.1 Hz, 200ms duration), LTP was induced using high-frequency stimulation (HFS, 100 Hz, 1 s duration x 4, 60s interval). In order to isolate excitatory inputs, all recordings were carried out in the presence of picrotoxin (100uM).

Statistics

Data are expressed as mean ± SEM. Student's t test was used as noted. Significance was taken as $p<0.05$.

Results

Selective LTP deficit at medial perforant path-dentate granule cell synapses is accompanied by decreased serine-phosphorylated GSK3β.

Using acute brain slices and high frequency stimulation (HFS), the LTP magnitude at CA3-CA1 synapses in slices from FX mice is not different from WT mice (WT: 197±12% of baseline fEPSP slope vs FX: 215±23% of baseline fEPSP slope, $p>0.05$) (Fig. 1A). In contrast, there is a significant LTP deficit in FX mice at MPP-DGC synapses (WT: 183±14% vs FX: 130±8%, $p<0.05$) (Fig. 1B). These results confirm previous reports (Huber et al., 2002; Eadie et al., 2012), and importantly, demonstrate hippocampal region-specific deficits in NMDAR-dependent synaptic plasticity in FX mice.

In hippocampal homogenates from FX mice, inhibitory serine-phosphorylation of GSK3β (p-GSK3β) is decreased, indicating increased activity (Min et al., 2009; Yuskaitis et al., 2010b). However, it is not known whether the decrease in p-GSK3β is hippocampal region specific. Because GSK3 negatively regulates NMDAR-dependent LTP (Hooper et al., 2007; Peineau et al., 2007; Zhu et al., 2007), the decrease in p-GSK3 should occur in DG but not CA1, if pathologically increased GSK3 activity is causing the specific deficit in LTP at MPP-DGC synapses. Western blot analysis of synaptosomal fractions revealed that p-GSK3β levels are unchanged in CA1 (135±8% of control, $p>0.05$) but decreased in DG (53±2% of control, $p<0.05$) (Fig. 1C and D) from FX mice compared to WT mice, whereas total GSK3β levels were equivalent. These results raise the possibility that hyperactive GSK3 in DG contributes to the deficit in LTP at MPP-DGC synapses.

Pharmacological blockade of GSK3 rescues the deficit in LTP at MPP-DGC synapses.

Reduced p-GSK3β levels coupled with the LTP deficit specifically in DG suggest that pharmacological GSK3β inhibition might reverse the synaptic dysfunction. Because lithium rescues some behavioral phenotypes in FX mice and it inhibits GSK3 (Yuskaitis et al., 2010b; Liu et al., 2011), we first investigated whether bath application of LiCl (20 mM) rescues the LTP deficit. Indeed, LiCl, applied 30 min prior to HFS, significantly increases the LTP magnitude at MPP-DGC synapses in slices from FX mice (FX: 130±8% vs FX+Li: 162±15%, $p<0.05$) (Fig. 2B) without affecting the LTP magnitude at WT synapses (WT: 183±14% vs WT+Li: 183±12%, $p>0.05$) (Fig. 2A). In fact, the LTP magnitude is equivalent in WT and FX+Li treated slices (WT: 183±14% vs FX+Li: 162±15%, $p>0.05$) (Fig. 2C and D), indicating a complete rescue of the LTP deficit.

In order to confirm that lithium is rescuing LTP specifically through inhibition of GSK3 rather than off-target effects, we utilized CT99021, a highly selective GSK3 inhibitor (Bain et al., 2007). Slices were perfused with either vehicle or CT99021 (2 μM) for at least 30 min prior to HFS. Similar to LiCl treatment, CT99021 completely rescued the deficit in LTP in FX slices but had no significant effect on WT slices (WT: 146±5% vs WT+CT99021: 164±12%, $p>0.05$) (FX: 115±7% vs FX+CT99021: 146±10%, $p<0.05$) (WT: 146±5% vs FX+CT99021: 146±10%, $p>0.05$) (Fig. 2E-H). Collectively, these results conclusively demonstrate that inhibition of GSK3 reverses the deficit in LTP at MPP-DGC synapses.

Pharmacological blockade of GSK3 reverses the deficit in steady-state depolarization in FX slices during HFS.

Because LTP induction requires sufficient depolarization to relieve the voltage-dependent Mg^{2+} block from NMDARs, we next investigated whether the deficit in LTP and subsequent rescue by GSK3 inhibition was due to alterations in the depolarization during HFS. We measured steady-state depolarization during the fourth round of HFS in WT and FX slices and found a significant decrease in FX slices compared to WT slices, which was reversed by LiCl (Fig. 3A and B) and CT99021 (Fig. 3C and D). These findings raise the possibility that GSK3 inhibition rescues the LTP deficit by increasing neurotransmitter release. However, we found no significant differences in the paired pulse ratio, an indirect measure of presynaptic neurotransmitter release probability (Dobrunz and Stevens, 1997), between WT and FX slices under any condition or interstimulus interval (Fig. S1 A and B), indicating that enhanced neurotransmitter release may not be the mechanism underlying rescued LTP.

Synaptic deficits in FX mice are not rescued by mGluR5 inhibition.

Because inhibition of mGluR reverses many of the synaptic and behavioral phenotypes in FX mice (Dolen et al., 2007; Michalon et al., 2012), we investigated whether MPEP, an mGluR5 antagonist, would also reverse the dentate gyrus associated deficits in synaptic plasticity and learning and memory. We show using bath application of MPEP (100 uM) that mGluR5 inhibition fails to rescue LTP magnitude in FX slices (WT: 143±8%; WT+MPEP 127±9%, $p>0.05$) (FX: 116±11%; FX+MPEP: 101±7%, $p>0.05$) (WT:143±8%; FX+MPEP: 101±7%, $p<0.05$) (Fig. 4A-C).

Pharmacological blockade of GSK3 does not reverse the deficit in NMDAR hypofucntion during steady-state depolarization in FX slices during HFS

Because decreased NMDAR transmission has been reported in FX mice (Yun and Trommer, 2011; Eadie et al., 2012) and GSK3 has been implicated in NMDAR internalization (Chen et al., 2007), we wondered whether inhibition of GSK3 reverses deficits in LTP by reversing NMDAR hypofucntion in FX mice. Before testing this, we firstconfirmed previous reports demonstrating NMDAR hypofunction (Eadie et al., 2010; Yun and Trommer, 2011). To do this we measured SSD in WT mice with and without APV, an NMDAR antagonist, to determine the contribution of NMDARs to SSD during HFS. We show that during SSD, NMDARs contribute a smaller percentage to the total amount of depolarization in slices from FX mice compared to slices from WT mice (WT:63±6%; FX: 42±2%, $p<0.05$) (Figure 5A). To determine whether GSK3 inhibition increased NMDAR transmission and therefore the contribution of NMDARs to SSD we measured SSD in the presence of CT99021. We show that CT99021 does not enhance NMDAR function during SSD in slices from FX mice (WT:63±6%; FX+CT: 41±2%, $p<0.05$) (FX:42±6%; FX+CT: 41±2%, $p>0.05$) (Figure 5A). As a positive control for the effectiveness of CT99021 in these experiments, we show that CT99021 enhances SSD in FX slices (Arbitrary units: FX:137±16; FX+MPEP: 166±33, $p<0.05$) (Figure 5B).

FX mice display a deficit in LTP at TA-CA1 synapses.

GSK3 is located in both the pre-synaptic terminal (Ahmad-Annuar et al., 2006) and the post synaptic density (Hooper et al., 2007; Peineau et al., 2007). Because GSK3

inhibition rescues deficits in steady-state depolarization which can be a measure of pre-synaptic function, we wondered whether the hyperactive GSK3 impairs LTP through a pre-synaptic mechanism. We reasoned that if pre-synaptic hyperactive GSK3 from MPP inputs contributes to LTP deficits, then LTP at other MPP synapses should also be impaired in FX mice. To test this, we assessed the magnitude of LTP at temporammonic-CA1 synapses (MPP synapses onto distal dendrites of CA1). We show that FX mice display a deficit in LTP at TA-CA1 synapses (WT:143±1%; FX: 120±5%, $p<0.05$) (Figure 6) suggesting a possible pre-synaptic role for hyperactive GSK3 in FX.

Discussion

Here we report a significant decrease in inhibitory serine p-GSK3 levels specifically in synaptosomes from the dentate gyrus of FX mice that is associated with a deficit in NMDAR-dependent LTP at MPP-DGC synapses. Pharmacological inhibition of GSK3, but not an inhibitor of mGluR5, completely reverses both synaptic strongly suggesting that GSK3 should be considered as a therapeutic target for the treatment of cognitive dysfunction in FX.

GSK3 inhibitors have been proposed as potential therapeutic candidates for treating FX, based to a large extent on the multiple beneficial effects resulting from administration of the GSK3 inhibitor lithium to FX mice (Mines and Jope, 2011). Lithium treatment of FX mice reverses locomotor hyperactivity, audiogenic seizure hypersensitivity, increased spine density, enhanced mGluR-mediated LTD, reactive astrocytes, macroorchidism, excess protein synthesis, and social behavior deficits (Min et al., 2009; Mines et al., 2010; Yuskaitis et al., 2010a; Yuskaitis et al., 2010b; Choi et al.,

2011; Liu et al., 2011; Liu et al., 2012). Furthermore, lithium improved deficient passive avoidance behavior, the only reported test of lithium's effect on cognitive impairments in FX mice. These actions of lithium are likely due to inhibition of GSK3 because GSK3 inhibitors other than lithium also have been reported to control locomotor hyperactivity, susceptibility to audiogenic seizures, trace conditioning, delayed non-matching-to-place radial arm maze and neurogenesis in FX mice (Min et al., 2009; Guo et al., 2012). Thus it appears that GSK3 inhibitors, including lithium, are able to normalize many abnormal characteristics of FX mice and thus are potential therapeutic interventions for FX. We found that the impaired MPP-DGC LTP in FX mice was repaired by administration of GSK3 inhibitors, suggesting that GSK3 inhibition might also reverse cognitive deficits by normalization of LTP. These findings are in accordance with reports that hyperactive GSK3 impairs LTP (Hooper et al., 2007; Zhu et al., 2007), and that GSK3 is hyperactive in the hippocampus of FX mice (Min et al., 2009; Yuskaitis et al., 2010b), specifically in synapstosomes from the dentate gyrus, reported here.

Because aberrant mGluR5 function is known to regulate several of the phenotypes of FX mice and acute administration of the mGluR5 antagonist MPEP to FX mice reverses deficits in several behaviors and mEPSC frequency in the amygdala (Yan et al., 2004; de Vrij et al., 2008; Min et al., 2009; Suvrathan et al., 2010; Gross et al., 2011; Thomas et al., 2012), we tested if administration of MPEP also reversed deficits in LTP. Acute administration of MPEP was completely ineffective in reversing the impaired MPP-DGC LTP, in accordance with a report that MPEP fails to rescue LTP deficits in amygdala (Suvrathan et al., 2010). Collectively, these results indicate that although mGluR5 is a promising therapeutic target (Dolen et al., 2007; Michalon et al., 2012), GSK3 inhibitors

must also be considered for treatment of FX. Additonally, because acute GSK3, but not acute mGluR5, inhibition reverses these deficits, mGluR5 is not directly driving the pathologically hyperactive GSK3 in FX mice. However, a recent study showed that chronic, but not acute, mGluR5 inhibition reversed novel object recognition deficits (Busquets-Garcia et al.), so aberrant mGluR function in FX could indirectly modulate GSK3 activity.

To fully understand how hyperactive GSK3 leads to synaptic in FX, identification of downstream targets of GSK3 is needed. Our results suggest that inhibition of GSK3 does not rescue LTP by reversing deficits in NMDAR transmission. It is possible that inhibition of GSK3 may rescue LTP through effector targets downstream of NMDAR activation. GSK3 regulates AMPAR trafficking (Wei et al., 2010), however this mechanism is unlikely since there are no deficits in baseline AMPAR transmissison in FX mice. Alternatively, our data showing deficits in TA-CA1 LTP imply that hyperactive GSK3 in the pre-synaptic terminal might be responsible for impaired NMDAR-dependent LTP. It will be of great interest in future studies to elucidate the precise mechanisms by which hyperactive GSK3 decreases plasticity and learning and memory, as these mechanisms represent novel targets for therapeutic development for the treatment of intellectual disability in FX.

Acknowledgments

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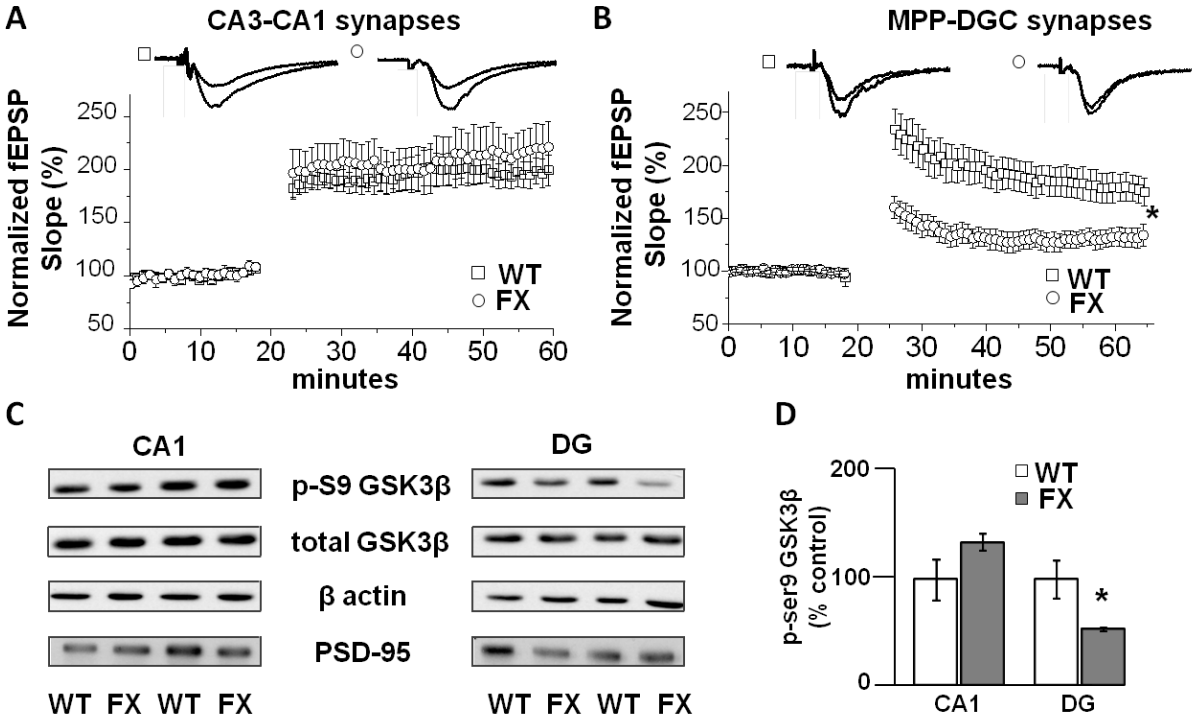

Figure 1. Deficits in LTP at MPP-DGC synapses in FX mice are accompanied by decreased inhibitory serine-phosphorylation of GSK3β. (A) Summary plots of the magnitude of LTP induced by HFS at CA3-CA1 Schaffer collateral synapses in slices from WT (n=7) and FX (n=6) mice. (B) Summary plots of the magnitude of LTP induced by HFS at MPP-DGC synapses in slices from WT (n=6) and FX (n=9) mice. (C) Representative Western blots showing a reduction in phospho-serine9-GSK3β (p-S9 GSK3) protein in dentate gyrus (DG) but not in CA1 from FX versus WT mice. Total GSK3β protein levels are not different between groups. β-Actin and PSD-95 were used as cytosolic and synaptic protein loading controls. (D) Quantitation of the ratio of p-S9 GSK3β to total GSK3β normalized to values of WT mice (CA1, n=4, and DG, n=6, for each genotype). *$p<0.05$ (Student's t test).

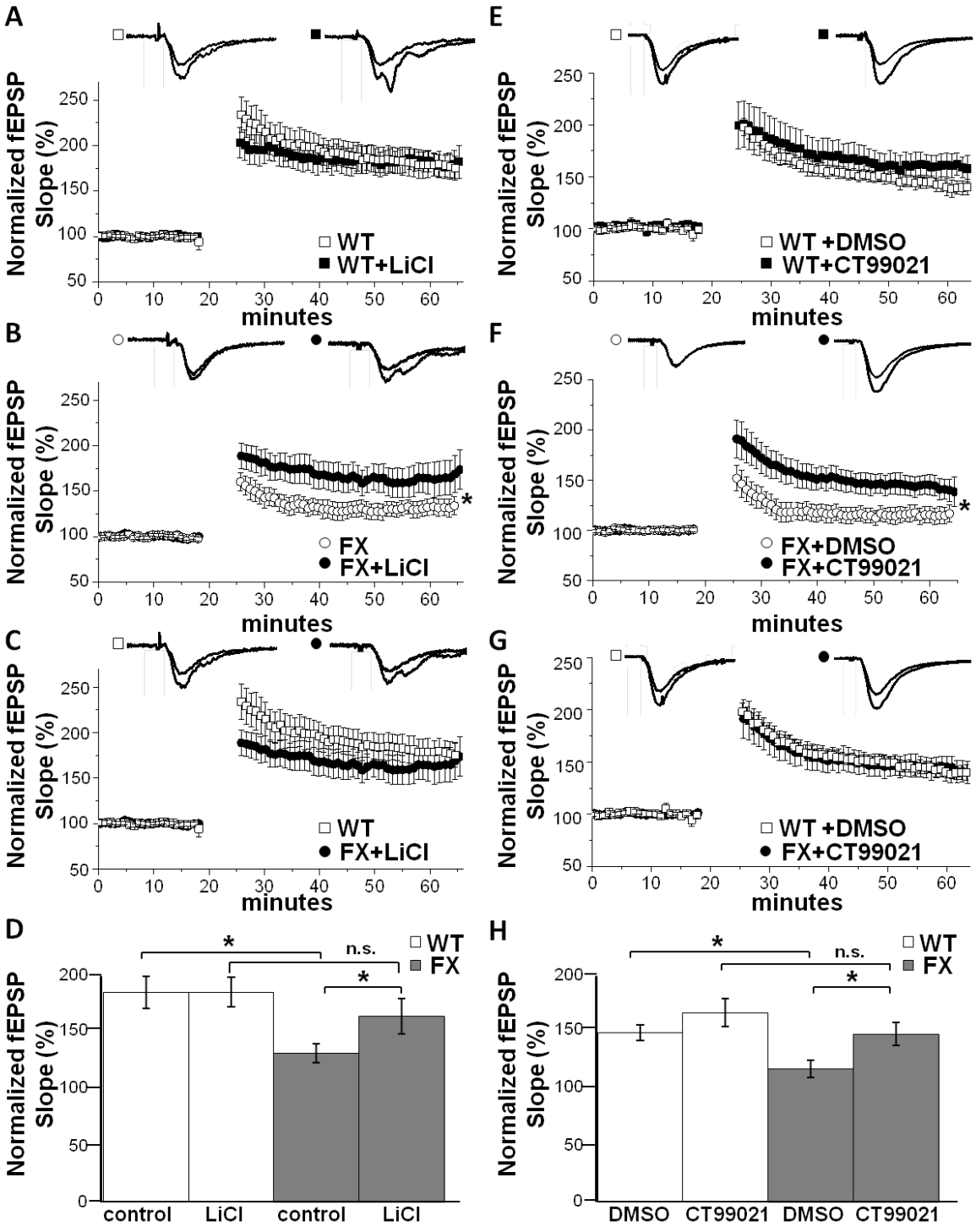

Figure 2. Pharmacological blockade of GSK3 reverses the deficit in LTP at MPP-DGC synapses. Summary plots of the magnitude of LTP induced by HFS at MPP-DGC synapses in slices from (A) WT mice with (n=6) and without (n=6) bath application of LiCl (20 mM), and (B) FX mice with (n=6) and without (n=9) bath application of LiCl

(20 mM). (C) Data from WT mice (from A) and FX mice (bath application of LiCl from B) replotted to compare the magnitudes of LTP.(D) Summary of data A-C. (E) WT mice with (n=8) and without (n=12) bath application of CT99021 (2 uM). (F) FX mice with (n=7) and without (n=7) bath application of CT99021. (G) WT (from E) and FX (bath application of CT99021 from F) replotted to compare the magnitudes of LTP. *$p<0.05$ (Student's t test). (H) Summary of data E-F

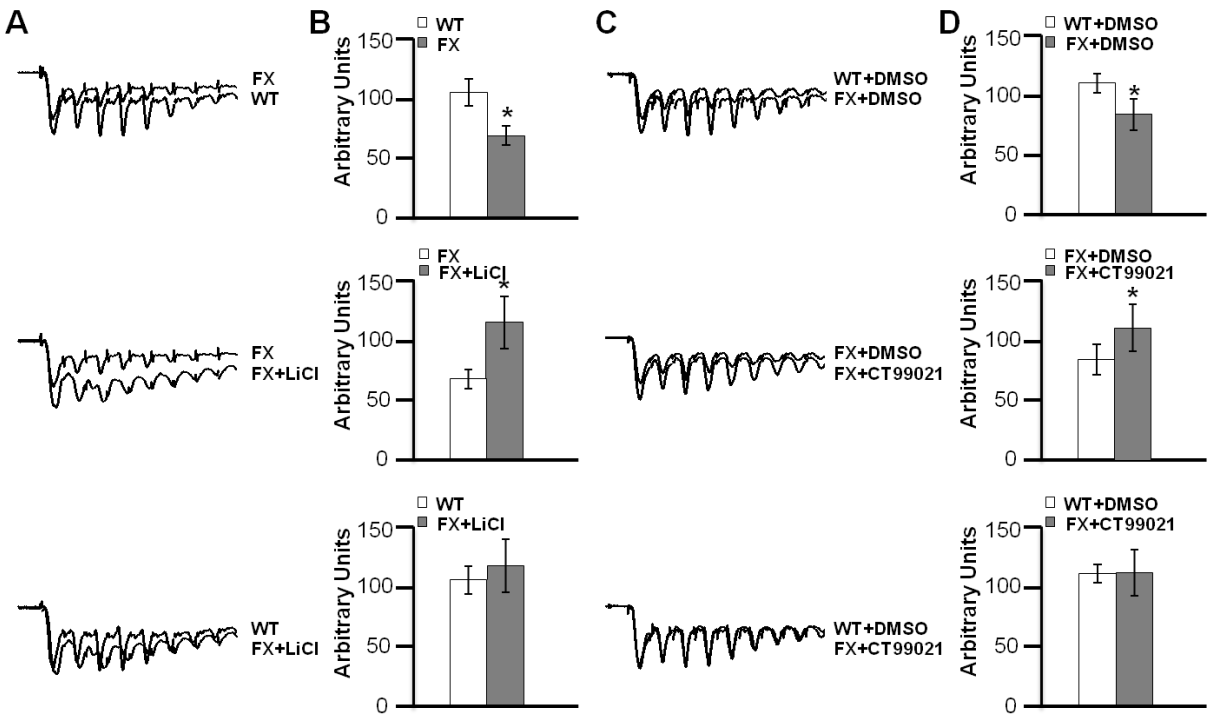

Figure 3. Pharmacological blockade of GSK3 does not alter steady-state depolarization. (A) Averaged traces during the 4th tetanus from experiments in Fig 2A-C show reduced steady-state depolarization during tetanus in FX slices. (B) Pooled data from all LTP experiments in Fig 2 show LiCl treatment reverses the deficit in steady state depolarization in FX slices. (WT: 110±12 vs FX: 72±8) (FX: 71±8 vs FX+Li: 123±23) (WT: 110±12 vs FX+Li: 123±23) *$p<0.05$ (Student's t test). (C) Averaged traces during the 4th tetanus from experiments in Fig 2D-E show reduced steady-state depolarization during tetanus in FX slices. (D) Pooled data from experiments in Fig 2 show CT99021 treatment reverses the deficit in steady state depolarization in FX slices. (WT: 116±8 vs FX: 71±8) (FX: 88±13 vs FX+CT99021: 117±20)(WT: 116±8 vs FX+CT99021: 117±20) *$p<0.05$ (Student's t test)

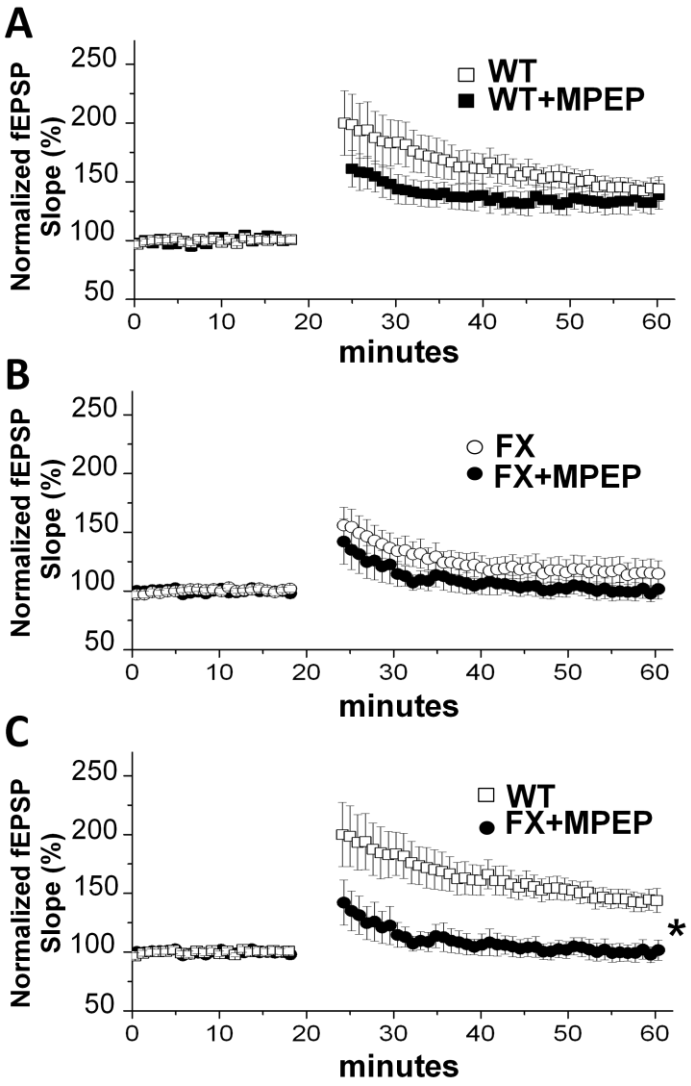

Figure 4. Synaptic deficits in FX mice are not altered by mGluR inhibition. Summary plots of the magnitude of LTP induced by HFS at MPP-DGC synapses in slices from (A) WT mice with (n=5) and without (n=5) bath application of MPEP (100 uM), and (B) FX mice with (n=7) and without (n=6) bath application of MPEP. (C) Data from WT (from A) and FX (bath application of MPEP from B) mice replotted to compare the magnitude of LTP. *$p<0.05$ (Student's t test).

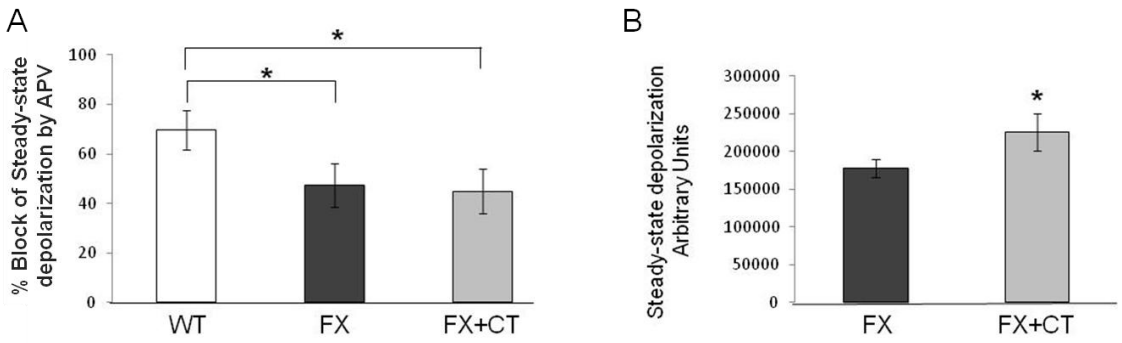

Figure 5. GSK3 inhibition does not reverse NMDAR hypofuction during SSD. (A) Percent of SSD remaining after bath application of APV in WT mice (n=7) and FX mice with (n=9) and without (n=9) bath application of CT99021 and (B)Steady state depolarization in FX with (n=9) and without (n=9) CT99021. *$p<0.05$ (Student's t test).

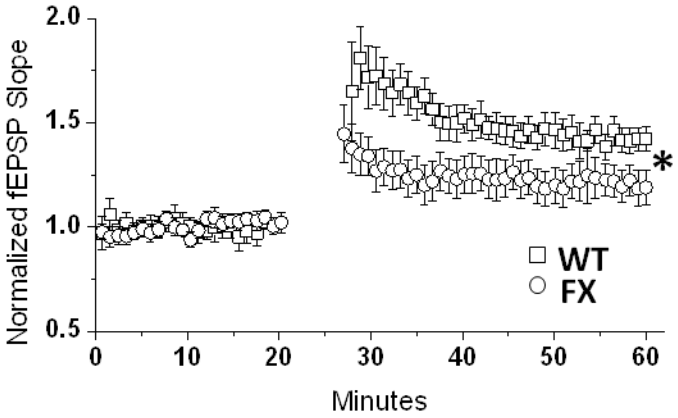

Figure 6. FX mice display deficit in LTP at TA-CA1 synapses. Summary plot of LTP induced using HFS at TA-CA1 synapses in slices from WT (n=6) and FX (n=9) mice. *p<0.05 (Student's t test).

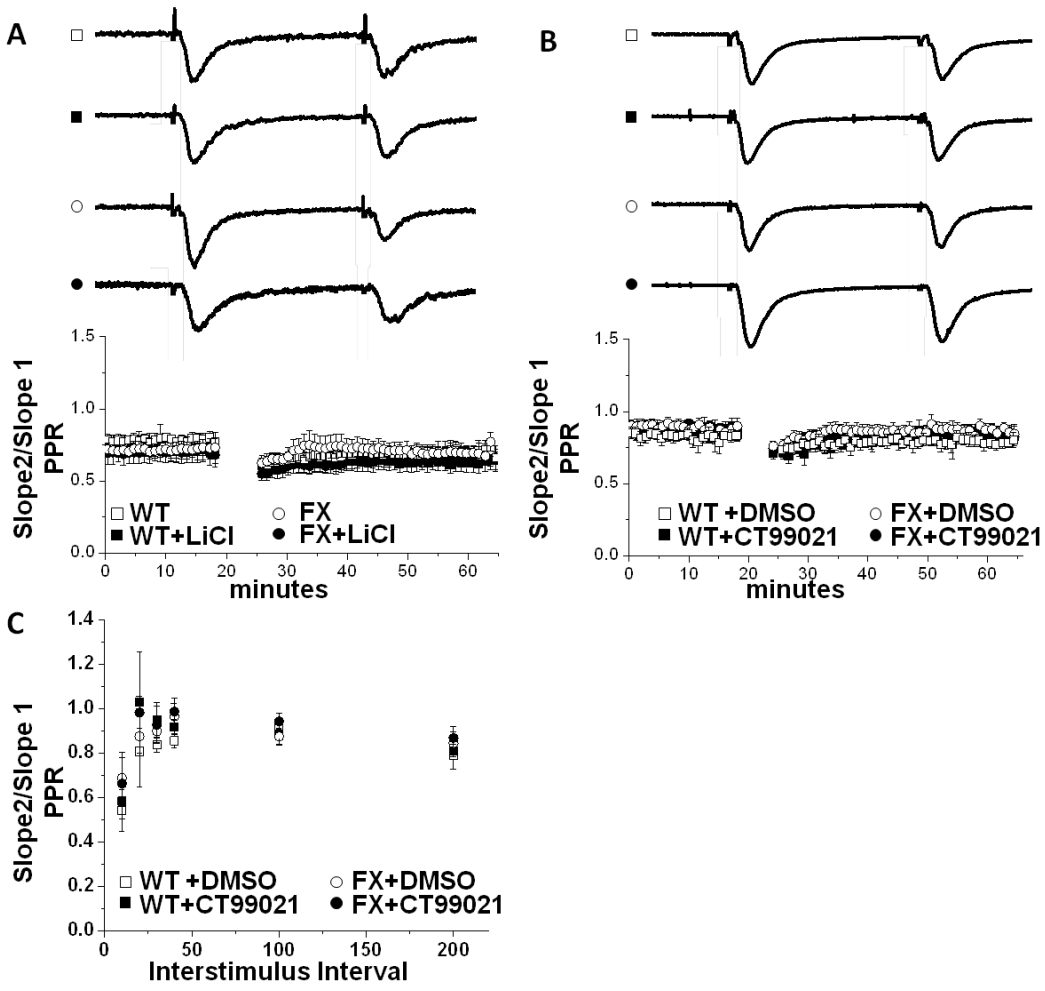

Figure S1. Inhibition of GSK3 does not alter paired-pulse ratio. (A) Summary plots showing no difference in the paired pulse ratio from experiments in Figures 2A, 2B, 2C. (WT: 0.77±.09 vs WT+Li: 0.66±0.06 vs FX: 0.79±0.09 vs FX+Li: 0.65±0.06 $p>0.05$) (B) Summary plots showing no difference in the paired pulse ratio from experiments in Figures 2D, 2E, 2F (WT: 0.79±0.02 vs WT+CT99021: 0.81±0.04 vs FX: 0.86±0.04 vs FX+CT99021 :0.83±0.02) $p>0.05$ (One-way ANOVA).

INCREASED LONG-TERM POTENTIATION AT MEDIAL-PERFORANT PATH-DENTATE GRANULE CELL SYNAPSES INDUCED BY SELECTIVE INHIBITION OF HISTONE DEACETYLASE 3 REQUIRES FRAGILE X MENTAL RETARDATION PROTEIN

by

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In preparation for *Journal of Neuroscience*

Format adapted for dissertation

Abstract

Non-selective inhibition of histone deacetylases (HDACs), enzymes that remove acetyl groups from histone core proteins, enhances cognition and NMDAR-dependent long-term potentiation at hippocampal CA3-CA1 synapses. It is not known whether HDACs modulate plasticity at other hippocampal synapses, nor is it known whether HDAC inhibition can reverse deficits in synaptic plasticity in disease models. To determine whether inhibition of HDACs, and specifically HDAC3, a class I HDAC isoform known to negatively regulate hippocampus-dependent learning and memory, enhances LTP at medial perforant path-dentate granule cell (MPP-DGC) synapses, we examined the magnitude of LTP in the presence of a non-selective HDAC inhibitor, trichostatin A (TSA), or the selective HDAC3 inhibitor, RGFP966. We find that non-selective HDAC inhibition increased the magnitude of LTP at MPP-DGC synapses in wild-type mice, similar to reports at CA3-CA1 synapses. This effect was mimicked by selective HDAC3 inhibition, implicating a role for this HDAC isoform in the negative regulation of synaptic plasticity. We next investigated whether selective HDAC3 inhibition was able to reverse the deficit in LTP at MPP-DGC synapses in a mouse model of Fragile X Syndrome. Importantly, no enhancement in LTP was observed at MPP-DGC synapses in slices from *Fmr1-/-* mice, indicating that the enhancing effect of HDAC3 inhibition on LTP in wild-type mice requires FMRP.

Introduction

Histone deacetylases (HDACs), enzymes that encourage chromatin condensation by removing acetyl groups from histones, decrease transcription of genes required for memory formation (Kouzarides, 2007). Previous studies demonstrated that non-selective HDAC inhibition enhances NMDAR-dependent long-term potentiation (LTP) at hippocampal CA3-CA1 synapses and hippocampus-dependent learning and memory in wild-type (WT) mice (Levenson et al., 2004; Vecsey et al., 2007; Stefanko et al., 2009). HDAC inhibition also improves learning and memory in an Alzheimer's disease mouse model and in mice with p25-induced neurodegeneration (Fischer et al., 2007; Kilgore et al., 2010). Although these findings support the therapeutic potential of HDAC inhibitors in treating cognitive deficiencies, their non-specific effects limit clinical use (Tsankova et al., 2006; Bruserud et al., 2007; Renthal et al., 2007), motivating studies to determine which HDAC isoforms can be selectively targeted to improve memory function.

To-date, two Class I HDAC isoforms have been identified as negative regulators of memory formation. Over-expression of HDAC2, but not HDAC1, impairs LTP at CA3-CA1 synapses associated with deficits in contextual fear conditioning and Morris water maze, while synaptic plasticity and memory are facilitated in HDAC2 knockout mice (Guan et al., 2009). Genetic deletion or selective pharmacological inhibition of HDAC3, the isoform most highly expressed in hippocampus (Broide et al., 2007), enhances object recognition and location memory (McQuown et al., 2011), but whether the behavioral improvements are associated with increased LTP was not explored.

The beneficial effects of HDAC inhibition on synaptic plasticity have been studied primarily at hippocampal CA3-CA1 synapses in WT mice. Therefore, it is not known whether HDAC inhibitors increase LTP at other hippocampal synapses or in animals with known deficits in hippocampal plasticity. In a mouse model of Fragile X Syndrome (*Fmr1-/-* mice), the most common inherited form of mental retardation (Bakker, 1994), loss of Fragile X Mental Retardation Protein (FMRP) causes a deficit in NMDAR-dependent LTP at medial perforant path-dentate-gyrus granule cell (MPP-DGC) synapses that is accompanied by impaired dentate gyrus associated cognitive tasks (Yun and Trommer, 2011; Eadie et al., 2012; Franklin et al., 2014). The LTP deficit can be reversed by acute application of glycine or D-serine (Bostrom et al., 2013) in brain slice recordings or by selective inhibition of glycogen synthase kinase-3 (GSK3) (Franklin et al., 2014). Acute GSK3 inhibition *in vivo* also reverses dentate gyrus specific behavioral deficits (Franklin et al., 2014), mechanistically linking impaired LTP and behavior. Here, we sought to determine whether HDAC3 inhibition increases the LTP magnitude at MPP-DGC synapses in WT mice, and whether it also reverses the LTP deficit at these synapses in *Frm1-/-* mice, with hopes of identifying a novel therapeutic target to treat cognitive impairment in Fragile X Syndrome.

Materials and methods

Animals

Male wild-type (WT) C57Bl/6 mice and male *Fmr1*-/- mice (ages 2-6 months) on a pure C57Bl/6 background, were used in all experiments. Male and female C57Bl/6 *Fmr1* +/-

mice were mated to generate *Fmr1-/-* and WT littermates. Mice were housed on a 12h light/dark cycle in temperature controlled rooms. The care and use of all mice followed an approved protocol by the University of Alabama at Birmingham Institutional Animal Care and Use Committee and in accordance National Institutes of Health guidelines.

Hippocampal slice preparation

Mice were decapitated following isoflurane anesthesia and brains were rapidly removed and placed in modified ice-cold artificial cerebrospinal fluid (aCSF) [in mM: 85 NaCl, 2.5 KCl, 4 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 glucose and 75 sucrose saturated in 95% O_2 and 5% CO_2]. Coronal slices (400 M) of dorsal hippocampus (defined as Bregma -1.46mm to-2.46; Franklin and Paxinos atlas) were prepared using a vibratome (Vibratome 1000 Plus; St. Louis MO) then immediately transferred into a holding chamber containing standard aCSF [in mM: 124 NaCl, 3 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 15 glucose saturated in 95% O_2 and 5% CO_2] where the slices remained until experimentation or drug treatment. In experiments where pre-incubation of drug was required, slices were stored in standard aCSF for 30 min following slicing then transferred to a holding chamber that contained standard aCSF with either DMSO or RGFP966. All slices, regardless of treatment, rested at least 1 hr prior to recording.

Electrophysiology

Brain slice electrophysiology experiments were performed as previously reported (Franklin et al., 2014). Briefly, for extracellular field dendritic field potential (fEPSP)

recordings, slices were transferred to a submersion chamber and perfused with standard aCSF at 26-28º F. Baseline fEPSPs were generated by stimulating the medial perforant path input onto dentate granule cell synapses (0.1 Hz, 200uS) (MPP-DGC). Correct electrode placement was confirmed visually and by the presence of paired-pulse depression characteristic of MPP-DGC synapses (McNaughton 1980, Colino and Malenka 1993), through the duration of the experiment. LTP was induced using high-frequency stimulation (HFS, 100 Hz, 1 s duration x 4, 60s interval). All recordings were performed in the presence of the $GABA_AR$ antagonist picrotoxin (100M) so that fEPSPs could be measured in isolation, as previously reported (Franklin et al., 2014).

Reagents

Trichostatin A (TSA) (Tocris Bioscience; Ellisville, MO) was dissolved in DMSO and used at a final concentration of 1.65□M (Levenson et al., 2004). RGFP966 (generous gift from Repligen Corp) is an HDAC3 inhibitor with an IC_{50} of 0.8uM and is specific to HDAC3 up to 15□M (Malvaez et al., 2013). It was dissolved in DMSO and used at a final concentration of 10 □M in all experiments.

Statistics

Data are expressed as mean ± SEM. Significant differences were determined using Student's *t* tests at $P < 0.05$. *n* represents animal number. Animals were included in a data set only when both control and drug experiments were successfully completed. Experiments were discarded from the data set if there was >10% change in fEPSP slope during baseline recording.

Results

We sought to determine whether HDAC inhibition increases the LTP magnitude at MPP-DGC synapses similarly to what has been previously reported at CA3-CA1 synapses in slices from adult WT mice (Levenson et al., 2004; Guan et al., 2009). Extracellular dendritic fEPSPs were recorded at MPP-DGC synapses in acute slices from adult WT mice. LTP was induced using high frequency stimulation in the presence or absence of TSA, a non-selective HDAC inhibitor. Slices were continuously perfused with either DMSO (vehicle) or TSA for 1 hr prior to delivering HFS to induce LTP. We found that in the presence of TSA the magnitude of LTP at MPP-DGC synapses is significantly increased compared to DMSO alone (DMSO: 136±2% of baseline fEPSP slope vs TSA: 162±5 % of baseline fEPSP slope; p=.01) (Figure 1A-1B).

We next assessed whether the enhanced LTP magnitude in TSA is associated with an increase in presynaptic neurotransmitter release. To this end, we measured the paired-pulse ratio (PPR), an indirect measure of presynaptic release probability, in DMSO and TSA treated slices pre- and post-tetanus and found significant differences between groups (pre-HFS, DMSO: 0.95± .05 vs TSA: 0.97±.06; p=.38; post-HFS, DMSO: 0.87± .03 vs TSA: 0.89±.067; p=.44) (Figure 1C). This suggests that an increase in neurotransmitter release is likely not responsible for the enhanced LTP magnitude. We also found no difference in the magnitude of the depolarization during HFS when comparing DMSO and TSA groups (arbitrary units, DMSO: 1.0± .09 vs TSA: 1.11±.13; p=.26) (Figure 1D). Taken together these results suggest a likely post-synaptic mechanism for the enhanced magnitude of LTP following HDAC inhibition.

Given that selective pharmacological inhibition of HDAC3 improves object recognition and location memory (McQuown et al., 2011), behaviors requiring the dentate gyrus (Goodrich-Hunsaker et al., 2008), we next tested whether selective HDAC3 inhibition would increase the LTP magnitude at MPP-DGC synapses, mimicking the effect of the non-selective HDAC inhibitor TSA. To accomplish this, slices were incubated for at least 3 hr in DMSO (vehicle) or 10□M RGFP966 prior to inducing LTP with HFS. We found that RGFP966 robustly enhanced the LTP magnitude at MPP-DGC synapses (DMSO: 152±23% of baseline fEPSP slope vs RGFP966: 216±91% of baseline fEPSP slope; p=.005) (Figure 2A-2B). However, in contrast to TSA where significant potentiation does not occur until 45 min post-tetanus (DMSO: 142±33% of baseline fEPSP slope vs TSA: 164±7 % of baseline fEPSP slope; p=.03) (Figure 1B), with selective HDAC3 inhibition, significant potentiation occurred as soon as 10 min post-tetanus (DMSO: 176±35% of baseline fEPSP slope vs RGFP966: 244±181% of baseline fEPSP slope; p=.046). Similar to TSA, there were no differences in PPR (pre-HFS, DMSO: 0.95± .004 vs RGFP966: 0.98±.03; p=.30; post-HFS, DMSO: 0.93± .02 vs RGFP: 1.12±.44; p=.19) (Figure 2C) or depolarization during HFS between the DMSO and RGFP966 groups (in arbitrary units, DMSO: 1.0±.13 vs TSA: 0.8±.15; p=.29) (Figure 2D).

Given the robust effects of HDAC3 inhibition on the LTP magnitude at MPP-DGC synapses in WT mice, we next sought to determine whether the inhibitor would rescue deficits in LTP at MPP-DGC synapses in *Fmr1-/-* mice. Slices from *Fmr1-/-* mice were incubated for at least 3 hrs in either DMSO or RGFP966, consistent with the protocol used in experiments with slices from WT mice. Surprisingly, HDAC3 inhibition

with RGFP966 was completely ineffective at increasing the LTP magnitude at MPP-DGC synapses in *Fmr1-/-* mice (DMSO: 135±3% of baseline fEPSP slope vs RGFP966: 146±7% of baseline fEPSPs slope; p=.24) (Figure 3).

Discussion

Here we report that inhibition of class I HDACs, and more specifically, that inhibition of HDAC3, enhances the LTP magnitude at MPP-DGC synapses in adult WT mice. This finding confirms that modulation of LTP by HDACs is not selective for CA3-CA1 synapses, but also occurs at least at one other synapse in the hippocampal formation. Moreover, this finding raises the question as to whether the increase in LTP magnitude following HDAC inhibition is a general mechanism impacting many, if not all, excitatory synapses throughout the CNS. These findings are also consistent with the interpretation that the increase in LTP magnitude at MPP-DGC synapses we observed following HDAC3 inhibition could be mechanistically linked to the previously reported improved object recognition and location memory in WT mice following pharmacological HDAC3 inhibition, because these behaviors require the dentate gyrus (Goodrich-Hunsaker et al., 2008).

While our finding that HDAC3 inhibition is unable to enhance plasticity in *Fmr1-/-* mice suggests HDAC inhibitors may not be useful treatment options in Fragile X Syndrome, these results are exciting in that they provide unique insight into a potential mechanism for enhanced plasticity at excitatory synapses in WT mice. FMRP is an RNA–binding protein known to suppress translation of proteins, many of which are involved in synaptic plasticity (Ashley et al., 1993; Siomi et al., 1993; Feng et al., 1997;

Brown et al., 2001; Laggerbauer et al., 2001; Todd et al., 2003; Zalfa et al., 2003; Khandjian et al., 2004; Lu et al., 2004; Weiler et al., 2004). Interestingly, the FMR1 promoter region contains binding sites for both CREB and Nrf2 (Smith et al., 2006), transcription factors which are modulated by HDAC inhibition. In fact, HDAC inhibition requires CREB activation for enhancement of LTP (Vecsey et al., 2007) and also activates Nrf2 (Wang et al., 2012). Therefore it is possible that HDAC inhibition modulates LTP by increasing FMRP transcription through CREB and Nrf2 activation. Subsequently, increased FMRP could contribute to enhanced LTP by preventing translation of proteins that normally constrain LTP expression. When FMRP is absent, the brake on translation of "LTP suppressing" proteins is removed and LTP is no longer enhanced.

An interesting observation is that selective HDAC3 inhibition induces significant potentiation as early as 10 min post-tetanus, while significant potentiation does not occur until 45 min post-tetanus using the non-selective HDAC inhibitor TSA. One possible explanation for the difference in timing is that some HDAC isoforms may act to positively regulate LTP, such as HDAC4 (Kim et al., 2012). Perhaps the delayed onset of the increase in LTP magnitude after TSA is the net result of inhibition of both negative and positive regulators of early plasticity. However, when RGFP966 is applied, only HDAC3 is inhibited, therefore there are no opposing effects from other HDACs and LTP is robustly enhanced immediately after induction.

In summary, our data demonstrate that HDACs, specifically HDAC3, are negative regulators of synaptic plasticity at synapses in dentate gyrus. While selective inhibition of HDAC3 does not appear to be beneficial for synaptic dysfunction in a mouse model of

Fragile X Syndrome due to the requirement of FMRP to enhance plasticity, our data do not rule out the possibility of using HDAC3 as a therapeutic target in other cognitive disorders where MPP-DGC synapses are affected, like the Ts65Dn model of Down's syndrome (Kleschevnikov et al., 2004). Furthermore, the question of whether enhanced LTP is an absolute requirement for reversal of cognitive impairments in Fmr1-/- mice remains. We have demonstrated a crucial role for FMRP in HDAC3 modulation of LTP however future studies are necessary to dissect the precise mechanism by which HDAC3 and FMRP interact.

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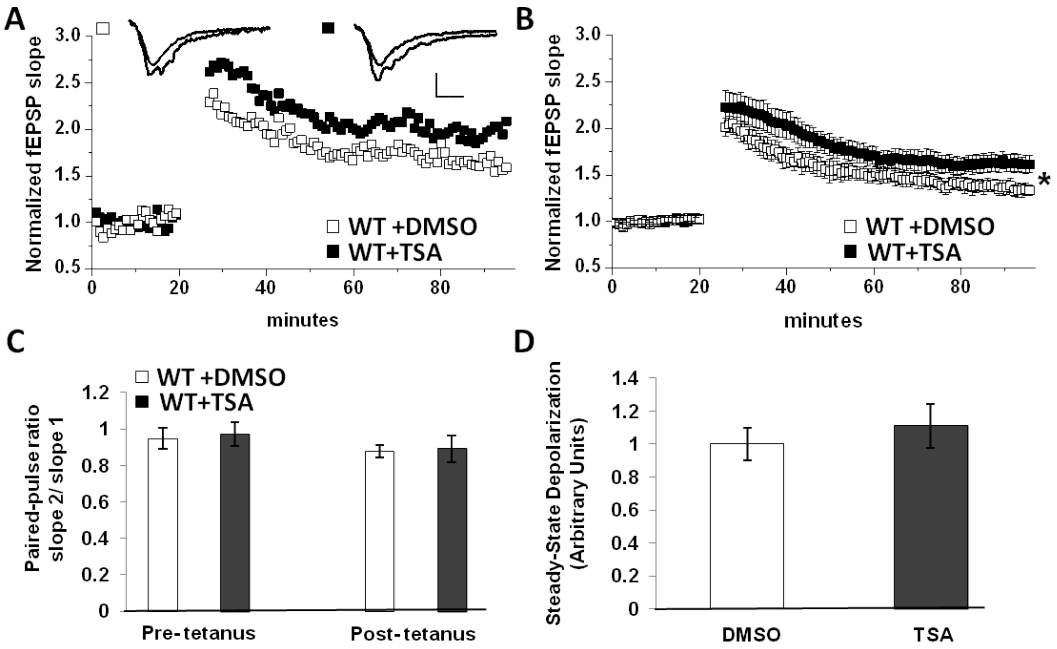

Figure 1. Non-Selective HDAC inhibition enhances LTP at MPP-DGC Synapses. (A) Single representative experiment and (B) summary plot of the average LTP magnitude at MPP-DGC synapses in WT slices treated with DMSO (n=7) or TSA(n=7). Scale bar 0.2mV/100uS (C) Average PPR in DMSO and TSA treated groups (from experiments in B) are not significantly different. (D) No difference in depolarization during HFS between DMSO and TSA treated groups (from experiments in B). (*$p<0.05$ Student's t test)

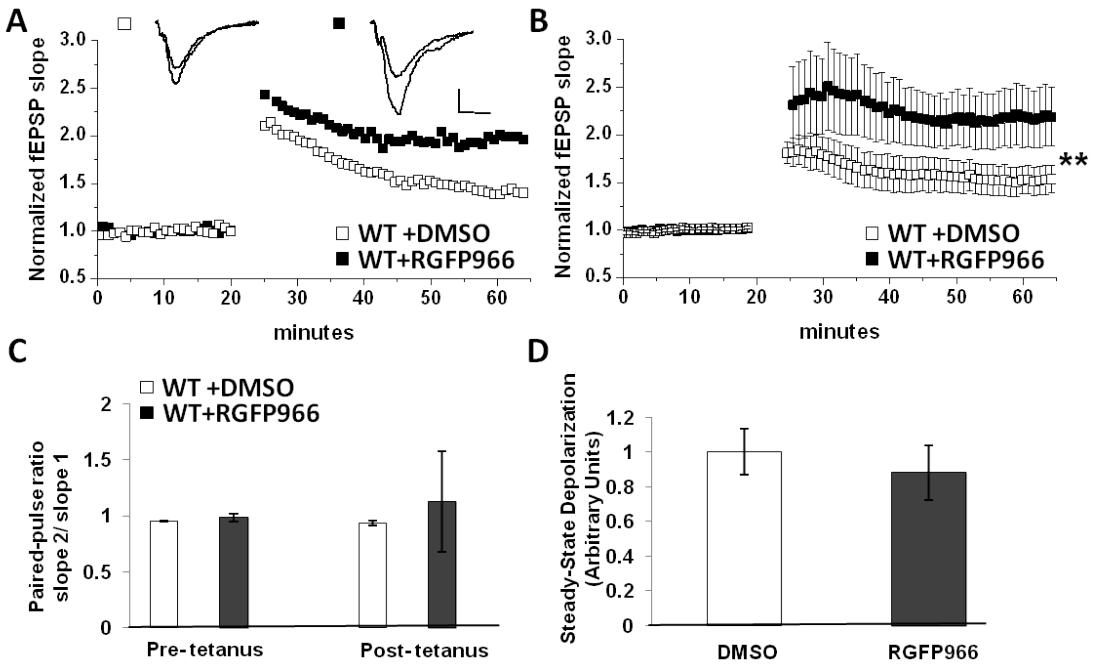

Figure 2. Selective HDAC3 inhibition enhances LTP at MPP-DGC Synapses. (A) Single representative experiment and (B) summary plot of the average LTP magnitude at MPP-DGC synapses in WT slices treated with DMSO (n=10) or RGFP966 (n=10). Scale bar 0.2mV/100uS (C) Average PPR in DMSO and TSA treated groups (from experiments in B) are not significantly different. (D) No difference in depolarization during HFS between DMSO and TSA treated groups (from experiments in B). (**p<0.01 Student's t test)

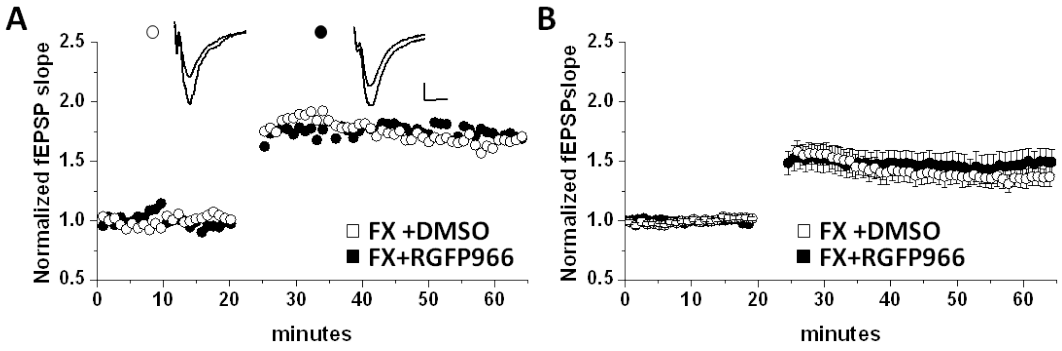

Figure 3. Selective HDAC3 inhibition does not enhance LTP at MPP-DGC Synapses in *Frm1*-/- mice. (A) Single representative experiment and (B) summary plot of the average LTP magnitude induced by HFS at MPP-DGC synapses in slices from *Frm1-/-* mice treated with DMSO (n=6) or RGFP966(n=6). Scale bar 0.2mV/100uS

SUMMARY AND DISCUSSION

This thesis research has lead to several significant findings. We find that acute inhibition of GSK3 with lithium or CT99021 reverses the deficit in LTP at MPP-DGC synapses in FX mice. We also report a deficit in steady-state depolarization (SSD) during high frequency tetanus and NMDAR hypofunction during SSD. GSK3 inhibition rescues SSD but fails to rescue NMDAR hypofunction during SSD. Importantly, blockade of mGluRs with MPEP, which rescues many of the phenotypes observed in FX mice, fails to rescue LTP at MPP-DGC synapses, suggesting a novel therapeutic target. Furthermore, we show that inhibition of HDAC3, a downstream target of GSK3, enhances LTP at MPP-DGC synapses in WT mice but fails to enhance LTP in FX mice.

Fragile X Syndrome (FX) is a debilitating disorder that causes severe mental retardation and is the leading genetic cause of autism. Presently, there are no therapies available to treat cognitive impairments in affected individuals. Because FX mice are a model of mental retardation it was expected that cognitive impairments would be readily identified. However, FX mice have been assessed in many cognitive tasks in which they behave normally or show only mild impairments, such as the Morris water maze, radial arm maze, operant conditioning paradigms and contextual and conditioned fear memory (Bakker, 1994; Kooy et al., 1996; D'Hooge et al., 1997; Fisch et al., 1999; Paradee et al., 1999; Dobkin et al., 2000; Peier et al., 2000; Mineur et al., 2002; Yan et al., 2004; Baker et al., 2010). Recent studies have shifted focus to dentate gyrus dependent cognition and

have demonstrated that FX mice display robust deficits in novel object recognition and pattern separation (Ventura et al., 2004; Pacey et al., 2011; Bhattacharya and Klann, 2012; Eadie et al., 2012) Interestingly, these are associated with deficits in NMDAR-LTP and NMDAR hypofunction at MPP-DGC synapses (Yun and Trommer, 2011; Eadie et al., 2012). These seminal findings have provided researchers with a model in which to study underlying pathological mechanisms contributing to cognitive deficits in FX. Previous studies have identified a pathological role for hyperactive GSK3 in physical and behavioral impairments in FX (McBride et al., 2005; Min et al., 2009; Yuskaitis et al., 2010; Choi et al., 2011; Liu et al., 2011; Liu et al., 2012) . However, the role of GSK3 in synaptic and cognitive deficits has yet to be investigated. Because synaptic alterations are the cellular basis for learning and memory (Malenka and Bear, 2004), the present study was undertaken to determine whether hyperactive GSK3 contributes to synaptic deficits in FX mice in hopes of identifying novel therapeutic targets for the treatment of cognitive impairment.

GSK in Cognition

Given that LTP is one of the cellular correlates of learning and memory (Malenka and Bear, 2004), our finding that inhibition of GSK3, using both lithium and CT99021, reverses deficits in LTP at MPP-DGC synapses raises the possibility that inhibition of GSK3 may also reverse deficits in dentate specific cognition. Indeed, our collaborators show that treatment with two different GSK3 inhibitors reverses deficits in novel object recognition, categorical spatial processing and coordinate spatial processing (Franklin et al., 2014). We believe that due to the established role of GSK3 in bidirectional

modulation of NMDAR-dependent plasticity (Peineau et al., 2007) that GSK3 inhibition is reversing deficits in cognition through the normalization of LTP.

Interestingly, deficits in temporal order of visual objects, a CA1 dependent task (Goodrich-Hunsaker et al., 2005), was also observed. We report normal inhibitory serine 9 phosphorylation levels of GSK3 CA1 synaptosomes and expression of NMDAR-dependent LTP at CA3-CA1 (Godfraind et al., 1996; Paradee et al., 1999; Franklin et al., 2014) synapses suggesting that neither GSK3 hyperactivity, nor impaired plasticity contributes to temporal ordering deficits. However, several possibilities implicating GSK3 hyperactivity and/or synaptic deficits exist. One possible explanation is that normal function of the hippocampal trisynaptic circuit is required in the awake, behaving animal, and deficits in LTP at MPP-DGC synapses could cause deficits in function of downstream CA3-CA1 synapses. In *in vitro* hippocampal brain slice experiments, proper function of the trisynaptic circuit does not impact the ability of CA3-CA1 synapses measured in isolation to normally express NMDAR-dependent plasticity induced by electrical stimulation. In support of this, GSK3 inhibition, which reverses LTP deficits at MPP-DGC synapses, also normalizes temporal ordering deficits indicating that systemic GSK3 inhibition improves the overall function of the trisynaptic circuit.

An alternative explanation that could account for deficits in CA1-dependent cognitive tasks is decreased LTP at TA-CA1 synapses in FX mice. While there are no clear links between TA-CA1 LTP and cognition currently, TA-CA1 synapses have been implicated in several learning and memory models (Lisman and Otmakhova, 2001; Treves, 2004; Hasselmo, 2005; Rolls and Kesner, 2006). It has been hypothesized that TA-CA1 synapses function as an error detector (Izumi and Zorumski, 2008) or final

matching component of information directly from entorhinal cortex (EC) with information coming through the trisynaptic circuit (Lisman and Otmakhova, 2001). TA-CA1-LTP has now been observed and characterized *in vivo* (Aksoy-Aksel and Manahan-Vaughan, 2013) which will allow for more sophisticated studies dissecting the role of TA-LTP in cognitive tasks.

Since GSK3 inhibition normalized temporal ordering in FX mice, if TA-CA1 LTP deficits underlie cognitive impairment we would expect GSK3 inhibitors to reverse LTP deficits as well. In our western blot analysis of serine 9 phospho-GKS3 levels in area CA1 included both stratum radium (where CA3 projections synapse) and stratum lacunosum moleculare (where EC projections synapse) and we found no significant difference in serine 9 phospho-GSK3 suggesting GSK3 is not hyperactive at TA-CA1 synapses. It is possible that GSK3 is in fact hyperactive at these synapses but a decrease in inhibitory serine 9 phosphorylation was not observed because any change in serine 9 phospho-GSK3 could have been diluted out due to the presence of stratum radiatum in our samples.

Further studies probing serine 9 phospho-GSK in different layers of CA1 would be necessary to determine the phosphorylation levels at CA3-CA1 versus TA-CA1 synapses.

It is important to also mention that a few studies have now observed impairments in LTP at CA3-CA1 synapses induced using weak stimulation protocols (Lauterborn et al., 2007; Hu et al., 2008; Shang et al., 2009) in *Fmr1-/-* mice. It is likely that while the induction protocols utilized to elicit LTP in these studies induce forms of plasticity share

a similar expression mechanism with NMDAR-dependent LTP, they are mediated by different signaling cascades (Shang et al., 2009). Whether these forms of LTP are linked any form of cognition has yet to be investigated.

mGluR Theory of Fragile X

The mGluR theory of Fragile X, proposed almost a decade ago, hypothesized that enhanced group I mGluR signaling significantly contributes to FX pathology (Bear et al., 2004). Numerous studies have demonstrated that mGluR5 mediates many of the phenotypes observed in FX mice, including hyperactivity, increased dendritic spines, enhanced mGluR-LTD, and enhanced protein synthesis (Dolen et al., 2007; Dolen and Bear, 2008; Choi et al., 2011; Krueger et al., 2011). Based on these results, mGluR inhibitors are now being tested in clinical trials in hopes of providing therapeutic benefit. However, it is not known whether inhibition of mGluR will prove beneficial in reversing dentate-associated synaptic and cognitive deficits. It is possible that hyperactive GSK3 is a downstream consequence of enhanced mGluR signaling. Alternatively, GSK3 hyperactivity may be an independent pathological pathway recruited in the absence of FMRP. Importantly, we show that LTP deficits are not reversed at MPP-DGC synapses. These results are in accordance with a previous study demonstrating mGluR inhibition fails to rescue LTP deficits in amygdala (Suvrathan et al., 2010). If the LTP deficits are in fact subserving cognitive deficits in FX mice we would expect that MPEP would fail to reverse novel object recognition, categorical spatial processing, and coordinate spatial processing. In agreement with this hypothesis, our collaborators demonstrated that acute mGluR inhibition fails to rescue dentate-associated cognitive impairments in FX mice

(Franklin et al., 2014). Taken together, these results strongly suggest that increased GSK3 function in FX mice is not a direct consequence of enhanced mGluR function**.** These data, together with previously published work, establish GSK3 as a viable therapeutic target in FX, and raise the question that combined therapy directed at mGluRs and GSK3 could be a more effective treatment for cognitive deficits and behavioral abnormalities in FX patients.

Potential Mechanisms

Our data demonstrating that inhibition of GSK3 reverses cognitive and synaptic deficits in FX mice solidifies GSK3 as a therapeutic target in the treatment of FX. To gain insight into how hyperactive GSK3 leads to synaptic and cognitive impairment in FX and to identify possible novel therapeutic targets, it is critical to identify downstream targets of GSK3 that contribute to the pathophysiology.

NMDARs

NMDAR transmission is impaired at MPP-DGC synapses (Yun and Trommer, 2011; Eadie et al., 2012) in FX mice likely due to decreased surface expression of NMDARs (Bostrom et al., 2013). Importantly, augmenting NMDAR transmission with acute application of d-serine or glycine, NMDAR co-agonists, reverses deficits in LTP at MPP-DGC synapses in FX mice suggesting that NMDAR hypofunction causes deficits in LTP (Bostrom et al., 2013). GSK3 is involved in NMDAR internalization in pyramidal neurons in cortex (Chen et al., 2007) raising the possibility that hyperactive GSK3 leads to increased NMDAR internalization and subsequent decreased transmission and LTP. Furthermore, NMDAR transmission is suppressed through tonic dephoshorylation by

protein phosphatase 1 (PP1) (Westphal et al., 1999). PP1 also dephosphoryates ser 9 of GSK3β, thereby increasing activity. GSK3 in turn, phosphorylates and activates PP1 creating a positive feedback loop (Szatmari et al., 2005). Therefore, hyperactive GSK3 could also lead to NMDA hypofunction by increasing PP1 mediated inhibition of NMDARs. We indirectly assessed NMDAR function during HFS used to induce LTP by measuring the SSD before and after pharmacological NMDAR inhibition. In accordance with previous findings of NMDA hypofunction, we show that NMDARs contribute a smaller percentage to total SSD in FX mice versus WT mice. Surprisingly, this is not reversed with GSK3 inhibitors, suggesting hyperactive GSK3 is not responsible for NMDAR hypofunction.

Because this is an indirect measure of NMDA function, directly testing NMDAR transmission using whole-cell patch clamp electrophysiology to measure the input-output relationship is necessary to completely rule out NMDARs as downstream targets of pathologically hyperactive GSK3 in FX mice.

Neurogenesis

In addition to synaptic deficits, neurogenesis is also impaired in dentate gyrus in FX mice (Eadie et al., 2009; Guo et al., 2011). Importantly, decreased neurogenesis is reversed with lithium and specific GSK3 inhibitors (Guo et al., 2011). This is not surprising given previous studies demonstrating constitutively active GSK3 impairs (Eom and Jope, 2009) and deletion of GSK3 enhances neurogenesis implicating GSK3 as a key regulator of neurogenesis (Kim et al., 2009). These findings raise the possibility that inhibition of GSK3 reverses synaptic and cognitive deficits by normalizing neurogenesis

in FX mice. However, it is unlikely that acute GSK3 inhibition (minutes to an hour) is able increase cell survival and rescue neurogenesis in the short time scale of our experiments. Furthermore neurogenesis is impaired selectively in ventral hippocampus in FX mice whereas synaptic deficits are found in dorsal dentate gyrus (Eadie et al., 2009; Yun and Trommer, 2011; Eadie et al., 2012). Therefore GSK3 inhibition reverses deficits in LTP through a mechanism other than rescued neurogenesis. It is also unlikely that enhanced neurogenesis contributes to improved performance in pattern separation tasks because these tasks are primarily dependent on dorsal hippocampus. Ventral hippocampus is associated with anxiety-related behavior (Eadie et al., 2009) therefore increased neurogenesis following chronic *in vivo* administration of GSK3 inhibitors is likely a mechanism for reversed behavioral performance in anxiety related tasks in FX mice (Yuskaitis et al., 2010).

Pre-synaptic GSK3

Our results demonstrating a deficit in LTP at TA-CA1 synapses raises an interesting possibility that hyperactive GSK3 may occur pre-synaptically rather than post-synaptically. Our data showing a deficit in SSD during HFS, which can be a measure of pre-synaptic neurotransmitter release, that is reversed by GSK3 inhibition is in agreement with this idea. However, SSD can also be a measure of post-synaptic receptors density. Furthermore, we and others show there are no changes in paired pulse ratios (PPR) at MPP-DGC synapses (Yun and Trommer, 2011; Eadie et al., 2012) which is an indirect measure of pre-synaptic neurotransmitter release (Dobrunz and Stevens, 1997). Therefore, if there are pre-synaptic release deficits, they are only apparent when the

system is challenged with a high frequency train. Interestingly, one study has demonstrated an increase in PPR at TA-CA1 synapses in FX mice, however our results do not replicate this finding. A possible explanation for the discrepancy is that (El Idrissi et al., 2010) the previous study was conducted in FX mice on an FVB background as opposed to C57bl/6 background. Several strain specific differences have been observed in FX models (discussions at FRAXA Investigators Meeting).

Because of the conflicting data regarding pre-synaptic function in FX mice, additional experiments are necessary to determine whether hyperactive GSK3 is located pre- or post- synaptically. One way to assess pre-synaptic GSK3 function is to probe entorhinal cortext for p-GSK levels. If GSK3 is hyperactive in entorhinal cortex in FX mice compared to WT controls then Western blot analysis should reveal decreased serine 9 phosohporylation of GSK3β. An alternative way to test if hyperactive GSK3 is located pre-synaptically would be to perform whole cell LTP experiments with GSK3 inhibitors in the whole-cell pipette solution. If LTP deficits are reversed in FX mice then this concludes that hyperactive GSK3 is located post-synaptically. However, there are several caveats to this experiment. The most problematic is the experimental phenomenon of "LTP washout" during whole-cell recordings when LTP is no longer able to be induced. LTP washout occurs when the induction protocol is not administered within 10 minutes of break-in. This becomes an issue when GSK3 inhibitors are administered through the recording pipette. It is not known if 10 minutes is sufficient enough time for CT99021 or lithium to enter the cell and effectively inhibit GSK3, especially when investigating LTP at TA-CA1 synapses when inhibitors much reach the distal dendrites of CA1. A negative result in this experiment requires confirmation that 10 minute drug application is

sufficient for GSK3 inhibition. Post-synaptic inhibition of GSK3 prevents LTD so as a control it can be determined if 10 minutes is long enough for GSK3 inhibitors to block LTD.

HDAC3

Histone Deacetylase 3 (HDAC3) is a member of the Class I family of histone deacetylases (HDACs). Several studies have demonstrated that non-selective inhibition of HDACs enhance LTP and learning and memory(Levenson et al., 2004; Kouzarides, 2007; Vecsey et al., 2007; Stefanko et al., 2009; Kilgore et al., 2010), implying that HDACs negatively regulate synaptic plasticity and cognition. Furthermore, selective inhibition of HDAC3 enhances dentate-asscociated cognitive tasks (Stefanko et al., 2009; McQuown et al., 2011). Importantly, studies recently demonstrated that GSK3 can phoshorylate and increase HDAC3 activity (Bardai and D'Mello, 2011) raising the possibility that pathologically enhanced GSK3 activity could cause deficits in plasticity and cognition in FX mice due to increased HDAC3 activity.

We show that non-selective HDAC inhibition and selective inhibition of HDAC3 enhance LTP at MPP-DGC synapses in WT mice. This finding demonstrates that HDAC modulation of LTP is not selective for CA3-CA1 hippocampal synapses and raises the possibility that inhibition of LTP after HDAC inhibitors occurs at all excitatory synapses. This is in accordance with another study demonstrating enhanced LTP in amygdala following non-selective HDAC inhibition (Yeh et al., 2004). Furthermore, because LTP is thought to be the cellular correlate of learning and memory (Malenka and Bear, 2004), it is likely that enhanced LTP contributes to enhanced novel object recognition and

placement, as these are dentate specific tasks (Goodrich-Hunsaker et al., 2005). While we observed an enhancement of LTP at MPP-DGC synapses in WT animals, we were surprised to find that selective HDAC3 inhibition failed to rescue or enhanced LTP in FX mice. These results demonstrate that enhancement of LTP following selective HDAC3 inhibition requires FMRP.

One possible explanation for the lack of enhanced LTP at MPP-DGC synapses in FX mice that centers around the transcription factor cAMP response element-binding (CREB) is discussed in detail in chapter 2. However, an alternative explanation linking hyperactive GSK3 and CREB in FX has yet to be explored. CREB can be activated via phosphorylation of serine 133. However, as a result of phosphorylation at this site, a binding motif is created for GSK3. This allows GSK3 to bind to CREB and inhibit activity via phosphorylation of ser 129 (Fiol et al., 1994). Because GSK3 is hyperactive in FX mice, it is possible that inhibitory phosphorylation of CREB is increased thereby decreasing CREB activity. In support of this idea, several studies have demonstrated that CREB has diminished function in FX models (Berry-Kravis and Sklena, 1993; Berry-Kravis and Ciurlionis, 1998; Dockendorff et al., 2002; Kelley et al., 2007). Importantly, CREB is required for the enhancement of LTP observed at CA3-CA1 synapses following non-selective HDAC inhibition (Vecsey et al., 2007). Therefore, in the absence of FMRP, GSK3 activity is increased, CREB activity is decreased, possibly accounting for the lack of enhanced LTP following HDAC inhibition.

While it is exciting to speculate potential mechanisms, understanding the precise function of FMRP in enhanced LTP following selective HDAC3 inhibition requires further studies. RNA-sequencing transcriptome analysis could potentially identify key

genes that are differentially expressed following HDAC3 inhibition in WT versus FX mice which could direct future investigations.

CONCLUSION

Taken together, these findings establish that synaptic plasticity and cognitive deficits in FX mice can be improved by intervention with inhibitors of GSK3. This suggests that GSK3 inhibitors may prove therapeutically beneficial in the treatment of cognitive impairments in FX. In support of this, lithium treatment improves cognition in FX patients (Berry-Kravis et al., 2008). However, untoward side-effects and toxicity issues arising from lithium treatment may limit it's in the treatment of FX. Future studies aimed at dissecting the precise mechanism of pathological GSK3 function are necessary to identify novel targets which will lead to development of refined therapeutic strategies.

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APPENDIX

IACUC APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: January 27, 2014

TO: LORI L MCMAHON WAKEFIELD, Ph.D.
MCLM-964
(205) 934-3523

FROM:

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Role of GSK3 in Hippocampal Deficits in Fragile X
Sponsor: Internal
Animal Project_Number: 140209549

As of February 3, 2014 the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and number of animals:

Species	Use Category	Number In Category
Mice	A	912
Mice	B	145

Animal use must be renewed by February 2, 2015. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

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

Refer to Animal Protocol Number (APN) 140209549 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee (IACUC)
CH19 Suite 403
933 19th Street South
(205) 934-7692
FAX (205) 934-1188

Mailing Address:
CH19 Suite 403
1530 3rd Ave S
Birmingham, AL 35294-0019

Admission to Candidacy
Research Compliance Verification Form

Instructions
Complete this form, including all applicable forms and the signatures of the student, the student's advisor, and the Graduate Program Director. For research approval forms, contact the Institutional Review Board (IRB) (http://www.uab.edu/irb or 934-3789), or the Institutional Animal Care and Use Committee (IACUC) (http://www.uab.edu/iacuc or 934-7692).

Human Subjects
The University of Alabama at Birmingham defines a human subject as not only a living human being, but also human tissue, blood samples, pathology or diagnostic specimens, study of medical records, observation of public behavior, and all questionnaires or surveys.

Does the research proposed by the student involve human subjects? ☐ Yes (continue below) ☒ No
This research is:
Approved _____ Date _____

IRB Protocol No. _____

Attach a copy of your IRB approval. Your own name must appear on the original approval or on an attached amendment.

Animal Subjects
The University of Alabama at Birmingham defines a laboratory animal as any vertebrate animal (e.g., traditional laboratory animals, farm animals, wildlife, and aquatic animals) and certain higher invertebrate animals used in research, teaching, or testing at UAB or sponsored through UAB but conducted off-site (i.e., field research or at collaborating institutions, etc.).

Does the research proposed by the student involve animal subjects? ☒ Yes (continue below) ☐ No

This research is:
Approved ✓ Date 5-11-11

IACUC Protocol No. 110509362

Attach a copy of your IACUC Notice of Approval, showing your research subject and the animal project number. If your own name does not appear on the Notice of Approval, take this form to the IACUC office for verification of approval.

The IACUC office verifies that Aimee Franklin (name of candidate) is covered under the attached approval.

Signature of IACUC representative _____ Date: 10-13-11

NOTE: The student's advisor, the student, and the Graduate Program Director agree that no research will be initiated until an application is submitted for review and approved by the appropriate review boards (IRB and/or IACUC) if the proposed thesis or dissertation project requires approval. If approval already exists, this student's name must be added to the existing protocol before candidacy will be approved by the Graduate School. It is the responsibility of the student's advisor and the student to comply with federal and UAB regulations associated with this research. Documentation of continuous, appropriate approval will be required before degree conferral; all required IRB and/or IACUC approvals must be current at the time final versions of theses or dissertations are submitted to the Graduate School.

Student's Signature	Physiology & Biophysics Dept.	8-23-11 Date
Signature of Student's Advisor	Physiology & Biophysics Dept.	8/23/11 Date
Graduate Program Director	Physiology & Biophysics Dept.	8/23/11 Date

Updated 10/31/08