Elevated glycogen synthase kinase-3 activity in Fragile X mice: Key metabolic regulator with evidence for treatment potential

Wenzhong William Min a, Christopher J. Yuskaitis b, Qijiang Yan a, Christopher Sikorski a, Shengqiang Chen a, Richard S. Jope b, Robert P. Bauchwitz a, * 

a St. Luke’s-Roosevelt Institute for Health Sciences, Columbia University, 432 W. 58th St., Rm. 411, New York, NY 10019, USA 

b Department of Psychiatry and Behavioral Neurobiology, 1720 7th Ave South, Sparks Center 1057, University of Alabama at Birmingham, Birmingham, AL 35294-0017, USA

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ABSTRACT

Significant advances have been made in understanding the underlying defects of and developing potential treatments for Fragile X syndrome (FXS), the most common heritable mental retardation. It has been shown that neuronal metabotropic glutamate receptor 5 (mGluR5)-mediated signaling is affected in FX animal models, with consequent alterations in activity-dependent protein translation and synaptic spine functionality. We demonstrate here that a central metabolic regulatory enzyme, glycogen synthase kinase-3 (GSK3) is present in a form indicating elevated activity in several regions of the FX mouse brain. Furthermore, we show that selective GSK3 inhibitors, as well as lithium, are able to revert mutant phenotypes of the FX mouse. Lithium, in particular, remained effective with chronic administration, although its effects were reversible even when given from birth. The combination of an mGluR5 antagonist and GSK3 inhibitors was not additive. Instead, it was discovered that mGluR5 signaling and GSK3 activation in the FX mouse are coordinately elevated, with inhibition of mGluR5 leading to inhibition of GSK3. These findings raise the possibility that GSK3 is a fundamental and central component of FXS pathology, with a substantial treatment potential.

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1. Introduction

Fragile X syndrome (FXS) is the most common hereditary form of mental retardation. The syndrome is caused by a trinucleotide repeat (CGG) expansion that results in epigenetic silencing of the gene FMR1, preventing the expression of the encoded protein, Fragile X mental retardation protein (FMRP) as previously reviewed (Bardoni and Mandel, 2002). FMRP is an mRNA-binding protein that negatively regulates the translation of its cargos into protein (Laggerbauer et al., 2001; Li et al., 2001; Schaeffer et al., 2001). The primary symptom of FXS is mental retardation, but several hyperarousal behaviors are common, including attention deficits, anxiety, hypersensitivity to stimuli, hyperactivity, and childhood seizures (Baumgardner et al., 1995; Bregman et al., 1988; Fisch et al., 1999; Musumeci et al., 1999).

Animal models of Fragile X syndrome have been vital for studying the molecular mechanisms of the disorder and for the development of effective therapeutics. The first transgenic mouse model of Fragile X syndrome was generated more than 13 years ago by interrupting the FMR1 gene (Bakker and Consortium, 1994), thereby creating the mutant fmr1 allele used in these studies, fmr1tm1Cgr (“FX” or “ko”). Of the many interesting phenotypes from this FX mouse model reported over the years (Bernardet and Crusio, 2006; Yan et al., 2004), we focus in this study on two: elevated audiogenic seizure susceptibility and increased open field center square activity. The audiogenic seizure (AGS) and open field phenotypes have proven to be both robust and reproducible on several strain backgrounds and in various laboratories; consequently, these have been successfully employed in prior pharmacologic studies of the FX mouse (Yan et al., 2005a).

Indirect evidence has linked glycogen synthase kinase-3 (GSK3) to FXS and raised the possibility that lithium may be therapeutically beneficial. Lithium inhibits GSK3 (Klein and Melton, 1996; Stambolic et al., 1996), which in turn leads to inhibition of microtubule-associated protein (MAP1B) phosphorylation in mammalian cells (Garcia-Perez et al., 1998; Lucas et al., 1998). MAP1B mRNA has been consistently identified as being bound and translationally regulated by mammalian FMRP (Brown et al., 2001; Darnell et al., 2001). Studies of the Drosophila dfxr gene, a homolog of the human FMR1, FXR1, and FXR2 genes, found that it regulated Futsch, a homolog of human MAP1B (Hummel et al., 2000; Zhang et al., 2001). Furthermore, a loss-of-function mutation of Futsch reverted some dfxr mutant phenotypes (Dockendorff et al., 2002; Zhang et al., 2001). These facts led to proposals that lithium treatment...
might be of value in human FXS (Bauchwitz, 2002). Consistent with such proposals that lithium effects on GSK3 could be beneficial in FXS were experiments in flies and mice that showed a positive effect of lithium in reverting certain FX phenotypes (McBride et al., 2005; Yan et al., 2005b).

The ubiquitous serine/threonine kinases, GSK3α and GSK3β, are paralogous proteins arising from independent genes, but are commonly referred to as the two isoforms of GSK3 (Woodgett, 1990). The use of the terms “GSK3” or “GSK3β” henceforth is meant to refer to both GSK3 paralogs, since all GSK3 inhibitory agents, including lithium, affect both proteins. Among the many cellular functions regulated by GSK3 are gene expression, cellular architecture, and apoptosis, resulting from its more than forty currently known substrates (Jope and Johnson, 2004). GSK3 is constitutively active, with signaling cascades often leading to its inhibition. Because of this constitutive activity and the large number of substrates and cellular functions under the influence of GSK3, its activity must be tightly regulated. The most important mechanism regulating the activity of GSK3 is phosphorylation on serine-21 of GSK3α and serine-9 of GSK3β, which greatly inhibits the activity of GSK3, as previously reviewed (Jope and Johnson, 2004).

Although lithium has long been reported to have effects on phosphoinositide metabolism (Berridge et al., 1989), recently its therapeutic actions in bipolar disorder have been ascribed to inhibition of GSK3 (Jope, 2003; Phiel and Klein, 2001). Lithium directly inhibits GSK3, and this direct action is amplified in vivo by a subsequent increase in the inhibitory serine-phosphorylation of GSK3 (De Sarno et al., 2002). Several selective small molecule ATP-competitive inhibitors of GSK3 have been developed (Doble and Woodgett, 2003; Kozikowski et al., 2006; Martinez et al., 2002; Wagman et al., 2004). For unknown reasons, ATP-competitive inhibitors do not lead to an increase in serine-phosphorylation of GSK3, so their effects cannot be detected by this means. It is thought that with the inhibitor in the ATP binding pocket, the N-terminal of GSK3 containing the regulatory serine is looped back onto GSK3 and is unable to be phosphorylated, whereas it is free and phosphorylatable with lithium bound.

Antagonists of metabotropic glutamate receptors (mGlurS) also have shown promise as therapeutic agents for FXS. MPEP (2-methyl-6-phenylethynyl-pyridine) administration rescued Fragile X specific behavioral deficits in FMRP knockout mice (Yan et al., 2005a) and electrophysiological abnormalities in hippocampal slices from FMRP knockout mice (Chuang et al., 2005). In dfxr (dFMR) knockout flies, treatment with MPEP rescued defects of memory, behavior, and neuropathology (McBride et al., 2005). Defects in axonal guidance were rescued with MPEP in a zebrafish model (Tucker et al., 2006). Therefore, it is a point of both basic and clinical research interest to assess whether mGlurS antagonists show additive effects with lithium or specific ATP-competitive GSK3 inhibitors in FX animal models.

The present study was undertaken to determine if lithium and other GSK3 inhibitors might be beneficial in FXS using a mouse model. The results demonstrate that 1) GSK3 is hyperactive in the FX mouse brain, 2) elevated FX GSK3 activity can be restored to normal by lithium treatment as well as by administration of an mGluR5 antagonist, and 3) significant therapeutic effects are attained by administration of GSK3 inhibitors.

2. Materials and methods

2.1. Animals and in vivo tests

Male mice of inbred FVB/NJ (“FVB”) and F1 hybrid (“hybrid” or “HYB”) C57Bl/6 × FVB/NJ strains, with or without a disruption of the Fmr1 gene (fmr1tm1Cgr allele; “FX”, “fmr1” or “ko”), were genotyped and tested in audiogenic seizure (“AGS”) and open field activity assays as previously described (Yan et al., 2005a), except that open field results were also scored using the Viewer tracking program (Biobserve, Bonn, Germany). Tests were performed during the light phase (7AM–7PM). Mice were given water and fed ad libitum with Purina Mouse Diet 20, or the same chow containing lithium carbonate as indicated below (Bio-Serve, NJ). Lithium levels can rise to potentially toxic levels on sodium restricted diets (Linden and Rich, 1983). Purina Mouse Diet 20 is 0.25% sodium and there was no effect from inclusion of additional salt (as solid blocks) in the diet. The use of animals was reviewed by the Institutional Animal Care Usage Committee (IACUC) and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Pharmacologic agents

Lithium chloride was obtained as an 8 M solution (EMD). Lithium citrate was from a clinical preparation (Roxane, 8 mmol/ml lithium). Lithium chow (Bio-Serve, NJ) contained 3.2 mg/kg lithium carbonate in Picolab Mouse Diet 20 (5058-M, Purina). The resulting pellets were sterilized by irradiation. Serum lithium measurements were made with a Vitros 950 chemistry system (Johnson and Johnson) for which samples were brought to a starting volume of 50 μl with 7% BSA as necessary, and then serially diluted in the same reagent. Selective ATP-competitive inhibitors of GSK3 used were SB-216763 (Coghlan et al., 2000; Bain et al., 2007) and AR-A014418 (Bhat et al., 2003; Bain et al., 2007). AR-A014418 (Sigma) was generally made 25 mg/ml in DMSO; we were able to achieve 28 mg/ml as the maximum concentration, which was used as “high dose” in one experiment in combination with SB-216763 (Fig. 7B). SB-216763 (Tocris) was dissolved in DMSO to 20 mg/ml. The mChR5 antagonist MPEP (Gasparini et al., 1999), synthesized by Technically, Inc. for the FRAXA Research Foundation, was made fresh for each experiment at a starting concentration of 5 mM in water (Yan et al., 2005a), or in DMSO if to be combined with a drug soluble in that reagent. Intrapерitoneal (ip) injections for MPEP were as previously described (Yan et al., 2005). All DMSO ip injections were 7.5 μl of 3 mg/ml MPEP, as indicated, and made especially as previously described (Kim et al., 1998) using a Hamilton 700RN 10 μl syringe with a custom 45° bevel, 0.275 inch replaceable needle. This needle allowed icv injections through a single guide cannula (2 mm projection beyond pedestal; model C312GS-4/SPC 2 mm, PlasticsOne, Roanoke, VA), which was used for some repeat dosing experiments. (Repeat dosing results using the cannula did not differ from those using direct injection without the cannula.) Briefly, for direct icv injections, mice were anesthetized by placement for approximately 10 s into a 50 ml tube containing a few drops of isoflurane (Aerane). Drug was then injected by Hamilton syringe held at 45° to the skull, with the bevel of needle facing up, rubbing the point of needle slightly against the skull to find the bregma, and then inserting the needle 2.5–3 mm into the bregma. Preliminary tests with dyes indicated that a more complete diffusion into the brain occurred at 45 min, so this time was used prior to testing rather than the 30 min used after ip injections. Chronic icv and ip dosing was once daily (q.d.) for 5 consecutive days, during which mice were weighed daily and drug volumes adjusted accordingly.

2.3. Tissue preparation

Mice of 30 days of age were decapitated and brains were rapidly frozen. Dissection of brain regions were made in ice in a 50 mm syringe with a custom cannula (Tri–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP–40, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 5 μg/ml pepstatin, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium vanadate, 50 mM sodium fluoride, and 100 mM EDTA. The lysates were centrifuged at 20,800 g for 10 min to remove insoluble debris. Protein concentrations in the supernatants were determined in triplicate using the Bradford protein assay. When GSK3 is immunoprecipitated for activity measurements, the three inhibitors used in this study are dissociated and diluted out, so activity measurements cannot be used to detect their in vivo inhibition. Thus, the widely used strategy of confirming behavioral effects with two or more GSK3 inhibitors is the best method to ensure that GSK3 is inhibited by the agents.

2.4. Immunoblotting

Extracts were mixed with Laemmli sample buffer (2% SDS) and placed in a boiling water bath for 5 min. Proteins were resolved in SDS-polyacrylamide gels, and transferred to nitrocellulose. Blots were probed with antibodies to phospho-Ser9-GSK3α and transferred to nitrocellulose. Blots were probed with antibodies to phospho-Ser9-GSK3β, and total GSK3 levels were determined in a single guide cannula (2 mm projection beyond pedestal; model C312GS-4/SPC 2 mm, PlasticsOne, Roanoke, VA), which was used for some repeat dosing experiments. (Repeat dosing results using the cannula did not differ from those using direct injection without the cannula.) Briefly, for direct icv injections, mice were anesthetized by placement for approximately 10 s into a 50 ml tube containing a few drops of isoflurane (Aerane). Drug was then injected by Hamilton syringe held at 45° to the skull, with the bevel of needle facing up, rubbing the point of needle slightly against the skull to find the bregma, and then inserting the needle 2.5–3 mm into the bregma. Preliminary tests with dyes indicated that a more complete diffusion into the brain occurred at 45 min, so this time was used prior to testing rather than the 30 min used after ip injections. Chronic icv and ip dosing was once daily (q.d.) for 5 consecutive days, during which mice were weighed daily and drug volumes adjusted accordingly.

AGS data were analyzed using contingency tables and chi-square (with column and row correction) if the expected frequencies were low (Yan et al., 2005a). When groups were compared, the trend was not of relevance to this work (Yan et al., 2005a). For statistical analysis of open field behavior, significance was assessed by Fisher’s exact test. Figs. 1 and 6 show dose responses whose intention is to illustrate the clear trend of drug activity; statistical analysis of the trends was not performed since an estimate of ED50 was not of relevance to this work (Yan et al., 2005a). For statistical analysis of open field behavior, significance was assessed by ANOVA followed by a Dunnet’s post hoc test. Figs. 3 and 4 were analyzed by Student’s t-test.
Fragile X (FX) mice display an increased susceptibility to audiogenic seizures ("AGS") (Chen and Toth, 2001; Musumeci et al., 2000). The intensity and population frequency of such seizures is affected by the mouse strain background (Yan et al., 2004). Use of the fmr1<sup>tm1Cgr</sup> (FX) allele in the more AGS-sensitive FVB/NJ strain allows an increased percentage of mice displaying the AGS phenotype compared with other backgrounds, and therefore is more practical for anti-seizure drug testing (Yan et al., 2005a).

Throughout the present studies, almost all vehicle-treated FX mice on the FVB/NJ (FVB) strain background underwent audiogenic seizures compared with one-fifth that level among wild-type mice dying from status epilepticus (SE), a sustained tonic seizure (Fig. 1). These differences were even greater for mice dying from status epilepticus (SE), a sustained tonic seizure; this was found to be the case here for the lowest dose of ip lithium citrate used (120 mg/kg ≈ 1.71 mmol/kg lithium). Therefore, SB-216763 was utilized in place of lithium for these measurements. Fig. 3 illustrates that a low, acute dose of SB-216763 (4 mg/kg) produced a statistically significant reversal of FX hyperactivity and thigmotactic (wall hugging) behavior to wild-type levels, as well as a trend consistent with partial correction of the center square entry and center square duration phenotypes.

The ability of lithium and selective inhibitors of GSK3 to positively impact the mouse FX phenotype raised the possibility that GSK3 activity might be elevated in FX mice. To assess this, phosphorylation of GSK3 on serines known to inhibit activity when phosphorylated (phospho-Ser9-GSK3<sub>a</sub>) and phospho-Ser21-GSK3<sub>B</sub>) was measured in several brain regions of wild-type and FX mice. Both GSK3<sub>a</sub> and GSK3<sub>B</sub>) were found to be significantly dephosphorylated on the inhibitory serines in FX striatum and cortex, indicative of increases in active GSK3 (Fig. 4). GSK3<sub>a</sub> was also relatively dephosphorylated in the hippocampus. Total levels of each isoform of GSK3 were equivalent in wild-type and FX mouse brain regions.

In vivo lithium treatment increases the inhibitory serine-phosphorylation of GSK3 in wild-type mouse brain (De Sarno et al., 2002). Lithium treatment of FX mice elevated brain phospho-Ser9-GSK3<sub>B</sub>, confirming that lithium was capable of counteracting the hyperactive GSK3 in FX mice (Fig. 5). Total levels of GSK3<sub>B</sub> remained unaltered. These results support the behavioral results presented in Fig. 1.)
presented above, which showed that lithium produced the same effects as more selective GSK3 inhibitors (Bain et al., 2007).

We examined if repeated daily treatments with lithium or other GSK3 inhibitors provided longer-term beneficial effects on FX mouse phenotypes. Lithium citrate given once a day for 5 days effectively reduced audiogenic seizures (Fig. 6). In addition, treated mice did not show any evidence of diminished drug effect (“tolerance”). In contrast, daily treatment with AR-A014418 (icv) elicited tolerance after 5 days of administration (for all doses between 10 and 100 μg (Fig. 7A). Increasing the final dose of AR-A014418 did not overcome the reduced effectiveness, in contrast to prior findings for mGluR5 antagonists (Yan et al., 2005a). Similar results were obtained with SB-216763 given ip (Fig. 7B). Tolerance was still evident even with combined administration of high doses of SB-216763 and AR-A014418 given (icv) (Fig. 7B). In nurslings (mice under 21 days of age in this study), an initial body weight dependent survival was observed with chronic ip GSK3 inhibitor administration (Fig. S1), but not when given at lower doses directly to the CNS (icv). Normal survival with reduced body weights was observed when lithium was present in the chow of the lactating mother (Table S1; see also lithium chow results below). Administration of DMSO vehicle icv caused minimal effects on AGS (Fig. S2).

It was of interest to determine whether combinations of GSK3 inhibitors and mGluR5 antagonists could act in an additive fashion, or if not, whether there was some common point of action. As shown in Fig. 2, the co-administration of lithium and AR-A014418 did not produce an additive effect, consistent with both acting primarily on a GSK3 pathway to alter the behavior. Similarly, MPEP treatment in these studies reduced audiogenic seizures in FX mice; but when acute combinations of MPEP and AR-A014418 were given icv, they did not show an additive effect (Fig. 8). The combination of SB-216763 and MPEP ip also gave acute protection that was statistically similar to that of the more potent of the two compounds used (Fig. S3). However, the reduced effectiveness of AR-A014418 observed after repeated treatment (Fig. 7A) also carried over to impair the effectiveness of repeated administration of a non-tolerating dose of MPEP (Fig. 8, last column). Consistent with the AGS results, the administration of MPEP to mice exposed to lithium from birth also showed no significantly enhanced corrective effect in the open field assay (see below, and Fig. 10, lane 8).

The absence of an additive effect from combinations of GSK3 inhibitors and the mGluR5 antagonist MPEP suggested that these drug classes might have been impacting targets that function in a common signaling pathway. To assess this more directly, we examined whether MPEP treatment altered the serine-phosphorylation of GSK3 in mouse brain. Surprisingly, administration of MPEP (30 mg/kg, 30 min) significantly increased phosho-Ser21-GSK3α and phospho-Ser9-GSK3β in FX mouse brain, but did not change total levels of GSK3α/β (Fig. 9). This demonstrates that MPEP administration inhibits GSK3 in the FX mouse brain, revealing a common action among MPEP, lithium, and the other GSK3 inhibitors that ameliorate the FX phenotype.

The therapeutic effects of GSK3 inhibitors in FX mice led us to test if chronic lithium administration provided therapeutic benefits in FX mice. For chronic lithium treatment of rodents, lithium is most commonly administered in the food (De Sarno et al., 2002; Su et al., 2004). After 9 days of dietary lithium administration to FX male mice (FVB strain, from 20 to 30 days of age), the serum lithium level was 2.0 ± 0.27 mmol/l (mean ± SEM; n = 17; Table S1), close to the therapeutic range of 0.5–1.5 mmol/l in human patients, but lower than the 5.8 mmol/l level observed 30 min after the last of 5 daily lithium citrate treatments (ip, 300 mg/kg) that completely protected from audiogenic seizures (Fig. 6). Nonetheless, even at this lower lithium level, administration of dietary lithium over 9 days reduced audiogenic seizures in FX mice by 55% (Table S1). It also restored FX center square entry levels in the open field to normal (Fig. 10, lane 4). The open field behavior of wild-type mice receiving lithium chow was not affected (Fig. 10). Thus, a therapeutically relevant lithium administration protocol provided significant correction of phenotypes in FX mice.
We also tested if earlier chronic intervention with lithium was effective by providing lithium-containing food to the lactating mother beginning at birth and continuing for 29–30 days, which produced serum lithium levels of 2.84 ± 0.22 mmol/l in the one month old mice (Table S1). This early intervention with lithium reduced audiogenic seizures in FX mice to 38%. The 30-day dietary lithium treatment regimen also reduced FX mouse center square entries behavior to wild-type levels; a lithium diet between 20 and 30 days of age was equally efficacious (Fig. 10). Upon lithium withdrawal for 10 days after dietary treatment between 0 and 20 days of age, the FX center square entry phenotype returned (Fig. 10, lane 7); seizure sensitivity also returned by 10 h after drug injection (not shown). These results showed that the therapeutic action of lithium is reversible.

4. Discussion

This study has demonstrated in a mouse model of Fragile X Syndrome that both acute and chronic lithium administration reduced FX susceptibility to audiogenic seizures and modified open field behavior towards that of wild-type mice. These effects of lithium likely result from inhibition of GSK3, since 1) lithium is a well-documented inhibitor of GSK3 (Klein and Melton, 1996; Stambolic et al., 1996), 2) GSK3β haploinsufficiency in mice produces behavioral effects mimicking lithium (O’Brien et al., 2004), 3) the use of two additional selective inhibitors of GSK3, AR-A014418 and SB-216763, had effects similar to lithium in the present experiments, and 4) the effects of AR-A014418 and SB-216763 were not additive with those of lithium. Therefore, the evidence reported here suggests that GSK3 may be a valid target for therapeutic intervention in FXS. This extends previous reports of beneficial effects of lithium in animal models of FXS.
Fragile X syndrome is thought to involve dysfunction of localized signaling since it was reversed by the mGluR5 antagonist MPEP. The influence of lithium and GSK3 inhibitor SB-216763 in the open field also suggested a FX-specific effect because lithium present in the food substantially reduced center square entries, and SB-216763 reduced overall hyperactivity and thigmotaxis of the FX mice, whereas, these compounds did not reduce activity of wild-type mice (Bersudsky et al., 2007; O’Brien et al., 2004).

The results obtained here indicate that lithium application during early development of the FX mouse was not sufficient to prevent symptom emergence upon cessation of its use later in life. Even when exposed to lithium for the first month after birth, the withdrawal of lithium from the diet led to reemergence of the FX phenotypes. In contrast, some phenotypic features of the FX fly model were apparently resolved with early (larval) exposure to the mGluR5 antagonist MPEP (McBride et al., 2005). Lithium administration to FX mice prior to birth might allow a more complete assessment of this issue, although such studies may be hampered by the body weight dependent effects observed in this study. Lithium has been long known to be toxic to infants when exposed during human pregnancy and nursing (Kallen and Tandberg, 1983; Linden and Rich, 1983), and at high doses lithium produces body weight reductions and other negative effects in mice during the same developmental periods (Messiha, 1986). Nonetheless, lithium has been successfully used to treat bipolar disorder in pediatric populations; indeed, weight gain is the more common issue (Correll, 2007). Interestingly, lower doses of lithium have been reported to reduce testis weights in normal mice through 23 days of age (Messiha, 1986), suggesting that GSK3 elevation may be relevant to the FX macroorchidism phenotype as well. Despite the known risks of lithium during early mammalian development, it is important to note that the current study demonstrated that 1) lithium and the GSK3 inhibitors were effective in adults, indicating they did not need to be given to neonates in order to provide phenotypic benefits, and 2) weight loss and reduced survival did not occur in nursing mice when the GSK3 inhibitors SB-216763 or AR-A014418 were administered at low doses directly to the CNS.

The mechanism underlying the hyperactivation of GSK3 in FX mice is not known, but appears to be linked to increases in mGluR5 signaling since it was reversed by the mGluR5 antagonist MPEP. Fragile X syndrome is thought to involve dysfunction of localized metabolic regulators with evidence for treatment potential, Neuropharmacology (2008), doi:10.1016/j.neuropharm.2008.09.017
protein translation in neurons (Bear et al., 2007; Grossman et al., 2006; Miyashiro et al., 2003; Weiler et al., 1997). At least in part, such translation is influenced by signaling from group I metabotropic glutamate receptors (Aschrafi et al., 2005; Huber et al., 2002; Volk et al., 2007). Consistent with the importance of mGluR signaling, Fragile X behavioral phenotypes can be reverted in mouse, fly, and zebrafish animal models by mGluR5 antagonists (McBride et al., 2005; Tucker et al., 2006; Yan et al., 2005a). mGluR5 signaling could be responsible for the FX GSK3 elevations observed from densitometric measurements of immunoblots of GSK3 serine-phosphorylation in four brain regions from FX mice after MPEP or saline treatment. Str = striatum; Ctx = cortex; Cbl = cerebellum; Hipp = hippocampus. *p < 0.05 compared to saline values (Student’s t-test).

Despite the seeming independence of common mGluR5 signaling pathways and changes in GSK3 activity in the FX mouse as discussed above, we have also shown in this study that simultaneous blockade of both proteins did not lead to a notable additive effect. Consistent with the implied commonality of action, MPEP produced an elevation of inhibitory serine-phosphorylation of GSK3 in FX mouse hippocampus. It has been suggested in previous work that, in an assay in which both mGluR5 agonist (CHPG) and antagonist (MPEP) acted in the same manner, MPEP may have been antagonizing NMDAR (Bao et al., 2001); support for this possibility has been demonstrated in mouse and rat cortical cell cultures (Lea et al., 2005). Interestingly, it has recently been shown that NMDAR antagonism can lead to increased serine-phosphorylation of GSK3 (De Sarno et al., 2006). Nonetheless, the highest dose of MPEP used in this study had previously been shown to have no effect on NMDA-induced seizures (Pilc et al., 2002) nor NMDA striatal toxicity (Popoli et al., 2004). Further tests will be required to assess exactly how MPEP might be influencing GSK3 phosphorylation in the FX mouse.

GSK3 has a number of actions whereby hyperactive GSK3 in FX mice may contribute to the FX phenotype. In a Drosophila dfxr model, McBride et al. (2005) demonstrated that MPEP and other mGluR antagonists, as well as UCL, were able to revert some phenotypes (suppression of courtship and, if given during development, mushroom body defects) but not others (loss-of-circadian-regulated locomotor activity). Although it remains to be fully established how the primarily presynaptic mGluR receptor

**Fig. 8.** Combined mGluR5 antagonist and GSK3 inhibitor audiogenic seizure inhibition in FX mouse is not additive. MPEP (“M”; 42 µg) and AR-A014418 (“A”; 50 µg) were given to FVB FX male mice (i.c.v, in 3 µl DMSO) as described in Fig. 7. “1x” indicates a single daily dose; “5x” indicates a single dose given daily for 5 days. Values are presented as the percentage of total number of mice per group displaying AGS (n = 14, 16, 13, 8, 13 mice in each group, left to right). Statistical significance was determined as described in Fig. 7. **p < 0.01.

**Fig. 9.** MPEP administration increases GSK3 serine-phosphorylation in FX mouse brain. FX mice (male, FVB background, 30 days of age) were administered MPEP (30 mg/kg; ip; n = 5) or vehicle (n = 5) 30 min prior to sacrifice. The top panel shows representative hippocampal samples immunoblotted for phospho-Ser21-GSK3β, phospho-Ser9-GSK3β, and total levels of GSK3α and GSK3β. Quantitative values are expressed as a percentage of control. Student’s t-test).
negatively regulates glycogen synthase, which is rate-limiting for FX mouse and fly models (Qin et al., 2002; Zhang et al., 2005). GSK3 

in vivo synthase, such as by administration of the glycogen phosphorylase inhibitor 1,4-dideoxy-1,4-imino-α-arabinohexitol (DAB), produces impairments in learning and memory, possibly by affecting glutamate metabolism required for synaptic activation (Gibbs et al., 2007; O’Dowd et al., 1994). GSK3 inhibition of glycogen synthase in the FX brain may act in a similar manner, by reducing glycogen stores and thereby subsequent glycogenolysis, to inhibit learning and memory consolidation. Even a relatively subtle contribution to a deficit in learning and memory, as found in FX animal models (Yan et al., 2004), could have more severe repercussions on human higher order cognition (Supplemental information **).

The phenotypes employed in this study did not directly address effects of GSK3 inhibition on learning and memory; however, new research findings provide reasons to expect a beneficial effect in FXS. FX mice demonstrate increased long-term depression (LTD) (Huber et al., 2002; Koekkoek et al., 2005) and impaired long-term potentiation (LTP) (Li et al., 2002), which are widely believed to be electrophysiologic markers of learning and memory processes (Daoudal and Debanne, 2003). Very recent studies have shown that GSK3 promotes LTD (Peineau et al., 2007), while overexpression of GSK3β inhibits LTP (Zhu et al., 2007) and produces deficits in spatial learning (Hernandez et al., 2002). These results are exciting, as they provide electrophysiologic support for the connection suggested in these studies between elevated GSK3 activity in FX mice and the known enhancement of LTD in the same system. Thus, GSK3 may be a central regulatory point in FXS.

In summary, we have shown that GSK3 activity is elevated in the FX mouse brain. Furthermore, specific inhibition of GSK3 ameliorates at least two significant phenotypes in FX mice, seizure sensitivity and open field hyperactivity. The clinically available mood-stabilizer lithium was able to provide beneficial effects with both acute and chronic administration. These results present the first demonstration of GSK3 involvement in a model of mental retardation. We have linked GSK3 activation in the absence of FMRP to mGluR5 signaling. The relationship between GSK3 activation and mGluR5 may occur via interconnected neuronal networks involving other neurotransmitters. GSK3 activation can impact LTD, microtubule associated proteins, glycogen metabolism, and other processes in ways generally consistent with those found in FX animal models. The therapeutic potential shown by GSK3 inhibitors is encouraging because lithium has an extensive history of safe, long-term usage in human patients with psychiatric diseases. Furthermore, a new generation of selective inhibitors of GSK3 is currently being developed. Although for the most part only limited in vivo studies of these new inhibitors have been completed, as reported here they have the potential to modify GSK3-linked alterations influencing the Fragile X syndrome, as well as disorders with related symptoms such as epilepsy and autism, for which elevations in GSK3 activity may be discovered. Thus, at present lithium may be useful for obtaining therapeutic effects in FXS, while further development of selective GSK3 inhibitors may provide more advantageous interventions in the future.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.neuropharm.2008.09.017.

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