Neuropharmacology xxx (2008) 1–10

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

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

ARTICLE INFO

Article history:
Received 9 July 2008
Received in revised form 20 September 2008
Accepted 30 September 2008

Keywords:
GSK3
Fragile X
Lithium
mGluR
Behavior
Audiogenic seizures

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.

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, $fmr1^{tm1Cgr}$ ("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 *MAP1*B (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

* Corresponding author. Tel.: +1 717 482 2089; fax: +1 212 523 7623.
E-mail address: rpb3@columbia.edu (R.P. Bauchwitz).

0028-3908/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuropharm.2008.09.017

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 "GSK3α/β" 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 mGluR5 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/6J × FVB/NJ) strains, with or without a disruption of the *Fmr1* gene (*fmr1*^{tm1Cgr} 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-Cookson) was dissolved in DMSO to 20 mg/ml. The mGluR5 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. Intraperitoneal (ip) injections for MPEP were as previously described (Yan et al., 2005a). All DMSO ip injections were 70 μl total volume. Intracerebroventricular (icv) injections were in 3 or 5 μl DMSO, as indicated, and made essentially as previously described (Kim et al., 1998) using a Hamilton 700RN 10 μl syringe with a custom 45° bevel, 0.375 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 (Aerrane). 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 ip and icv 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. Dissected brain regions were homogenized in ice-cold lysis buffer containing 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium vanadate, 50 mM sodium fluoride, and 100 nM okadaic acid. 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-GSK3β, phospho-Ser21-GSK3α, and total GSK3α/β (Cell Signaling Technology, Beverly, MA). Immunoblots were developed using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA), followed by detection with enhanced chemiluminescence, and quantitation by densitometry. To compare differences between wild-type and FX brain regions, values are shown as the percents of densities of immunoblots of wild-type samples analyzed on the same gels.

2.5. *Statistical analysis*

AGS data were analyzed using contingency tables and chi-square (with column drops) or 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

Please cite this article in press as: Wenzhong William Min et al., Elevated glycogen synthase kinase-3 activity in Fragile X mice: Key metabolic regulator with evidence for treatment potential, Neuropharmacology (2008), doi:10.1016/j.neuropharm.2008.09.017

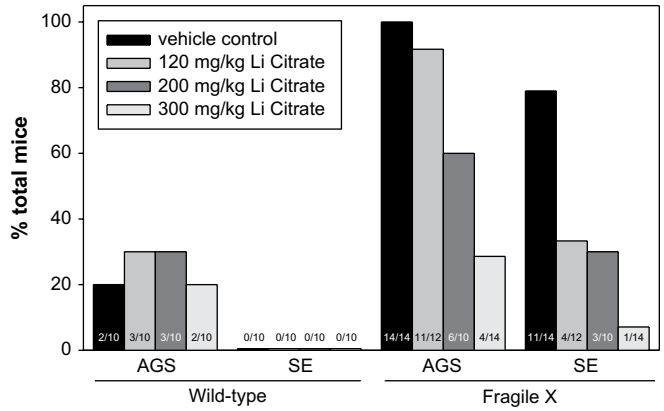

Fig. 1. Lithium produces a Fragile X specific reduction in audiogenic seizures. Lithium citrate (120, 200, or 300 mg/kg, equivalent to 1.71, 2.86, or 4.29 mmol lithium/kg, respectively) or vehicle control was administered ip to 29–30-day-old male FX (*fmr1*^{tm1Cgr}) or wild-type mice (FVB/NJ strain background) 30 min prior to exposure to a high intensity sound. Values are presented as the percentage of total number of mice per group displaying audiogenic seizures (AGS) or dying after status epilepticus (SE). The number within each bar represents the number of mice displaying AGS or SE over the total number of mice in that group.

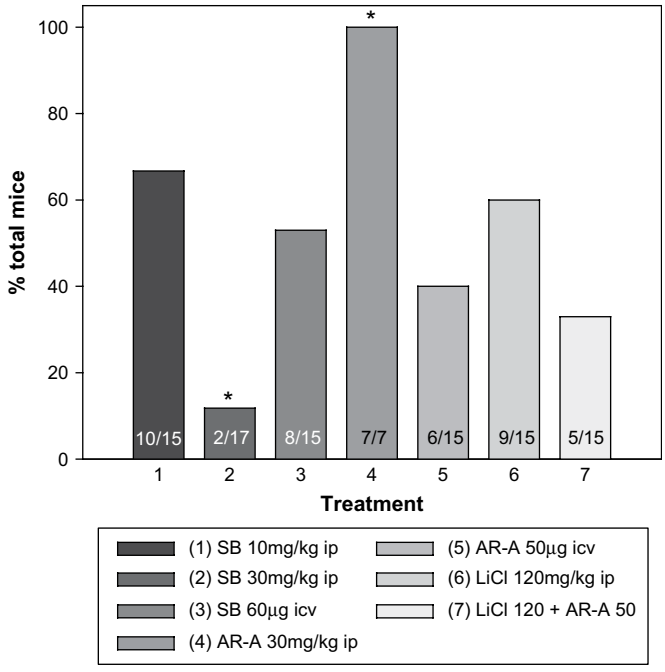

Fig. 2. GSK3 inhibitors reduce audiogenic seizures in FX mice. SB-216763 (SB) and AR-A014418 (AR-A) were administered to FVB FX mice by ip or icv injections. Lithium chloride (120 mg/kg; 2.83 mmol/kg) was administered ip. Testing for audiogenic seizures (AGS) was performed 30 min after ip or 45 min after icv injections. For co-administration, AR-A was given icv followed 15 min later by lithium (ip), and then AGS testing 30 min after the lithium dose. Values are presented as the percentage of total number of mice per group displaying AGS. The number within each bar represents the number of mice displaying AGS over the total number of mice in that group. Significance was determined using chi-square tests by comparison of results to mice treated with SB 10 mg/kg in order to emphasize the absence of significant difference between this treatment and most of the other treatments; the AR-A014418 non-response is equivalent to vehicle-treated mice (see Fig. 1). $^{*}p < 0.05$.

one factor ANOVA followed by Tukey–Kramer HSD test. For immunoblotting, FX band densities were compared as a group to those from wild-type extracts analyzed on the same gels by Student's *t*-test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3. Results

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*^{tm1Cgr} (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 controls (e.g. 100% of vehicle-treated FX mice seized in the experiments presented in Fig. 1). These differences were even greater for mice dying from status epilepticus (SE), a sustained tonic seizure used as a measure of response severity; again nearly all FX FVB mice died from SE, while none of the wild-type mice succumbed. Treatment of FX mice with lithium citrate provided a dose-dependent reduction in the number and lethality of audiogenic seizures. Furthermore, this effect of lithium was FX-specific, as audiogenic seizures in wild-type mice were not diminished by treatment with the same doses of lithium citrate (Fig. 1).

To determine if this protective action of lithium could be due to inhibition of GSK3, the effects of several selective GSK3 inhibitors were tested on audiogenic seizures in FX mice using either intraperitoneal (ip) or intracerebroventricular (icv) administration. Administration of the maleimide GSK3 inhibitor SB-216763 (Coghlan et al., 2000) ip or icv provided dose-dependent protection of FX mice from audiogenic seizures (Fig. 2). Reductions in audiogenic seizures also were attained by icv, but not ip, treatment with the highly selective thiazole GSK3 inhibitor AR-A014418 (Bhat et al., 2003), consistent with its poor ability to penetrate the blood–brain barrier (Vasdev et al., 2005). These results demonstrate that inhibition of GSK3 in the CNS greatly reduces FX audiogenic seizures.

To determine whether GSK3 inhibitors also corrected FX mouse behavioral phenotypes in the open field test, FX mice on a C57BL/6J strain background were employed because they have shown a hyperactivity phenotype in numerous laboratories (Bakker and Consortium, 1994; Peier et al., 2000), in addition to the common FX elevated center square entry phenotype. Intraperitoneal administration of lithium can produce substantial gastrointestinal distress (Schachtman et al., 2003), which in turn can make interpretation of results in the test difficult; 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 reversion 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-GSK3β and phospho-Ser21-GSK3α) was measured in several brain regions of wild-type and FX mice. Both GSK3α and GSK3β were found to be significantly dephosphorylated on the inhibitory serines in FX striatum and cortex, indicative of increases in active GSK3 (Fig. 4). GSK3α 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-GSK3β, confirming that lithium was capable of counteracting the hyperactive GSK3 in FX mice (Fig. 5). Total levels of GSK3β remained unaltered. These results support the behavioral results

Please cite this article in press as: Wenzhong William Min et al., Elevated glycogen synthase kinase-3 activity in Fragile X mice: Key metabolic regulator with evidence for treatment potential, Neuropharmacology (2008), doi:10.1016/j.neuropharm.2008.09.017

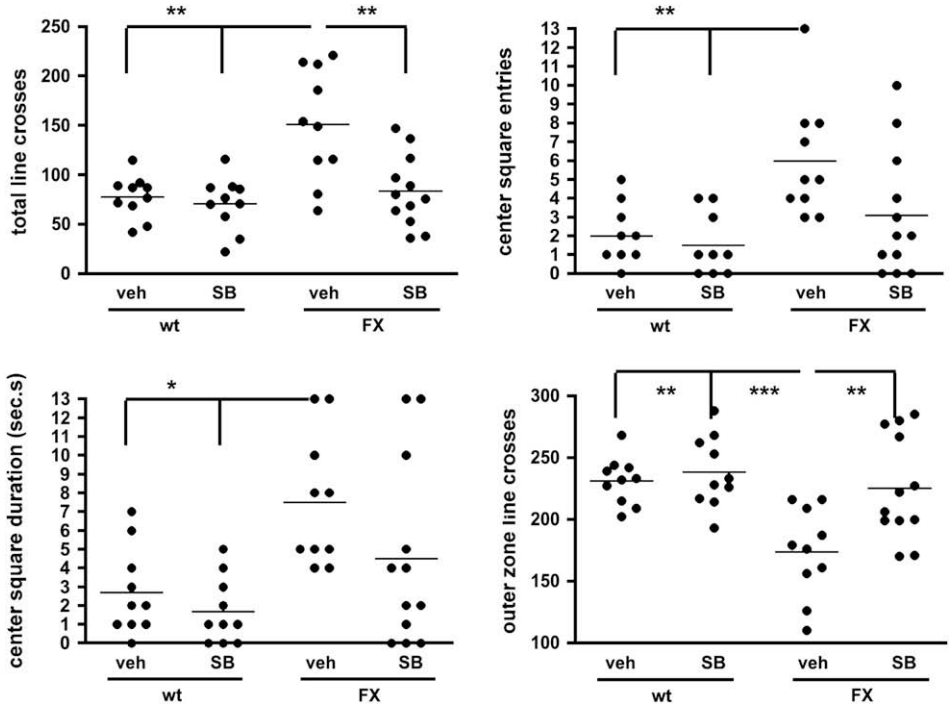

Fig. 3. GSK3 inhibitor SB-216763 normalizes FX open field behavior. SB-216763 (SB; 4 mg/kg, ip) or vehicle (veh; 20 μl DMSO, ip) was administered to male, 30-day-old wild-type or FX (C57BL/6J strain) mice 30 min before a 5 min open field test. Total line crosses are a measure of hyperactivity. Outer zone line crosses are a measure of thigmotaxis (wall hugging). Each dot in a column indicates the behavior of a single mouse. Statistical significance was assessed by one factor ANOVA followed by Tukey–Kramer HSD test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Symbols below the bars show comparisons to the samples bounding the symbols, and symbols above the bars apply to all samples to which the bars refer.

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 treatment 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-tolerizing 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 phospho-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.

Please cite this article in press as: Wenzhong William Min et al., Elevated glycogen synthase kinase-3 activity in Fragile X mice: Key metabolic regulator with evidence for treatment potential, Neuropharmacology (2008), doi:10.1016/j.neuropharm.2008.09.017

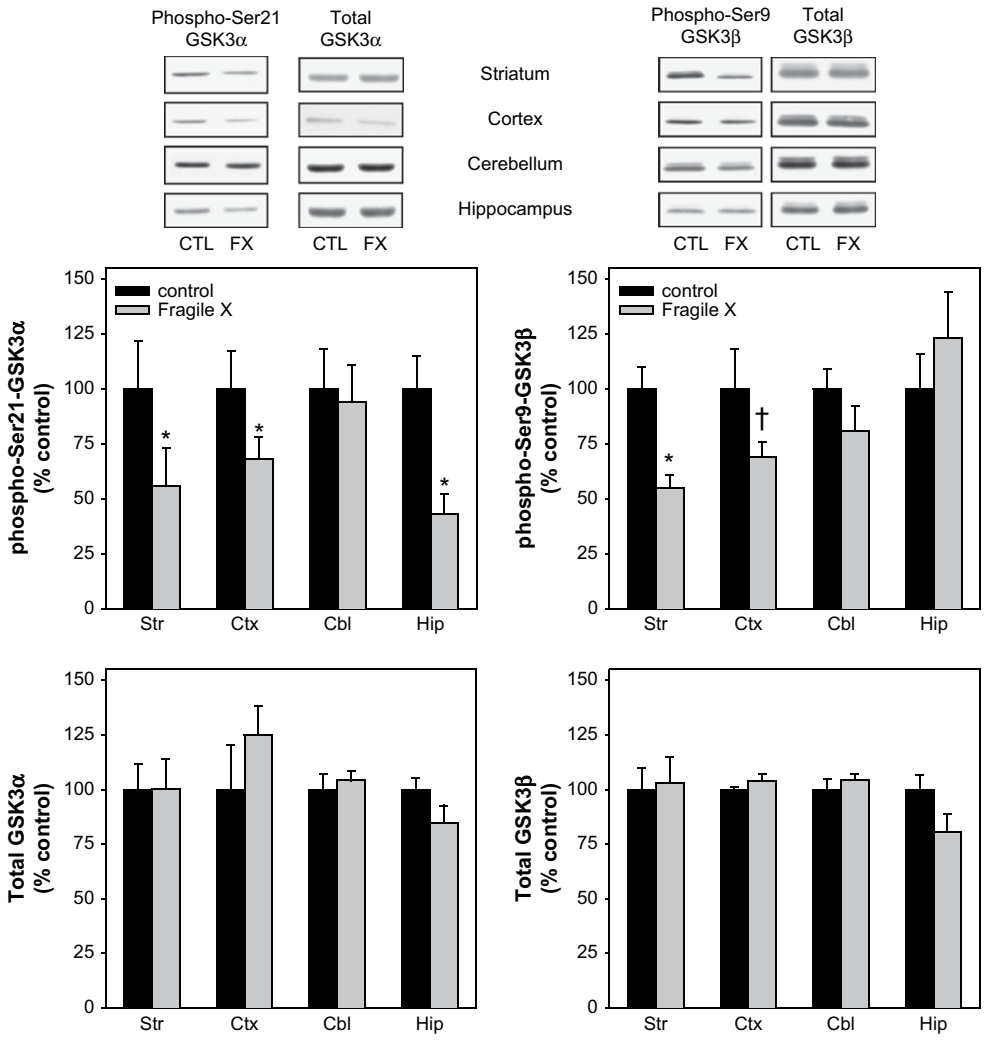

Fig. 4. GSK3 is hyperactive in the brains of FX mice. Immunoblots of striatum (Str), cerebral cortex (Ctx), cerebellum (Cbl), and hippocampus (Hip) extracts probed with antibodies specific to phospho-Ser21-GSK3α or total GSK3α (left) and phospho-Ser9-GSK3β or total GSK3β (right). Immunoblots were quantified by densitometry and are presented as the percents of densities of immunoblots of wild-type extracts analyzed on the same gels. $n = 8$ animals per group, of FVB/NJ strain background; $^{*}p < 0.05$; $\dagger p < 0.06$ compared to wild-type values (Student's *t*-test).

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.

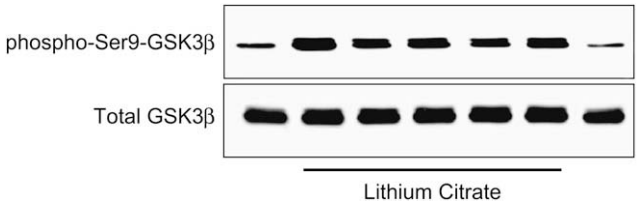

Fig. 5. Lithium increases FX GSK3β serine-phosphorylation *in vivo*. FX mice (F1 hybrid C57BL/6J × FVB/NJ background) were administered lithium citrate (ip; 300 mg/kg; 4.29 mmol/kg) and after 30 min brain extracts were prepared. Immunoblots show samples from each individual mouse probed for phospho-Ser9-GSK3β and total GSK3β. After lithium treatment, phospho-Ser9-GSK3β was 174 ± 10% of lithium-free values, and total GSK3β was 112 ± 2% of lithium-free values.

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

Please cite this article in press as: Wenzhong William Min et al., Elevated glycogen synthase kinase-3 activity in Fragile X mice: Key metabolic regulator with evidence for treatment potential, Neuropharmacology (2008), doi:10.1016/j.neuropharm.2008.09.017

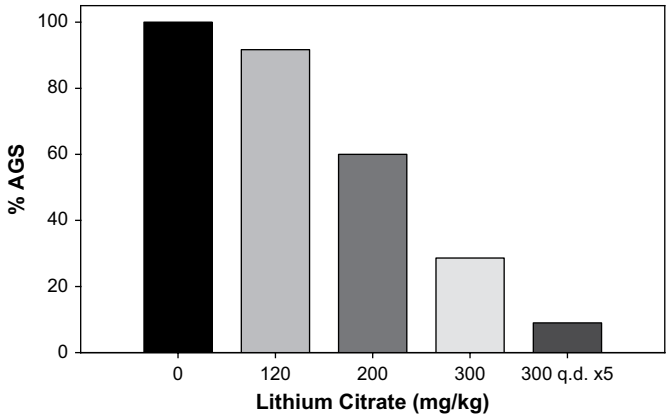

Fig. 6. Repeat administration of lithium reduces audiogenic seizures in FX mice. Lithium citrate was administered ip once (120, 200, or 300 mg/kg, equivalent to 1.71, 2.86, or 4.29 mmol lithium/kg, respectively) or once a day (q.d.) for five days (ip; 300 mg/kg) 30 min prior to exposure to a high intensity sound. Values are presented as the percentage of total number of mice per group displaying AGS. The number of mice in each test group was, left to right, 14, 12, 10, 14, 11.

(McBride et al., 2005; Yan et al., 2005b), which had been used in support of preliminary trials of lithium in FXS patients (R. Hagerman, personal communications). Of particular interest, the anti-epileptogenic effect of lithium appeared to be FX-specific in that lithium did not inhibit seizures in wild-type mice. 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

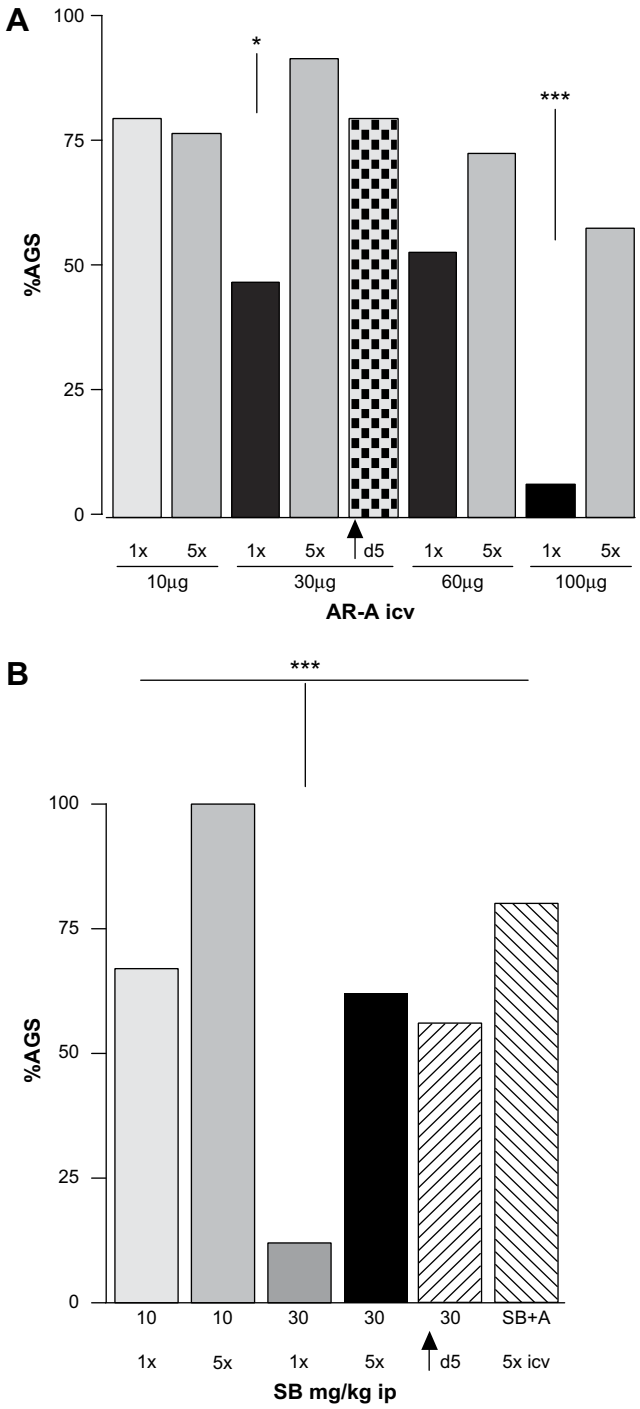

Fig. 7. Effects of GSK3 inhibitors on audiogenic seizures in FX mice. (A) FX male mice (FVB strain) were given one dose of GSK3 inhibitor AR-A014418 (AR-A, icv, in 3 µl DMSO) at 30 days of age, or a daily dose for five days beginning at 26 days of age. In one test (up arrow), the fifth dose of AR-A014418 was increased to 60 µg. 15 mice were tested in each group except for 13 mice each for the 10 µg 5× and 30 µg 5× groups. AGS testing was performed 45 min after the final dose administration. (B) GSK3 inhibitor SB-216763 (SB) was administered ip in 70 µl DMSO to FVB FX male mice at the ages as in panel A. In one test (up arrow), the SB-216763 dose was increased to 50 mg/kg on the fifth day. In another test ("SB + A"), combined high dose SB-216763 (100 µg) and AR-A014418 (140 µg) were given icv in 5 µl DMSO for 5 days. AGS testing was performed 30 min after the final ip dose, or 45 min after the final icv dose. Each test group had 15 mice except for 13 mice in the 30 µg 5× group, and 16 mice in the 30 µg elevated to 60 µg on day 5 ("up arrow") group. Statistical significance was determined by contingency test (chi-square with column drops and Fisher's exact test). $^{*}p < 0.05$; $^{***}p < 0.001$ compared with mice not given a GSK3 inhibitor, in which 100% of mice displayed AGS.

Please cite this article in press as: Wenzhong William Min et al., Elevated glycogen synthase kinase-3 activity in Fragile X mice: Key metabolic regulator with evidence for treatment potential, Neuropharmacology (2008), doi:10.1016/j.neuropharm.2008.09.017

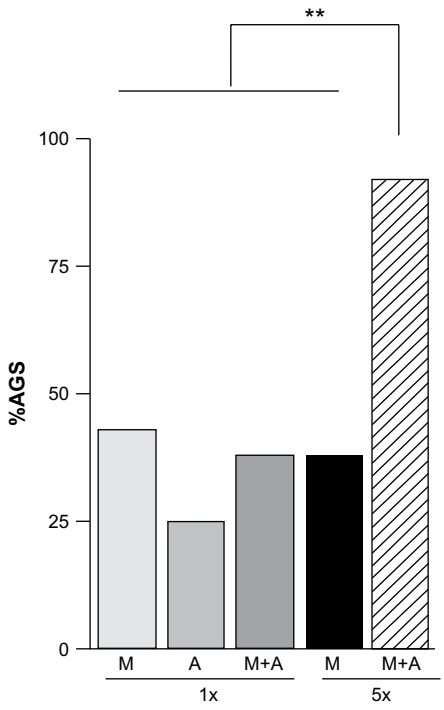

Fig. 8. Combined mGluR5 antagonist and GSK3 inhibitor audiogenic seizure inhibition in FX mice is not additive. MPEP ("M"; 42 μg) and AR-A014418 ("A"; 50 μg) were given to FVB FX male mice (icv, in 3 μl DMSO) as described in Fig. 7. "1×" indicates a single daily dose; "5×" indicates a single dose given 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. $^{**}p < 0.01$.

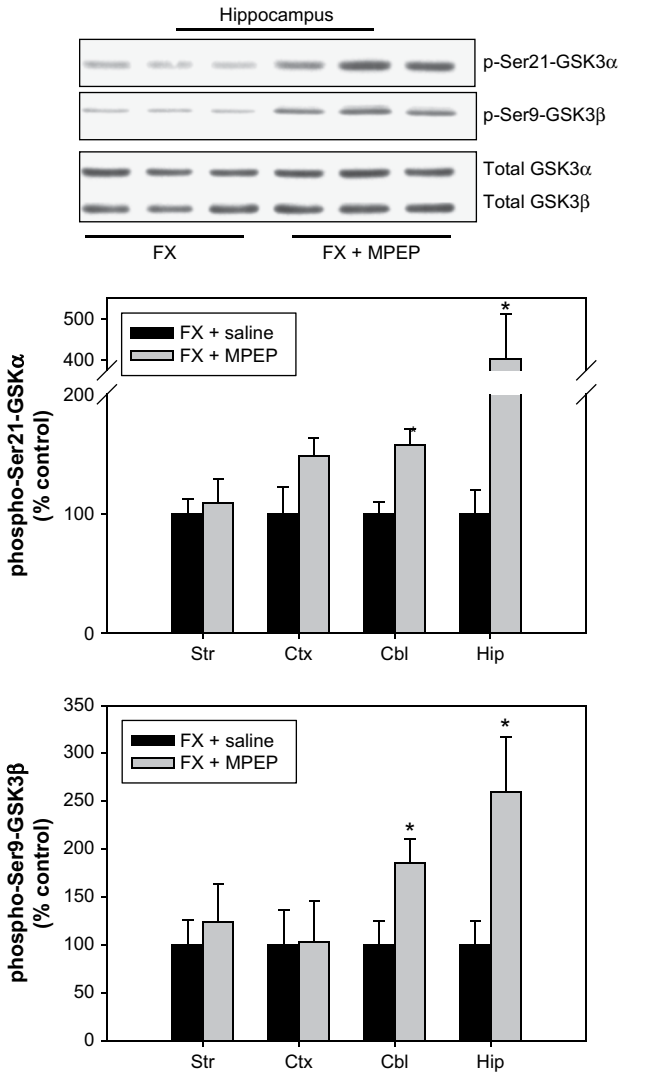

Fig. 9. MPEP administration increases GSK3 serine-phosphorylation in FX mouse brain. FX mice (male, FVB background, 30 days of age) were administered MPEP (30 mg/kg; ip; n = 5) or vehicle (n = 5) 30 min prior to sacrifice. The top panel shows representative hippocampal samples immunoblotted for phospho-Ser21-GSK3α, phospho-Ser9-GSK3β, and total levels of GSK3α and GSK3β. Quantitative values are 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; Hip = hippocampus. $^{*}p < 0.05$ compared to saline values (Student's t-test).

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 is known to activate the PI3K/Akt and MEK/ERK pathways (Banko et al., 2004; Hou and Klann, 2004), both of which can inhibit GSK3β (Angenstein et al., 1998; Liu et al., 2005). Therefore, elevated mGluR5 signaling would generally be expected to inactivate GSK3. Nonetheless, in this study, a deficit of FMRP in the FX brain was associated with elevation of a non-phosphorylated, active form of GSK3. These results imply that enhanced phosphorylation of GSK3β on Ser9 (and GSK3α on Ser21) in tissues expressing normal levels of FMRP may occur in a manner distinct from that generally expected from excessive mGluR group I signaling via the Akt or ERK1/2 kinases. Known alterations in FX monoamine and Rac1/Rho signaling could be responsible for the FX GSK3 elevations observed here (Bardoni and Mandel, 2002; Zhang et al., 2005); these possibilities are presented in more detail in the Supplemental information (*).

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 LiCl, 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

Please cite this article in press as: Wenzhong William Min et al., Elevated glycogen synthase kinase-3 activity in Fragile X mice: Key metabolic regulator with evidence for treatment potential, Neuropharmacology (2008), doi:10.1016/j.neuropharm.2008.09.017

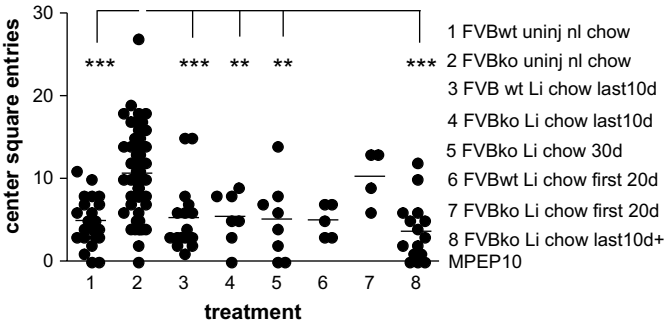

Fig. 10. Chronic lithium treatment reverts FX open field center square entry behavior to wild type. Open field testing was performed at 30 days of age with male FVB wild-type ("wt") and FX ("ko") mice that were fed normal chow or food containing 3.4 g/kg lithium carbonate ("Li") for the times indicated ("d" refers to the total number of days of treatment; "uninj" signifies the mice were not injected with any agent). "MPEP" indicates mice which received an ip injection of 10 mg/kg MPEP 30 min prior to open field testing. Each dot represents the behavior of a single mouse. Statistical significance was assessed by one factor ANOVA followed by Tukey–Kramer HSD test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Symbols below the bars show comparisons to the samples bounding the symbols.

effects that are present in the *dfxr* fly relate to those from post-synaptic mammalian mGluR group I receptors such as mGluR5, it was suggested that antagonism of *Drosophila* group II mGluRs might produce effects similar to that of lithium by influencing inositol trisphosphate (IP3) receptor-mediated calcium release, or by promoting activation of the transcription factor CREB (Grimes and Jope, 2001; McBride et al., 2005). A pure IP3-based explanation for the lithium results presented here are unlikely given the similar effects of the selective GSK3 inhibitors, which are not known to have any effect on IP3. CREB is known to be important for the establishment of long-term memory (Gass et al., 1998) and possibly for the effects of lithium in bipolar disorder, as previously reviewed (Bachmann et al., 2005). In both cases, the effects are delayed but persist. In these studies, however, it was found that the effect of lithium in the AGS and open field assays did not persist, which suggests the phenotype reversals observed here were more acute and transient than commonly expected from changes in gene transcription (Supplemental information **). As noted above, several studies have provided support for proposals that a negative impact on the dynamic regulation of synaptic protein translation, acting over times compatible with those employed in this study, is a critical determinant of FXS. The effects of GSK3 activity on translation (via eIF2B) and directly on proteins translationally mis-expressed in FXS (e.g. MAP1B) are discussed further in the Supplemental information (***).

Another potentially significant means by which GSK3 could be producing the effects observed in these studies is suggested by prior observations that cerebral glucose metabolism may be altered in the brains of people with FXS (Schapiro et al., 1995), as well as in FX mouse and fly models (Qin et al., 2002; Zhang et al., 2005). GSK3 negatively regulates glycogen synthase, which is rate-limiting for glycogen production and indirectly stimulated by lithium (Cheng et al., 1983). Glycogen is found at low levels in the brain, predominantly in astrocytes (Cataldo and Broadwell, 1986; Wiesinger et al., 1997). Glycogen use in the brain has been linked to neuronal activation; conversely, glycogen levels increase during sleep and anesthesia (Brown, 2004; Magistretti, 2006; Magistretti et al., 2000). Of particular note, recent findings suggest that inhibition of glycogenolysis, such as by administration of the glycogen phosphorylase inhibitor 1,4-dideoxy-1,4-imino-D-arabinaitol (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.

Acknowledgments

We wish to thank Michael Tranfaglia for discussions and advice on the *in vivo* pharmacologic testing, and Anna Zmijewska for experimental assistance. This work was funded by grants from the FRAXA Research Foundation and a grant from the National Institutes of Mental Health (MH38752).

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.

References

Angenstein, F., Greenough, W.T., Weiler, I.J., 1998. Metabotropic glutamate receptor-initiated translocation of protein kinase p90rsk to polyribosomes: a possible

factor regulating synaptic protein synthesis. Proceedings of the National Academy of Sciences of the United States of America 95, 15078–15083.
Aschrafi, A., Cunningham, B.A., Edelman, G.M., Vanderklish, P.W., 2005. The fragile X mental retardation protein and group I metabotropic glutamate receptors regulate levels of mRNA granules in brain. Proceedings of the National Academy of Sciences of the United States of America 102, 2180–2185.
Bachmann, R.F., Schloesser, R.J., Gould, T.D., Manji, H.K., 2005. Mood stabilizers target cellular plasticity and resilience cascades: implications for the development of novel therapeutics. Molecular Neurobiology 32, 173–202.
Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C.J., McLauchlan, H., Klevernic, I., Arthur, J.S., Alessi, D.R., Cohen, P., 2007. The selectivity of protein kinase inhibitors: a further update. Biochemical Journal 408, 297–315.
Bakker, C.E., Consortium, D.-B.F.X., 1994. Fmr1 knockout mice: a model to study fragile X mental retardation. Cell 78, 23–33.
Banko, J.L., Hou, L., Klann, E., 2004. NMDA receptor activation results in PKA- and ERK-dependent Mnk1 activation and increased eIF4E phosphorylation in hippocampal area CA1. Journal of Neurochemistry 91, 462–470.
Bao, W.L., Williams, A.J., Faden, A.I., Tortella, F.C., 2001. Selective mGluR5 receptor antagonist or agonist provides neuroprotection in a rat model of focal cerebral ischemia. Brain Research 922, 173–179.
Bardoni, B., Mandel, J.-L., 2002. Advances in understanding of fragile X pathogenesis and FMRP function, and in identification of X linked mental retardation genes. Current Opinion in Genetics and Development 12, 284–293.
Bauchwitz, R.P., 2002. Pharmacologic and genetic targets in fragile X treatment research. In: Eighth International Fragile X Foundation Conference.
Baumgardner, T.L., Reiss, A.L., Freund, L.S., Abrams, M.T., 1995. Specification of the neurobehavioral phenotype in males with fragile X syndrome. Pediatrics 95, 744–752.
Bear, M.F., Dolen, G., Osterweil, E., Nagarajan, N., 2007. Fragile X: translation in action. Neuropsychopharmacology 33, 84–87.
Bernardet, M., Crusio, W.E., 2006. Fmr1 KO mice as a possible model of autistic features. ScientificWorldJournal 6, 1164–1176.
Berridge, M.J., Downes, C.P., Hanley, M.R., 1989. Neural and developmental actions of lithium: a unifying hypothesis. Cell 59, 411–419.
Bersudsky, Y., Shaldubina, A., Belmaker, R.H., 2007. Lithium's effect in forced-swim test is blood level dependent but not dependent on weight loss. Behavioural Pharmacology 18, 77–80.
Bhat, R., Xue, Y., Berg, S., Hellberg, S., Ormo, M., Nilsson, Y., Radesater, A.C., Jerning, E., Markgren, P.O., Borgegard, T., Nylof, M., Gimenez-Cassina, A., Hernandez, F., Lucas, J.J., Diaz-Nido, J., Avila, J., 2003. Structural insights and biological effects of glycogen synthase kinase 3-specific inhibitor AR-A014418. Journal of Biological Chemistry 278, 45937–45945.
Bregman, J.D., Leckman, J.F., Ort, S.I., 1988. Fragile X syndrome: genetic predisposition to psychopathology. Journal of Autism and Developmental Disorders 18, 343–354.
Brown, A.M., 2004. Brain glycogen re-awakened. Journal of Neurochemistry 89, 537–552.
Brown, V., Jin, P., Ceman, S., Darnell, J.C., O'Donnell, W.T., Tenenbaum, S.A., Jin, X., Feng, Y., Wilkinson, K.D., Keene, J.D., Darnell, R.B., Warren, S.T., 2001. Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. Cell 107, 477–487.
Cataldo, A.M., Broadwell, R.D., 1986. Cytochemical identification of cerebral glycogen and glucose-6-phosphatase activity under normal and experimental conditions. II. Choroid plexus and ependymal epithelia, endothelia and pericytes. Journal of Neurocytology 15, 511–524.
Chen, L., Toth, M., 2001. Fragile X mice develop sensory hyperreactivity to auditory stimuli. Neuroscience 103, 1043–1050.
Cheng, K., Creacy, S., Larner, J., 1983. 'Insulin-like' effects of lithium ion on isolated rat adipocytes. II. Specific activation of glycogen synthase. Molecular and Cellular Biochemistry 56, 183–189.
Chuang, S.C., Zhao, W., Bauchwitz, R., Yan, Q., Bianchi, R., Wong, R.K., 2005. Prolonged epileptiform discharges induced by altered group I metabotropic glutamate receptor-mediated synaptic responses in hippocampal slices of a fragile X mouse model. Journal of Neuroscience 25, 8048–8055.
Coghlan, M.P., Culbert, A.A., Cross, D.A., Corcoran, S.L., Yates, J.W., Pearce, N.J., Rausch, O.L., Murphy, G.J., Carter, P.S., Roxbee Cox, L., Mills, D., Brown, M.J., Haigh, D., Ward, R.W., Smith, D.G., Murray, K.J., Reith, A.D., Holder, J.C., 2000. Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. Chemistry and Biology 7, 793–803.
Correll, C.U., 2007. Weight gain and metabolic effects of mood stabilizers and antipsychotics in pediatric bipolar disorder: a systematic review and pooled analysis of short-term trials. Journal of the American Academy of Child and Adolescent Psychiatry 46, 687–700.
Daoudal, G., Debanne, D., 2003. Long-term plasticity of intrinsic excitability: learning rules and mechanisms. Learning and Memory 10, 456–465.
Darnell, J.C., Jensen, K.B., Jin, P., Brown, V., Warren, S.T., Darnell, R.B., 2001. Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. Cell 107, 489–499.
De Sarno, P., Bijur, G.N., Zmijewska, A.A., Li, X., Jope, R.S., 2006. In vivo regulation of GSK3 phosphorylation by cholinergic and NMDA receptors. Neurobiology of Aging 27, 413–422.
De Sarno, P., Li, X., Jope, R.S., 2002. Regulation of Akt and glycogen synthase kinase-3 beta phosphorylation by sodium valproate and lithium. Neuropharmacology 43, 1158–1164.
Doble, B.W., Woodgett, J.R., 2003. GSK-3: tricks of the trade for a multi-tasking kinase. Journal of Cell Science 116, 1175–1186.
Dockendorff, T.C., Su, H.S., McBride, S.M., Yang, Z., Choi, C.H., Siwicki, K.K., Sehgal, A., Jongens, T.A., 2002. *Drosophila* lacking dfmr1 activity show defects in circadian output and fail to maintain courtship interest. Neuron 34, 973–984.
Fisch, G.S., Carpenter, N.J., Holden, J.J., Simensen, R., Howard-Peebles, P.N., Maddalena, A., Pandya, A., Nance, W., 1999. Longitudinal assessment of adaptive and maladaptive behaviors in fragile X males: growth, development, and profiles. American Journal of Medical Genetics 83, 257–263.
Garcia-Perez, J., Avila, J., Diaz-Nido, J., 1998. Implication of cyclin-dependent kinases and glycogen synthase kinase 3 in the phosphorylation of microtubule-associated protein 1B in developing neuronal cells. Journal of Neuroscience Research 52, 445–452.
Gasparini, F., Lingenhohl, K., Stoehr, N., Flor, P.J., Heinrich, M., Vranesic, I., Biollaz, M., Allgeier, H., Heckendorn, R., Urwyler, S., Varney, M.A., Johnson, E.C., Hess, S.D., Rao, S.P., Sacaan, A.I., Santori, E.M., Velicelebi, G., Kuhn, R., 1999. 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), a potent, selective and systemically active mGlu5 receptor antagonist. Neuropharmacology 38, 1493–1503.
Gass, P., Wolfer, D.P., Balschun, D., Rudolph, D., Frey, U., Lipp, H.-P., Schutz, G., 1998. Deficits in memory tasks of mice with CREB mutations depend on gene dosage. Learning and Memory 5, 274–288.
Gibbs, M.E., Lloyd, H.G., Santa, T., Hertz, L., 2007. Glycogen is a preferred glutamate precursor during learning in 1-day-old chick: biochemical and behavioral evidence. Journal of Neuroscience Research 85, 3326–3333.
Grimes, C.A., Jope, R.S., 2001. CREB DNA binding activity is inhibited by glycogen synthase kinase-3 beta and facilitated by lithium. Journal of Neurochemistry 78, 1219–1232.
Grossman, A.W., Aldridge, G.M., Weiler, I.J., Greenough, W.T., 2006. Local protein synthesis and spine morphogenesis: fragile X syndrome and beyond. Journal of Neuroscience 26, 7151–7155.
Hernandez, F., Borrell, J., Guaza, C., Avila, J., Lucas, J.J., 2002. Spatial learning deficit in transgenic mice that conditionally over-express GSK-3beta in the brain but do not form tau filaments. Journal of Neurochemistry 83, 1529–1533.
Hou, L., Klann, E., 2004. Activation of the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling pathway is required for metabotropic glutamate receptor-dependent long-term depression. Journal of Neuroscience 24, 6352–6361.
Huber, K.M., Gallagher, S.M., Warren, S.T., Bear, M.F., 2002. Altered synaptic plasticity in a mouse model of fragile X mental retardation. Proceedings of the National Academy of Sciences of the United States of America 99, 7746–7750.
Hummel, T., Krukkert, K., Roos, J., Davis, G., Klambt, C., 2000. *Drosophila* Futsch/22C10 is a MAP1B-like protein required for dendritic and axonal development. Neuron 26, 357–370.
Jope, R.S., 2003. Lithium and GSK-3: one inhibitor, two inhibitory actions, multiple outcomes. Trends in Pharmacological Sciences 24, 441–443.
Jope, R.S., Johnson, G.V., 2004. The glamour and gloom of glycogen synthase kinase-3. Trends in Biochemical Sciences 29, 95–102.
Kallen, B., Tandberg, A., 1983. Lithium and pregnancy. A cohort study on manic-depressive women. Acta Psychiatrica Scandinavica 68, 134–139.
Kim, D.H., Jung, J.S., Song, D.K., Suh, H.W., Huh, S.O., Kim, Y.H., 1998. Intracerebroventricular injection-induced increase in plasma corticosterone levels in the mouse: a stress model. Journal of Pharmacological and Toxicological Methods 39, 71–73.
Klein, P.S., Melton, D.A., 1996. A molecular mechanism for the effect of lithium on development. Proceedings of the National Academy of Sciences of the United States of America 93, 8455–8459.
Koekkoek, S.K., Yamaguchi, K., Milojkovic, B.A., Dortland, B.R., Ruigrok, T.J., Maex, R., De Graaf, W., Smit, A.E., VanderWerf, F., Bakker, C.E., Willemsen, R., Ikeda, T., Kakizawa, S., Onodera, K., Nelson, D.L., Mientjes, E., Joosten, M., De Schutter, E., Oostra, B.A., Ito, M., De Zeeuw, C.I., 2005. Deletion of FMR1 in Purkinje cells enhances parallel fiber LTD, enlarges spines, and attenuates cerebellar eyelid conditioning in Fragile X syndrome. Neuron 47, 339–352.
Kozikowski, A.P., Gaisina, I.N., Petukhov, P.A., Sridhar, J., King, L.T., Blond, S.Y., Duka, T., Rusnak, M., Sidhu, A., 2006. Highly potent and specific GSK-3beta inhibitors that block tau phosphorylation and decrease alpha-synuclein protein expression in a cellular model of Parkinson's disease. ChemMedChem 1, 256–266.
Laggerbauer, B., Ostareck, D., Keidel, E., Ostareck-Lederer, A., Fischer, U., 2001. Evidence that fragile X mental retardation protein is a negative regulator of translation. Human Molecular Genetics 10, 329–338.
Lea, P.M., Movsesyan, V.A., Faden, A.I., 2005. Neuroprotective activity of the mGluR5 antagonists MPEP and MTEP against acute excitotoxicity differs and does not reflect actions at mGluR5 receptors. British Journal of Pharmacology 145, 527–534.
Li, J., Pelletier, M.R., Perez Velazquez, J.L., Carlen, P.L., 2002. Reduced cortical synaptic plasticity and GluR1 expression associated with fragile X mental retardation protein deficiency. Molecular and Cellular Neuroscience 19, 138–151.
Li, Z., Zhang, Y., Ku, L., Wilkinson, K.D., Warren, S.T., Feng, Y., 2001. The fragile X mental retardation protein inhibits translation via interacting with mRNA. Nucleic Acids Research 29, 2276–2283.
Linden, S., Rich, C.L., 1983. The use of lithium during pregnancy and lactation. Journal of Clinical Psychiatry 44, 358–361.
Liu, F., Gong, X., Zhang, G., Marquis, K., Reinhart, P., Andree, T.H., 2005. The inhibition of glycogen synthase kinase 3beta by a metabotropic glutamate receptor 5 mediated pathway confers neuroprotection to Abeta peptides. Journal of Neurochemistry 95, 1363–1372.
Lucas, F.R., Goold, R.G., Gordon-Weeks, P.R., Salinas, P.C., 1998. Inhibition of GSK-3beta leading to the loss of phosphorylated MAP-1B is an early event in

Please cite this article in press as: Wenzhong William Min et al., Elevated glycogen synthase kinase-3 activity in Fragile X mice: Key metabolic regulator with evidence for treatment potential, Neuropharmacology (2008), doi:10.1016/j.neuropharm.2008.09.017

axonal remodelling induced by WNT-7a or lithium. Journal of Cell Science 111 (Pt 10), 1351–1361.
Magistretti, P.J., 2006. Neuron–glia metabolic coupling and plasticity. Journal of Experimental Biology 209, 2304–2311.
Magistretti, P.J., Pellerin, L., Martin, J.L., 2000. Brain energy metabolism. Neuropsychopharmacology: the fourth generation of progress. American College of Neuropsychopharmacology.
Martinez, A., Castro, A., Dorronsoro, I., Alonso, M., 2002. Glycogen synthase kinase 3 (GSK-3) inhibitors as new promising drugs for diabetes, neurodegeneration, cancer, and inflammation. Medicinal Research Reviews 22, 373–384.
McBride, S.M., Choi, C.H., Wang, Y., Liebelt, D., Braunstein, E., Ferreiro, D., Sehgal, A., Siwicki, K.K., Dockendorff, T.C., Nguyen, H.T., McDonald, T.V., Jongens, T.A., 2005. Pharmacological rescue of synaptic plasticity, courtship behavior, and mushroom body defects in a *Drosophila* model of fragile X syndrome. Neuron 45, 753–764.
Messiha, F.S., 1986. Lithium and the neonate: developmental and metabolic aspects. Alcohol 3, 107–112.
Miyashiro, K.Y., Beckel-Mitchener, A., Purk, T.P., Becker, K.G., Barret, T., Liu, L., Carbonetto, S., Weiler, I.J., Greenough, W.T., Eberwine, J., 2003. RNA cargoes associating with FMRP reveal deficits in cellular functioning in Fmr1 null mice. Neuron 37, 417–431.
Musumeci, S.A., Bosco, P., Calabrese, G., Bakker, C., De Sarro, G.B., Elia, M., Ferri, R., Oostra, B.A., 2000. Audiogenic seizures susceptibility in transgenic mice with fragile X syndrome. Epilepsia 41, 19–23.
Musumeci, S.A., Hagerman, R.J., Ferri, R., Bosco, P., Dalla Bernardina, B., Tassinari, C.A., De Sarro, G.B., Elia, M., 1999. Epilepsy and EEG findings in males with fragile X syndrome. Epilepsia 40, 1092–1099.
O'Brien, W.T., Harper, A.D., Jove, F., Woodgett, J.R., Maretto, S., Piccolo, S., Klein, P.S., 2004. Glycogen synthase kinase-3{beta} haploinsufficiency mimics the behavioral and molecular effects of lithium. Journal of Neuroscience 24, 6791–6798.
O'Dowd, B.S., Gibbs, M.E., Ng, K.T., Hertz, E., Hertz, L., 1994. Astrocytic glycogenolysis energizes memory processes in neonate chicks. Brain Research. Developmental Brain Research 78, 137–141.
Peier, A.M., McIlwain, K.L., Kenneson, A., Warren, S.T., Paylor, R., Nelson, D.L., 2000. (Over)correction of FMR1 deficiency with YAC transgenics: behavioral and physical features. Human Molecular Genetics 9, 1145–1159.
Peineau, S., Taghibiglou, C., Bradley, C., Wong, T.P., Liu, L., Lu, J., Lo, E., Wu, D., Saule, E., Bouschet, T., Matthews, P., Isaac, J.T., Bortolotto, Z.A., Wang, Y.T., Collingridge, G.L., 2007. LTP inhibits LTD in the hippocampus via regulation of GSK3beta. Neuron 53, 703–717.
Phiel, C.J., Klein, P.S., 2001. Molecular targets of lithium action. Annual Review of Pharmacology and Toxicology 41, 789–813.
Pilc, A., Klodzinska, A., Branski, P., Nowak, G., Palucha, A., Szewczyk, B., Tatarczynska, E., Chojnacka-Wojcik, E., Wieronska, J.M., 2002. Multiple MPEP administrations evoke anxiolytic- and antidepressant-like effects in rats. Neuropharmacology 43, 181–187.
Popoli, P., Pintor, A., Tebano, M.T., Frank, C., Pepponi, R., Nazzicone, V., Grieco, R., Pezzola, A., Reggio, R., Minghetti, L., De Berardinis, M.A., Martire, A., Potenza, R.L., Domenici, M.R., Massotti, M., 2004. Neuroprotective effects of the mGlu5R antagonist MPEP towards quinolinic acid-induced striatal toxicity: involvement of pre- and post-synaptic mechanisms and lack of direct NMDA blocking activity. Journal of Neurochemistry 89, 1479–1489.
Qin, M., Kang, J., Smith, C.B., 2002. Increased rates of cerebral glucose metabolism in a mouse model of fragile X mental retardation. Proceedings of the National Academy of Sciences of the United States of America 99, 15758–15763.
Schachtman, T.R., Bills, C., Ghinescu, R., Murch, K., Serfozo, P., Simonyi, A., 2003. MPEP, a selective metabotropic glutamate receptor 5 antagonist, attenuates conditioned taste aversion in rats. Behavioural Brain Research 141, 177–182.
Schaeffer, C., Bardoni, B., Mandel, J.L., Ehresmann, B., Ehresmann, C., Moine, H., 2001. The fragile X mental retardation protein binds specifically to its mRNA via a purine quartet motif. EMBO Journal 20, 4803–4813.
Schapiro, M.B., Murphy, D.G., Hagerman, R.J., Azari, N.P., Alexander, G.E., Miezejeski, C.M., Hinton, V.J., Horwitz, B., Haxby, J.V., Kumar, A., et al., 1995. Adult fragile X syndrome: neuropsychology, brain anatomy, and metabolism. American Journal of Medical Genetics 60, 480–493.
Stambolic, V., Ruel, L., Woodgett, J.R., 1996. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. Current Biology 6, 1664–1668.
Su, Y., Ryder, J., Li, B., Wu, X., Fox, N., Solenberg, P., Brune, K., Paul, S., Zhou, Y., Liu, F., Ni, B., 2004. Lithium, a common drug for bipolar disorder treatment, regulates amyloid-beta precursor protein processing. Biochemistry 43, 6899–6908.
Tucker, B., Richards, R.I., Lardelli, M., 2006. Contribution of mGluR and Fmr1 functional pathways to neurite morphogenesis, craniofacial development and fragile X syndrome. Human Molecular Genetics 15, 3446–3458.
Vasdev, N., Garcia, A., Stableford, W.T., Young, A.B., Meyer, J.H., Houle, S., Wilson, A.A., 2005. Synthesis and ex vivo evaluation of carbon-11 labelled *N*-(4-methoxybenzyl)-*N*′-(5-nitro-1,3-thiazol-2-yl)urea ([^{11}C]AR-A014418): a radiolabelled glycogen synthase kinase-3beta specific inhibitor for PET studies. Bioorganic and Medicinal Chemistry Letters 15, 5270–5273.
Volk, L.J., Pfeiffer, B.E., Gibson, J.R., Huber, K.M., 2007. Multiple Gq-coupled receptors converge on a common protein synthesis-dependent long-term depression that is affected in fragile X syndrome mental retardation. Journal of Neuroscience 27, 11624–11634.
Wagman, A.S., Johnson, K.W., Bussiere, D.E., 2004. Discovery and development of GSK3 inhibitors for the treatment of type 2 diabetes. Current Pharmaceutical Design 10, 1105–1137.
Weiler, I.J., Irwin, S.A., Klintsova, A.Y., Spencer, C.M., Brazelton, A.D., Miyashiro, K., Comery, T.A., Patel, B., Eberwine, J., Greenough, W.T., 1997. Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. Proceedings of the National Academy of Sciences of the United States of America 94, 5395–5400.
Wiesinger, H., Hamprecht, B., Dringen, R., 1997. Metabolic pathways for glucose in astrocytes. Glia 21, 22–34.
Woodgett, J.R., 1990. Molecular cloning and expression of glycogen synthase kinase-3/factor A. EMBO Journal 9, 2431–2438.
Yan, Q.J., Asafo-Adjei, P.K., Arnold, H.M., Brown, R.E., Bauchwitz, R.P., 2004. A phenotypic and molecular characterization of the fmr1-tm1Cgr Fragile X mouse. Genes, Brain and Behavior 3, 337–359.
Yan, Q.J., Rammal, M., Tranfaglia, M., Bauchwitz, R.P., 2005a. Suppression of two major Fragile X Syndrome mouse model phenotypes by the mGluR5 antagonist MPEP. Neuropharmacology 49, 1053–1066.
Yan, Q.J., Sikorski, C., Bauchwitz, R.P., 2005b. GSK3 inhibitors revert Fragile X mouse audiogenic seizure and open field exploratory behaviors to wild type. Society for Neuroscience Abstracts, 679.11.
Zhang, Y.Q., Bailey, A.M., Matthies, H.J., Renden, R.B., Smith, M.A., Speese, S.D., Rubin, G.M., Broadie, K., 2001. *Drosophila* fragile X-related gene regulates the MAP1B homolog Futsch to control synaptic structure and function. Cell 107, 591–603.
Zhang, Y.Q., Friedman, D.B., Wang, Z., Woodruff III, E., Pan, L., O'Donnell, J., Broadie, K., 2005. Protein expression profiling of the *Drosophila* fragile X mutant brain reveals up-regulation of monoamine synthesis. Molecular & Cellular Proteomics 4, 278–290.
Zhu, L.Q., Wang, S.H., Liu, D., Yin, Y.Y., Tian, Q., Wang, X.C., Wang, Q., Chen, J.G., Wang, J.Z., 2007. Activation of glycogen synthase kinase-3 inhibits long-term potentiation with synapse-associated impairments. Journal of Neuroscience 27, 12211–12220.

Please cite this article in press as: Wenzhong William Min et al., Elevated glycogen synthase kinase-3 activity in Fragile X mice: Key metabolic regulator with evidence for treatment potential, Neuropharmacology (2008), doi:10.1016/j.neuropharm.2008.09.017