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Lithium ameliorates altered glycogen synthase kinase-3 and behavior in a mouse model of Fragile X syndrome

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ABSTRACT

Fragile X syndrome (FXS), the most common form of inherited mental retardation and a genetic cause of autism, results from mutated *fragile X mental retardation-1* (*Fmr1*). This study examined the effects on glycogen synthase kinase-3 (GSK3) of treatment with a metabotropic glutamate receptor (mGluR) antagonist, MPEP, and the GSK3 inhibitor, lithium, in C57Bl/6 *Fmr1* knockout mice. Increased mGluR signaling may contribute to the pathology of FXS, and the mGluR5 antagonist MPEP increased inhibitory serine-phosphorylation of brain GSK3 selectively in *Fmr1* knockout mice but not in wild-type mice. Inhibitory serine-phosphorylation of GSK3 was lower in *Fmr1* knockout, than wild-type, mouse brain regions and was increased by acute or chronic lithium treatment, which also increased hippocampal brain-derived neurotrophic factor levels. *Fmr1* knockout mice displayed alterations in open-field activity, elevated plus-maze, and passive avoidance, and these differences were ameliorated by chronic lithium treatment. These findings support the hypothesis that impaired inhibition of GSK3 contributes to the pathogenesis of FXS and support GSK3 as a potential therapeutic target.

1. Introduction

Fragile X syndrome (FXS), the most common cause of inherited developmental intellectual impairment, is caused by loss of expression of the *fragile X mental retardation-1* (*Fmr1*) gene [1] that encodes fragile X mental retardation protein (FMRP) [2]. *Fmr1* also is the first identified autism-related gene [3]. *Fmr1* knockout mice [4] display several FXS- and autism-relevant behavioral phenotypes, including hyperactivity [4], impaired fear-conditioned memory [5,6], and social behavior deficits [7–10]. Thus, *Fmr1* knockout mice provide an animal model to study FXS and autism deficits and to test potential therapeutics.

Studies of *Fmr1* knockout mice have identified two drugs that may be therapeutic for FXS, lithium and metabotropic glutamate receptor (mGluR) antagonists, such as MPEP (2-methyl-6-phenylethynyl-pyridine). Lithium treatment ameliorated behavioral deficits in *Drosophila* [11] and mouse [12] models of FXS. Lithium is particularly interesting because it already is used in humans as a mood stabilizer [13], likely due to its inhibition of glycogen synthase kinase-3 (GSK3) [14], suggesting that GSK3 also may be the therapeutic target of lithium in FXS. GSK3 comprises two isoforms GSK3α and GSK3β, is constitutively partially-active, phosphorylates >50 substrates, and has effects on many cellular processes [15–18]. GSK3 is mainly regulated by phosphorylation on an N-terminal serine, Ser-21-GSK3α and Ser-9-GSK3β, to inhibit GSK3 activity. This inhibitory serine-phosphorylation is widely used as an indicator of changes in GSK3 activity because, when phosphorylated, it has been shown to act as a pseudosubstrate that folds into the primed substrate binding pocket of GSK3 to block access of substrates and thereby block their phosphorylation by GSK3 [15,16,18]. Lithium directly inhibits GSK3 [19,20] and also increases the inhibitory serine-phosphorylation of GSK3 [21,22]. We recently reported that the inhibitory serine-phosphorylation of GSK3 is impaired in FVB/NJ *Fmr1* knockout mice [12].

mGluR signaling activity is increased in models of FXS [23]. Treatment of *Fmr1* knockout mice with mGluR antagonists corrected heightened audiogenic seizure susceptibility, abnormal center-field behavior [24], and impaired pre-pulse inhibition [25]. mGluRs can regulate GSK3, as stimulation of mGluR5 receptors transiently increased serine-phosphorylation of GSK3 in hippocampal slices from wild-type mice [26]. In brains of FVB/NJ *Fmr1* knockout mice, serine-phosphorylation of GSK3 in the brain was increased by administration of MPEP [12]. It is not known,

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however, if this is a sustained effect or if similar changes occur in wild-type mice—two points which are addressed in the current study.

In the present study, we first extended upon our preliminary findings in *Fmr1* knockout mice after acute MPEP or lithium treatment [12]. MPEP increased inhibitory serine-phosphorylation of both isoforms of GSK3 selectively in *Fmr1* knockout mice but not wild-type mice, a distinction not previously characterized. Acute lithium treatment significantly increased inhibitory serine-phosphorylation of both isoforms of GSK3, but to variable degrees among brain regions. Importantly, we also show that *in vivo* chronic, therapeutically relevant lithium treatment both rescued hyperactive GSK3 and several behavioral deficits exhibited by C57BL/6 *Fmr1* knockout mice. Taken together, the results support the hypothesis that impaired regulation of GSK3 contributes to impairments in FXS and that lithium may have therapeutic effects.

2. Materials and methods

2.1. Animals and in vivo treatments

Adult, male C57BL/6 mice, ~3 months of age, with or without a disruption of the *Fmr1* gene, were used in all experiments. The *Fmr1* knockout mice were generated by breeding male and female C57BL/6J *Fmr1* heterozygous mice to generate *Fmr1* knockout and wild-type littermates. For chronic lithium treatment, mice were given water and saline *ad libitum* and were fed pelleted chow containing 0.2% lithium carbonate (Teklad, Madison, WI) for three weeks. This is widely considered as a therapeutically relevant treatment regimen because it involves chronic administration, it produces serum lithium levels within the 0.5–1.2 mM serum lithium concentration range that is therapeutic in humans, it significantly increases inhibitory serine-phosphorylation of GSK3 in mouse brain, and it causes no deleterious effects on the physical state of mice except for polyuria that can cause hyponatremia, which is counterbalanced by self-administration of the saline solution that is provided [21,27–31]. For acute treatments, mice were given an intraperitoneal (ip) injection of 4 mmole/kg lithium chloride (Sigma), a dose that is effective in behavioral tests and increases serine-phosphorylation of GSK3 in mouse brain [12,31], or 30 mg/kg MPEP (2-methyl-6-phenylethynyl-pyridine; from the FRAXA Research Foundation) dissolved in saline, a dose previously reported to increase mouse brain serine-phosphorylated GSK3 and reduce audiogenic seizures in FVB *Fmr1* knockout mice [12,24]. All mice were housed and treated in accordance with the National Institutes of Health and the University of Alabama at Birmingham Institutional Animal Care and Use Committee guidelines.

2.2. Tissue preparation and analyses

Mice were decapitated and brains were rapidly removed and frozen. Brain regions were dissected on ice and 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 14,000 rpm for 10 min to remove insoluble debris. Protein concentrations in the supernatants were determined in triplicate using the Bradford protein assay. Extracts were mixed with Laemmli sample buffer (2% SDS) and placed in a boiling water bath for 5 min. Proteins were resolved in SDS-polyacrylamide gels, and transferred to nitrocellulose. Blots were probed with antibodies to phospho-Ser9-GSK3β, phospho-Ser21-GSK3α (Cell Signaling Technology, Beverly, MA), and total GSK3α/β (Millipore, Bedford, 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. Immunoblots were quantitated by densitometry. Values from *Fmr1* knockout mice are shown as the percents of densities of immunoblots of wild-type, control samples analyzed on the same gels and statistical significance was calculated using Student's *t*-test. To measure brain-derived neurotrophic factor (BDNF), hippocampal extracts were diluted 5-fold with Dulbecco's PBS and acidified to pH 2.6. After 15 min of incubation at room temperature, the supernatants were neutralized to pH 7.6 and frozen. The level of brain-derived neurotrophic factor was measured by ELISA according to the manufacturer's instructions (Promega Corporation, Madison, WI).

2.3. Behavior

Passive avoidance behavior was tested using a two-compartment box consisting of one darkened chamber and one lighted chamber with a grid floor of stainless steel bars connected to an internal shock source (Gemini II Avoidance System, San Diego Instruments, San Diego, CA). On the training day, each mouse was placed in the lighted chamber with the interchamber door closed. After 60 s the door was opened and when the mouse crossed to the darkened side the door was closed and a 0.5 mA × 0.5 s scrambled foot-shock was delivered. Mice were left in the dark chamber for 30 s before being returned to their home cage. For the test trial 24 h later, the protocol was repeated but without application of a foot-shock, with a cutoff time of 9 min. For both training and testing, the latency was measured as the time when all four paws entered into the darkened chamber.

For the elevated plus-maze, mice were placed on the central platform of a plexiglass maze that is one meter high with two open arms and two closed arms (Med Associates, St. Albans, VT). During a 5 min trial, entries and time spent in the open and closed arms were measured. The tail suspension test used an automated system consisting of an open-front testing chamber with a hanging vertical metal bar attached to a strain gauge that detects any movement (Med Associates). The mouse tail was attached to the apparatus with adhesive tape, movement was measured for 6 min, and the duration of immobility during the last 4 min of the test was recorded by the computer. Depression-like immobility behavior was calculated as the time the movement force was below a preset threshold. For the forced swim test each mouse was placed in a clear plexiglass cylinder (10 cm diameter), such that the mouse could not touch the bottom or rim of the cylinder, containing fresh water (23–25 °C) and outfitted with photo-beam detectors (Kinder Scientific). Activity was monitored by computer for 6 min and immobility time and basic movements were measured.

Locomotion and center-square behavior were measured in an open-field arena (43.2 cm × 43.2 cm × 30.5 cm) fitted with 16 evenly spaced I/R sources and sensors juxtaposed around the periphery of the four sides of the chamber (Med Associates, St Albans, VT). The outer walls were wrapped with white paper to limit external stimuli and light gradients, the room lights were dimmed, and a white noise generator was used (55 dB) during all tests. Each chamber was connected to a computer that recorded beam breaks (50 ms sampling rate). Three consecutive beam breaks represented an ambulatory episode (horizontal activity) and the total distance of all ambulatory episodes was recorded. Statistical significance was assessed by one factor ANOVA followed by Tukey–Kramer HSD test or by Student's *t*-test.

3. Results

3.1. The mGluR5 antagonist MPEP selectively increases serine-phosphorylation of GSK3 in Fmr1 knockout mouse brain

mGluR5 antagonists, such as MPEP, may have therapeutic effects in FXS [24,25,32,33] and we previously reported that serine-phosphorylated GSK3 was increased 30 min after administration of MPEP in FVB *Fmr1* knockout mouse brain [12]. This finding raised the important questions of whether this is an FXS-selective effect or if it also occurs in wild-type mice, and if the response in *Fmr1* knockout mice is strain specific. Therefore, we tested if MPEP administration increased serine-phosphorylation of GSK3 in brain regions of C57BL/6 *Fmr1* knockout and wild-type mice, and tested if this was a transient or long-lasting effect. Initial time course experiments indicated a short duration of action, leading to the choice of 30 and 90 min for the current study. The serine-phosphorylation of GSK3α and GSK3β was measured in four brain regions of *Fmr1* knockout and wild-type mice 30 and 90 min after MPEP (30 mg/kg) administration. In *Fmr1* knockout mice, 30 min after MPEP administration, there were significant increases in phospho-Ser21-GSK3α in the striatum (148 ± 9% of untreated values), hippocampus (130 ± 17%), cerebral cortex (154 ± 26%), and cerebellum (191 ± 14%) (Fig. 1A–D). Similarly, significant increases in phospho-Ser9-GSK3β occurred after 30 min of MPEP treatment in *Fmr1* knockout striatum (167 ± 18%), hippocampus (211 ± 41%), cerebral cortex (178 ± 36%), and cerebellum (174 ± 5%) (Fig. 1E–H). The striatum was the only brain region with a sustained increase in inhibitory phospho-Ser9-GSK3β 90 min after MPEP treatment. No changes in total levels of either GSK3α or GSK3β occurred (Fig. 1A–H). These findings extend our previous report [12] that MPEP rescues hyperactive GSK3 in FVB *Fmr1* knockout mice to C57BL/6 *Fmr1* knockout mice, and demonstrate that it is a transient inhibition of GSK3. Most importantly, in marked contrast to *Fmr1* knockout mice, MPEP administration did not increase serine-phosphorylation of GSK3α or GSK3β in wild-type mice in any brain region, but instead tended to decrease GSK3 serine-phosphorylation, particularly in the striatum and cerebellum (Fig. 2A–H). No changes in total levels of GSK3α or GSK3β were caused by MPEP treatment of wild-type mice (Fig. 2A–H). These results demonstrate that MPEP causes a *Fmr1* knockout-specific increase in the inhibitory serine-phosphorylation of GSK3 in the brain, which may be due to increased mGluR5 receptor signaling and decreased serine-phosphorylated GSK3 in these mice.

3.2. Acute lithium treatment increases inhibitory serine-phosphorylation of both GSK3α and GSK3β in Fmr1 knockout mice

Our previous results demonstrated that acute 30 min lithium treatment increased the phospho-Ser9-GSK3β in whole brain extracts of F1 hybrid C57BL/6J × FVB/NJ background *Fmr1* knockout mice [12]. Here we extended that study by assessing the pharmacokinetics and brain regional differences in the lithium-induced increase in the inhibitory serine-phosphorylation of both GSK3α and GSK3β in *Fmr1* knockout and wild-type mice using the same dose of 4 mmole/kg lithium that was effective in rescuing behavioral impairments in *Fmr1* knockout mice [12]. Phospho-Ser21-GSK3α and phospho-Ser9-GSK3β were measured in the striatum, hippocampus, cerebral cortex, and cerebellum of mice 30, 90 and 180 min after an acute injection of 4 mmole/kg lithium. Both *Fmr1* knockout mice (Fig. 3A) and wild-type (Fig. 3B) displayed significant increases over untreated levels of both phospho-Ser21-GSK3α and phospho-Ser9-GSK3β in all brain regions at 30 min. Thus, the mechanisms regulating inhibitory phosphorylation of GSK3 following lithium treatment are intact in *Fmr1* knockout mice, an important therapeutic consideration since this mechanism likely amplifies the direct inhibitory effect of lithium on GSK3 to generate therapeutic outcomes [22].

Regional differences in the response to lithium existed between both genotypes, for example the largest increase in phospho-Ser9-GSK3β was detected the hippocampus of both wild-type and *Fmr1* knockout mice. In *Fmr1* knockout mice, a sustained lithium-induced increase to 90 min was observed in the cerebellum for both phospho-Ser21-GSK3α and phospho-Ser9-GSK3β and in the cortex for phospho-Ser21-GSK3α, while in wild-type mice only the cerebellum exhibited a sustained increase in phospho-Ser21-GSK3α after acute lithium treatment. Taken together this data extends to several brain regions our previous finding that acute lithium administration can counteract the lower inhibitory serine-phosphorylation of GSK3 in *Fmr1* knockout mice.

3.3. Decreased inhibitory serine-phosphorylation of GSK3 is a robust phenotype of Fmr1 knockout mice

To further address the possibility of background strain effects, we tested if there were differences in the inhibitory serine-phosphorylation or total levels of GSK3α and GSK3β in four brain regions of *Fmr1* knockout compared to wild-type C57BL/6 mice. Both GSK3α (Fig. 4A–D) and GSK3β (Fig. 4I–L) were less phosphorylated on the inhibitory serines in the striatum, cerebral cortex, and hippocampus, but not in the cerebellum, in *Fmr1* knockout mice compared with wild-type mice. Total levels of each isoform of GSK3 were equivalent in wild-type and *Fmr1* knockout mouse brain regions (Fig. 4E–H and M–P), indicating that loss of FMRP did not alter the expression of GSK3 but impaired inhibitory signaling to GSK3. This extends to *Fmr1* knockout mice on a C57BL/6 background our previous finding of a similar deficit in the inhibitory control of GSK3 in *Fmr1* knockout mice on a FVB background [12]. However, more robust changes in inhibitory serine-phosphorylation of GSK3 were evident in C57BL/6 *Fmr1* knockout mice compared to our previous results on the FVB background. In particular, phospho-Ser9-GSK3β was significantly decreased in the hippocampus of C57BL/6 *Fmr1* knockout mice, while no change was detected in mice on the FVB background. Taken together, this data demonstrates that decreased serine-phosphorylation of GSK3 in brain regions is a robust phenotype associated with loss of FMRP, yet differences in certain brain regions exist between background strains.

3.4. Chronic lithium treatment rescues impaired inhibitory serine-phosphorylation of GSK3 in Fmr1 knockout mice

In order to model its use in humans, we tested if chronic lithium treatment reversed the impairment in the inhibitory serine-phosphorylation of GSK3 in *Fmr1* knockout mice. Lithium is a selective inhibitor of GSK3 [19,20,34] that increases GSK3 serine-phosphorylation *in vivo* [21], and lithium ameliorates some behavioral deficits associated with loss of FMRP [11,12,35]. Compared to untreated, wild-type mice, chronic lithium treatment caused significant increases in both wild-type and *Fmr1* knockout mice in phospho-Ser21-GSK3α in the striatum (Fig. 4A, WT + Li: 170 ± 13%; FX + Li: 209 ± 23%), hippocampus (Fig. 4B, WT + Li: 174 ± 17%; FX + Li: 185 ± 15%), cerebral cortex (Fig. 4C, WT + Li: 181 ± 24%; FX + Li: 291 ± 33%), and cerebellum (Fig. 4D, WT + Li: 191 ± 34%; FX + Li: 245 ± 28%). Phospho-Ser9-GSK3β also was significantly increased after chronic lithium treatment in wild-type and *Fmr1* knockout mice, compared to untreated, wild-type mice, in the striatum (Fig. 4I, WT + Li: 181 ± 16%; FX + Li: 141 ± 9%), hippocampus (Fig. 4J, WT + Li: 149 ± 9%; FX + Li: 167 ± 19%), cerebral cortex (Fig. 4K, WT + Li: 166 ± 18%; FX + Li: 226 ± 16%), and cerebellum (Fig. 4L, WT + Li: 129 ± 12%; FX + Li: 181 ± 16%). Total levels of each isoform of GSK3 were not changed in any brain region by chronic lithium

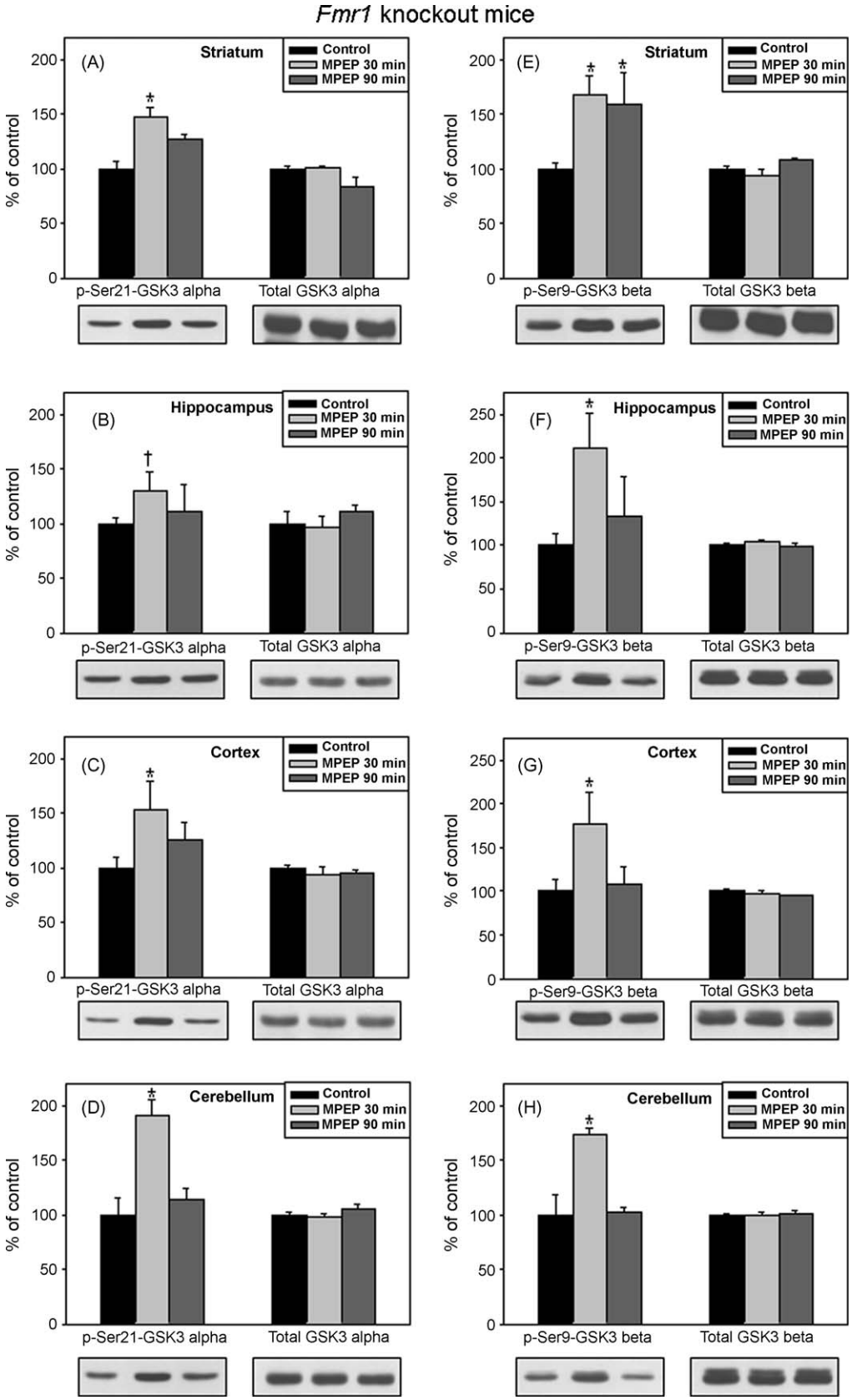


Fig. 1. Acute MPEP administration increases GSK3 serine-phosphorylation selectively in *Fmr1* knockout mice. *Fmr1* knockout mice were administered MPEP (30 mg/kg; ip) or vehicle 30 or 90 min prior to sacrifice. Extracts of striatum, hippocampus, cerebral cortex, and cerebellum were probed with antibodies to (A–D) phospho-Ser21-GSK3α or total GSK3α, and (E–H) phospho-Ser9-GSK3β or total GSK3β. Shown are representative immunoblots and quantitative values presented as the percent of values from untreated mice analyzed on the same gel. $n = 6$ mice for control and 30 min groups, and $n = 3$ for 90 min group; $^{*}p < 0.05$; $\dagger p < 0.06$ compared to untreated values.

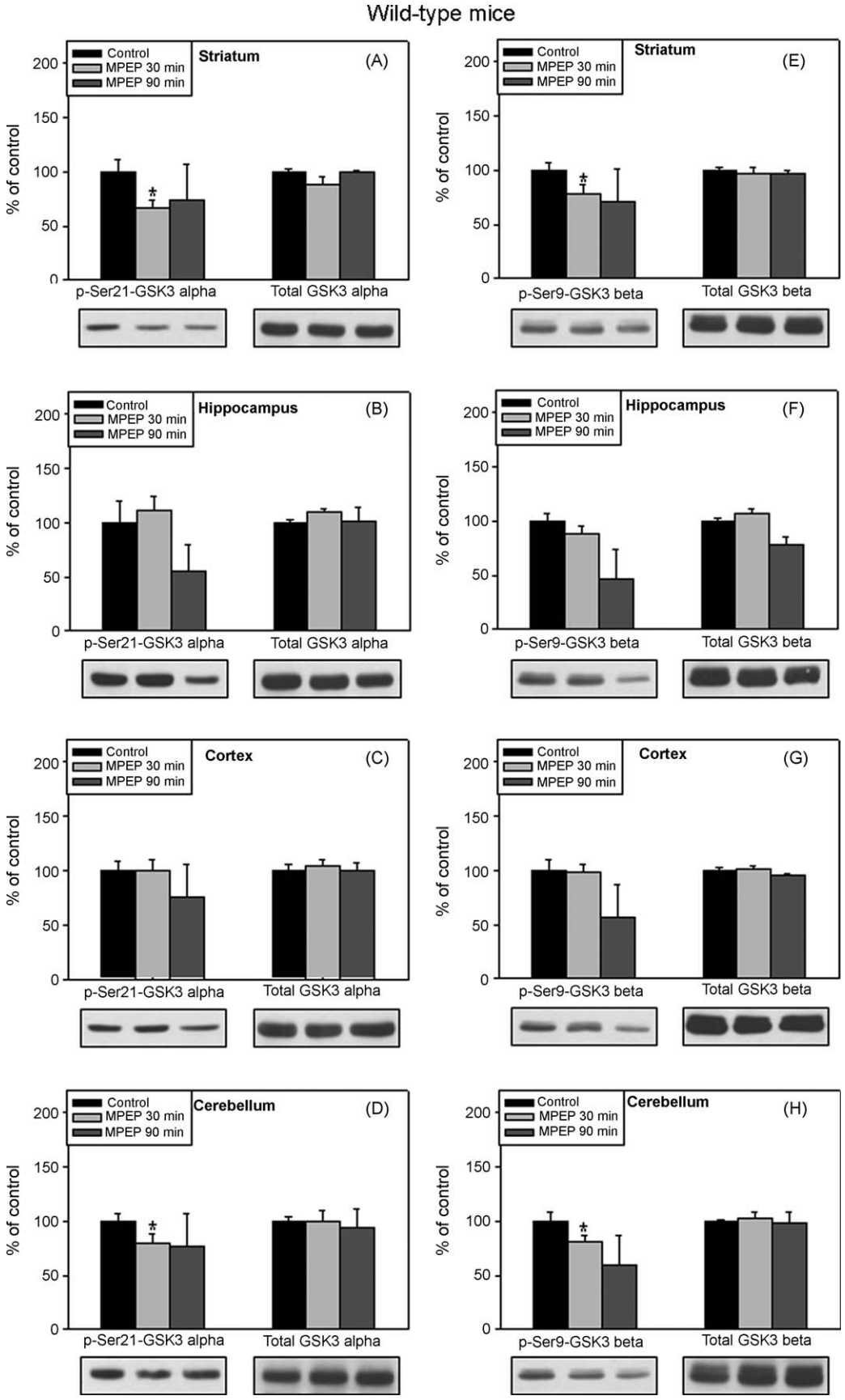


Fig. 2. Acute MPEP administration does not change GSK3 serine-phosphorylation in wild-type mice. Wild-type mice were administered MPEP (30 mg/kg; ip) or vehicle 30 or 90 min prior to sacrifice. Extracts of striatum, hippocampus, cerebral cortex, and cerebellum were probed with antibodies to (A–D) phospho-Ser21-GSK3α or total GSK3α, and (E–H,) phospho-Ser9-GSK3β or total GSK3β. Shown are representative immunoblots and quantitative values presented as the percent of values from untreated mice analyzed on the same gel. $n = 6$ mice for control and 30 min groups, and $n = 3$ for 90 min group; $^{*}p < 0.05$ compared to untreated values.

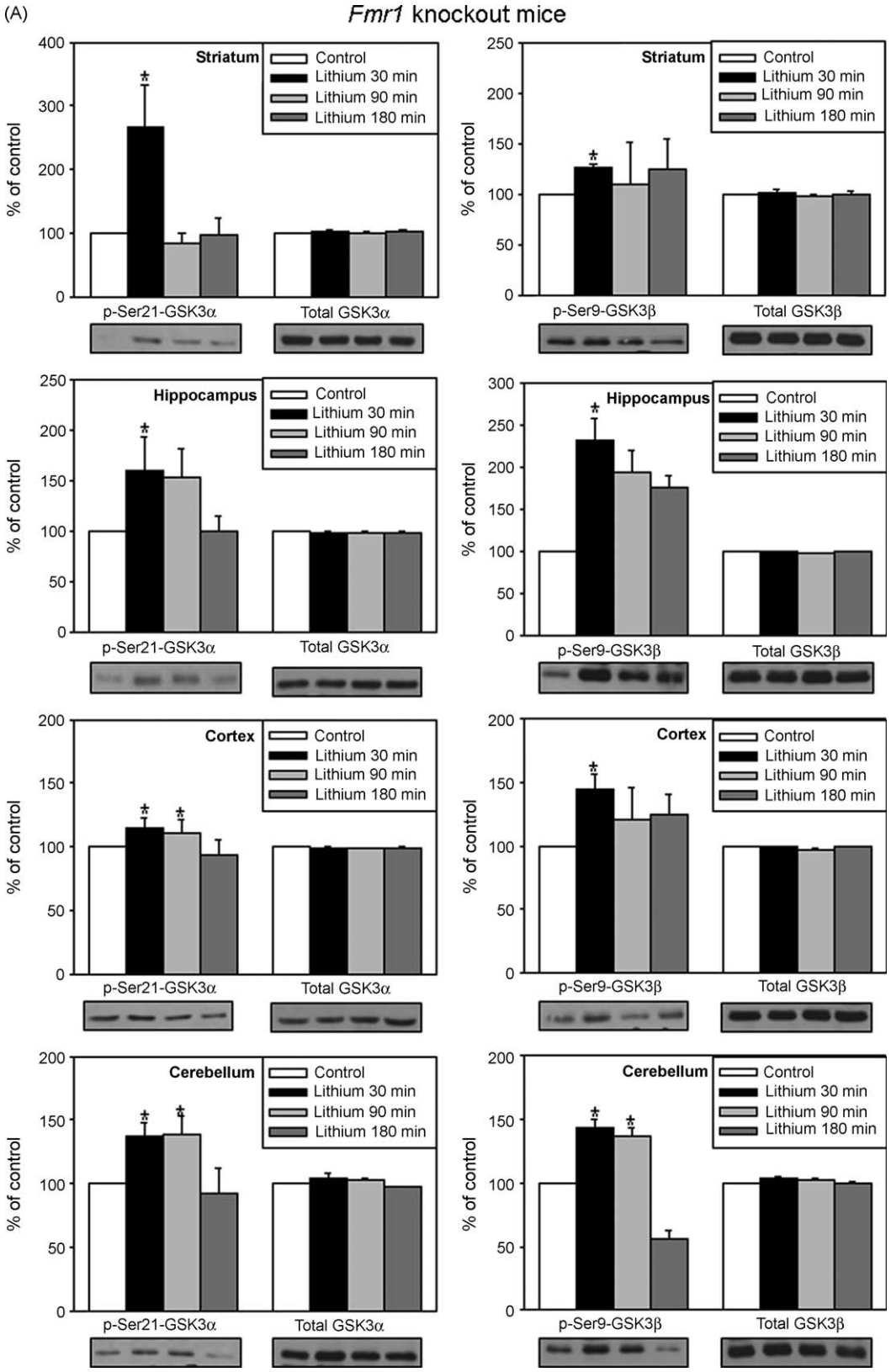


Fig. 3. GSK3 serine-phosphorylation modulated by acute lithium administration. (A) *Fmr1* knockout and (B) wild-type mice were administered lithium chloride (ip; 4 mmole/kg) in PBS for 30, 60, or 180 min. Extracts of striatum, hippocampus, cerebral cortex, and cerebellum were immunoblotted for phospho-Ser21-GSK3α, phospho-Ser9-GSK3β, total GSK3α, or total GSK3β. Shown are representative immunoblots and quantitative values presented as the percent of values from untreated mice analyzed on the same gel. $n = 4$ mice per group; $^{*}p < 0.05$ compared to untreated values.

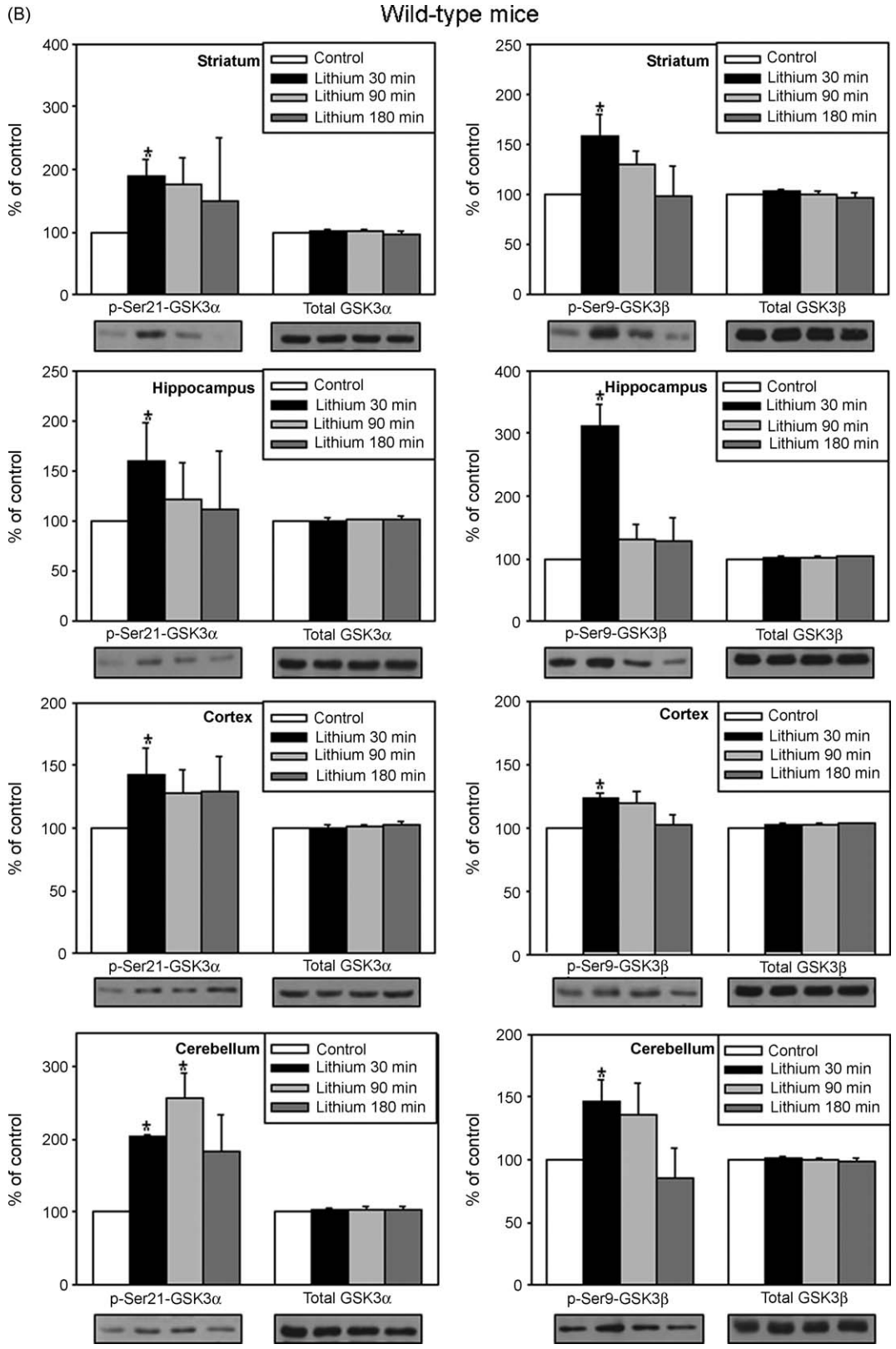


Fig. 3. (*Continued*).

treatment in wild-type or *Fmr1* knockout mice (Fig. 4E–H and M–P). These results extend our previous finding that an acute 30 min treatment with lithium increased phospho-Ser9-GSK3β in *Fmr1* knockout mouse brain [12], demonstrating that chronic lithium treatment provides a sustained rescue of deficient serine-phosphorylation of GSK3 in four brain regions of *Fmr1* knockout mice.

3.5. Chronic lithium treatment increases hippocampal BDNF

BDNF regulates learning and memory, synaptic plasticity, and activity levels in animal models [36]. Exogenous BDNF rescued plasticity associated with learning and memory in *Fmr1* knockout mice [37], and lithium has been reported to increase *in vivo* brain

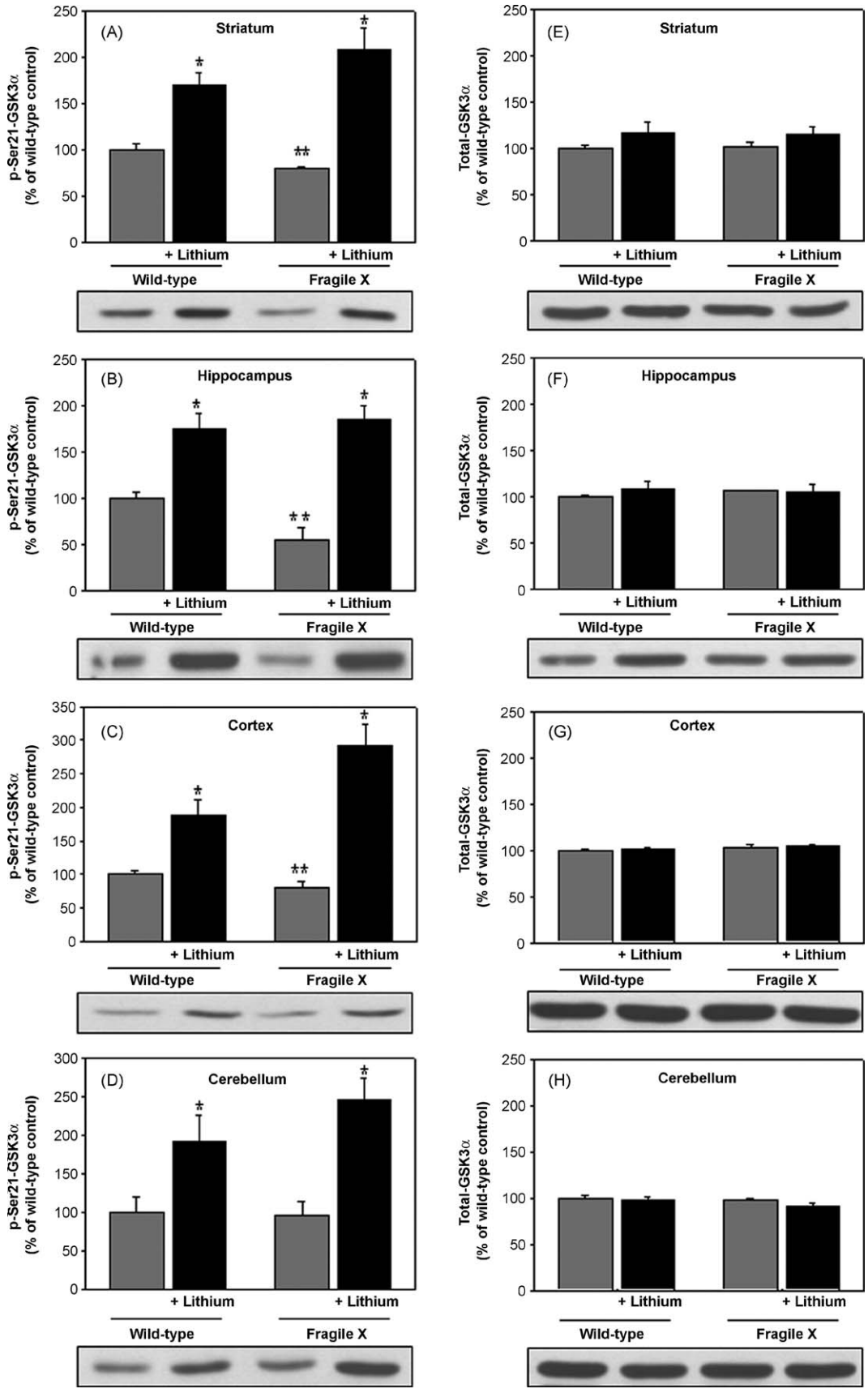
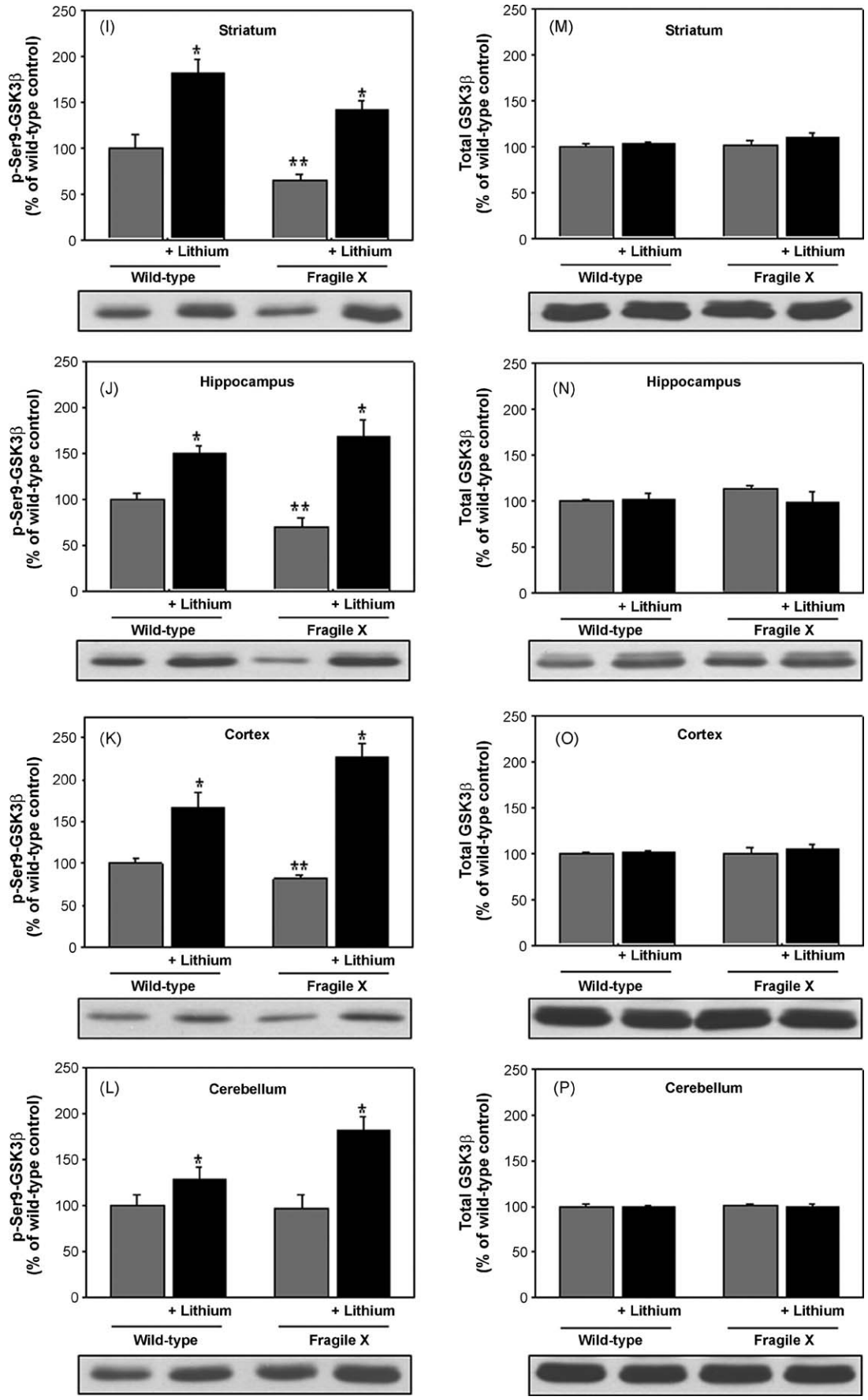


Fig. 4. Chronic lithium treatment rescues hyperactive GSK3 in *Fmr1* knockout mice. *Fmr1* knockout (Fragile X) and wild-type mice were treated with lithium for 3–4 weeks prior to sacrifice and compared to untreated littermates. Homogenates of the striatum, hippocampus, cerebral cortex, and cerebellum were probed with antibodies to (A–D) phospho-Ser21-GSK3α, (E–H) total GSK3α, (I–L) phospho-Ser9-GSK3β, (M-P) or total GSK3β. Immunoblots were quantified by densitometry and are presented as the percents of values from untreated wild-type mice. $n = 10$ mice per group; $^{**}p < 0.05$ comparing untreated Fragile X and wild-type values; $^{*}p < 0.05$ compared with matched sample without lithium treatment.



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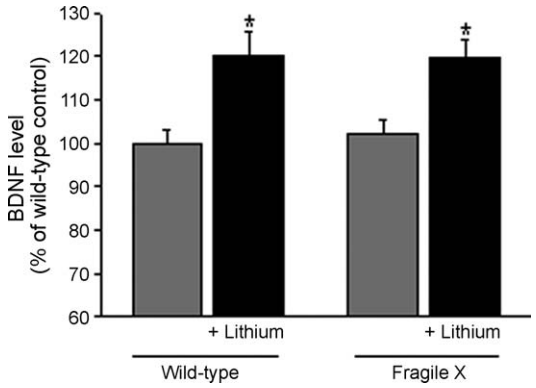

Fig. 5. Chronic lithium treatment increases hippocampal BDNF levels. *Fmr1* knockout and wild-type mice were treated with lithium for 3–4 weeks prior to sacrifice and compared to untreated littermates. BDNF levels were measured in hippocampal extracts by ELISA. Results are expressed as a percent of values in untreated wild-type controls; $n = 10$ mice per group; $^{*}p < 0.05$ compared with matched sample without lithium treatment.

levels of BDNF [38–40]. Therefore, we tested if lithium administration increased BDNF levels in *Fmr1* knockout mice. BDNF levels from hippocampal extracts of *Fmr1* knockout mice chronically treated with lithium (41 ± 6 pg/mg) were significantly higher (19%) than untreated *Fmr1* knockout mice (34 ± 4 pg/mg) (Fig. 5). Untreated wild-type mice had equivalent levels of BDNF (34 ± 4 pg/mg) as *Fmr1* knockout mice, and also had a significant increase in BDNF after chronic lithium treatment (40 ± 5 pg/mg). Thus, increases BDNF levels following chronic lithium administration may contribute to its therapeutic effects in *Fmr1* knockout mice.

3.6. Chronic lithium treatment rescues several FXS-specific behavioral phenotypes

Since GSK3 is hyperactive in *Fmr1* knockout mouse brain and this is reversed by chronic lithium treatment, we tested if lithium treatment rescued behavioral phenotypes in *Fmr1* knockout mice. A robust phenotype displayed by *Fmr1* knockout mice is hyperactivity [4,9,12,41,42], and we previously reported that acute administration of a GSK3 inhibitor, SB-216763, normalized hyperactivity of C57BL/6 *Fmr1* knockout mice [12]. Therefore, in the present study we addressed if chronic lithium treatment could rescue the hyperactivity of C57BL/6 *Fmr1* knockout mice. Throughout the 30 min measurement the *Fmr1* knockout mice traveled a greater distance than wild-type mice (Fig. 6A), which corresponded to a total distance of 8027 ± 899 cm traveled by the *Fmr1* knockout mice, a significant 71% increase compared with wild-type mice (4693 ± 391 cm) (Fig. 6B). This hyperactive behavior of *Fmr1* knockout mice was completely abolished by chronic lithium treatment, whereas lithium treatment only slightly reduced activity of wild-type mice. The current data importantly show that chronic lithium treatment specifically rescues the hyperactivity of *Fmr1* knockout mice without significantly reducing the activity of wild-type mice.

Fmr1 knockout mice also exhibit an increase in center-square behavior in the open-field [43]. Previously, we demonstrated that chronic lithium administration rescued the increased center-square behavior of *Fmr1* knockout mice on the FVB/NJ background [12]. In the present study, we also measured center-square behavior after chronic lithium treatment using mice on the C57BL/6 background. Center-square travel distance was greater in *Fmr1* knockout mice than wild-type mice (Fig. 6C and D). Chronic

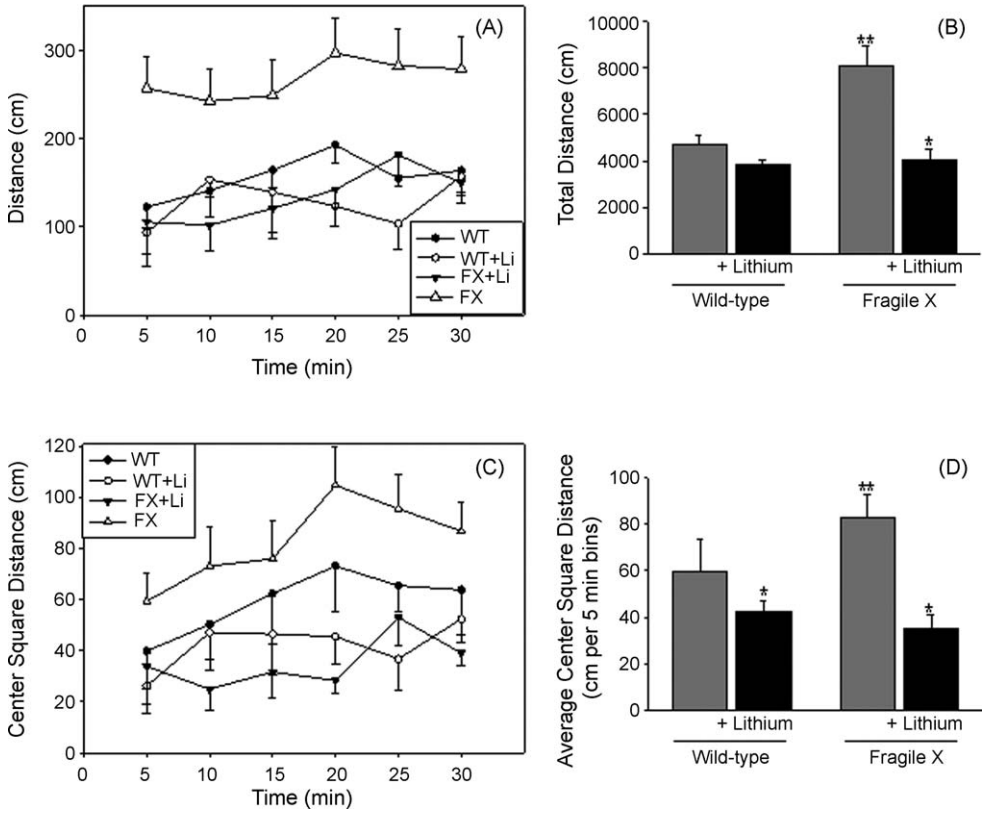

Fig. 6. Chronic lithium treatment rescues hyperactive behavior of *Fmr1* knockout mice. *Fmr1* knockout (FX) and wild-type (WT) mice were treated with lithium for 3–4 weeks prior to 30 min open-field testing of activity. (A) Distance traveled was analyzed in 5 min bins. (B) Total, cumulative distance traveled during the 30 min test. (C) Center-square behavior, defined as the central zone of the open-field, was measured as distance traveled in the central area in 5 min bins. (D) Average center-square distance per 5 min bin. $n = 5$ mice per group. $^{**}p < 0.05$ compared to untreated, wild-type values; $^{*}p < 0.05$ compared with matched sample without lithium treatment.

lithium treatment significantly reduced center-square behavior in both the wild-type and C57BL/6 *Fmr1* knockout mice. Taken together, the data suggests that chronic lithium treatment reduces the increased center-square behavior of *Fmr1* knockout mice, but lithium also reduces it in C57BL/6 wild-type mice, contrasting with results previously reported in FVB/NJ mice [12].

We tested if C57BL/6 *Fmr1* knockout mice displayed altered behavior in the elevated plus-maze, as previously was reported for FVB *Fmr1* knockout mice [5,7,44] and C57BL/6 *Fmr1* knockout mice [42,45]. This test measures the proclivity of mice to spend time in the two enclosed arms rather than the two open arms. *Fmr1* knockout mice spent significantly more total time (Fig. 7A) and

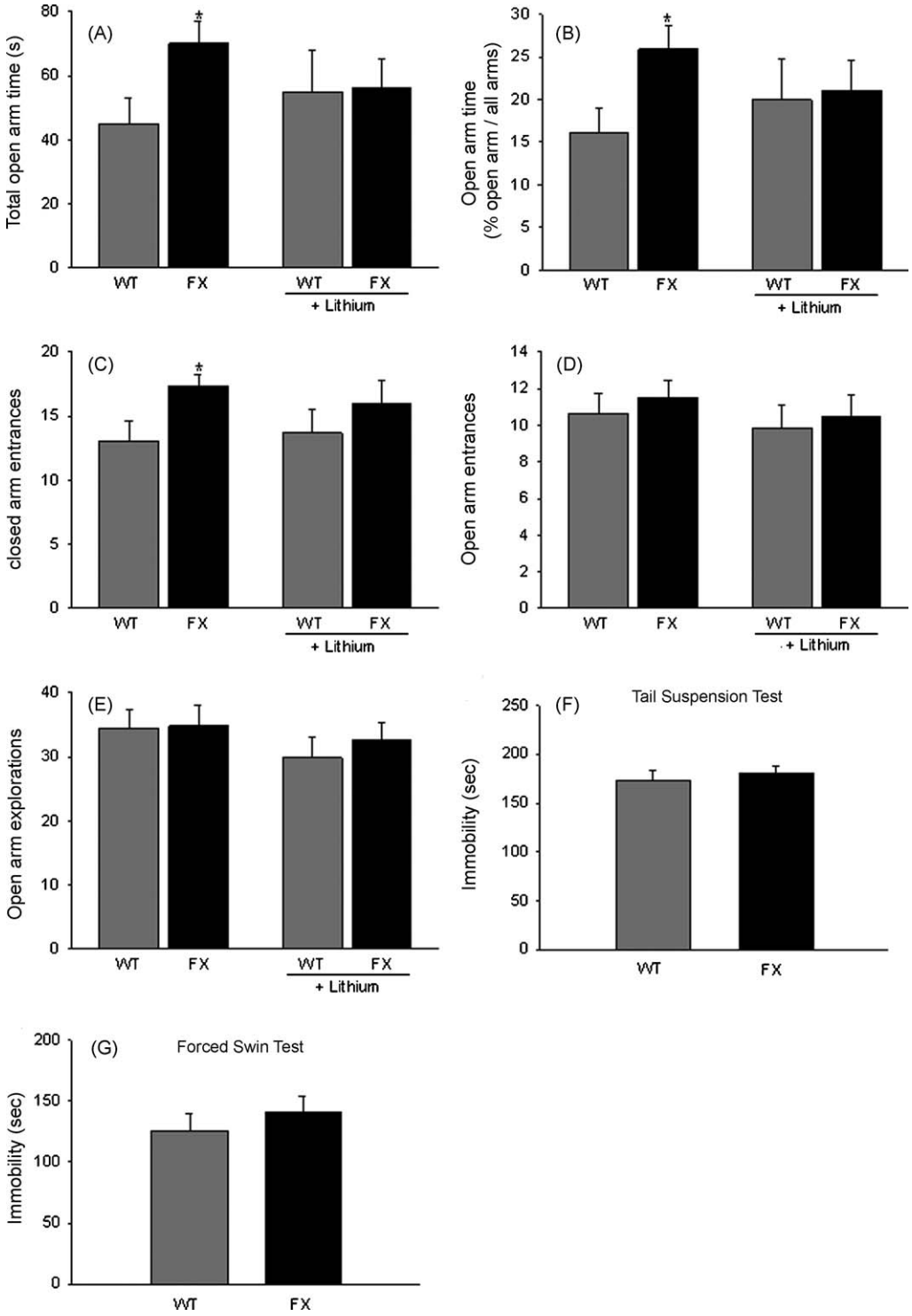

Fig. 7. Lithium partially rescues altered elevated plus-maze behavior of *Fmr1* knockout mice, and *Fmr1* knockout mice do not display depressive-like behaviors. *Fmr1* knockout (FX) and wild-type (WT) mice were treated with lithium for 3–4 weeks prior to testing. Behavior in the 5 min elevated plus-maze test was analyzed as (A) total time in the open arms, (B) percentage of time spent in the open arms compared with total time spent in all arms, (C) closed arm entries, (D) open arm entries, and (E) open arm explorations. (F) Immobility time was measured during the last 4 min of the 6 min tail suspension test. (G) Immobility time was measured by beam breaks in the forced swim test. $n = 10$ mice per group; $^{*}p < 0.05$ compared to untreated, wild-type values.

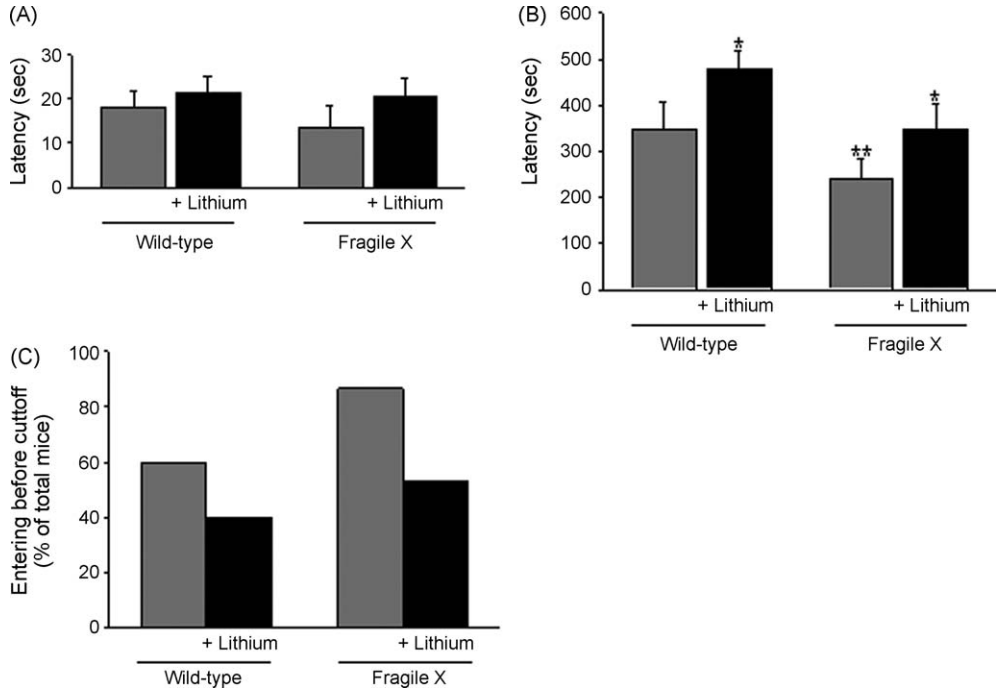

Fig. 8. Chronic lithium treatment rescues impaired passive avoidance behavior of *Fmr1* knockout mice. *Fmr1* knockout and wild-type mice were treated with lithium for 3–4 weeks prior to behavioral assessment. (A) On training day, the latency to enter the dark chamber was measured during a 60 s test. Mice remaining in the light chamber for 60 s were excluded from further analysis. (B) Latency to enter the dark chamber 24 h after training was measured, with a cutoff time of 9 min. (C) Percentage of mice in each group crossing into the dark chamber within the 9 min cutoff time. $n = 15$ mice per group; $^{**}p < 0.05$ compared to untreated, wild-type values; $^{*}p < 0.05$ compared with matched sample without lithium treatment.

higher percentage of time (Fig. 7B) on the open arms than did wild-type mice. Notably, this difference was partially ameliorated by lithium, as after chronic lithium treatment time spent on the open arm by *Fmr1* knockout mice was not significantly different from untreated wild-type or *Fmr1* knockout mice (WT 15 ± 3%; FX 26 ± 3%; WT + Li 14 ± 3%; FX + Li 21 ± 4%). Similar to the open-field, *Fmr1* knockout mice displayed hyperactive behavior in the elevated plus-maze as measured by closed arm entrances (Fig. 7C), which is the most reliable measure of activity in the elevated plus-maze [46]. However, the differences detected in open arm time between genotypes or treatments were not attributed to open arm entrances (Fig. 7D) or explorations (Fig. 7E), as no changes among these measures were observed. Taken together, these results indicate that lithium partially reduced the altered elevated plus-maze behavior of C57BL/6 *Fmr1* knockout mice.

We also examined the behaviors of *Fmr1* knockout and wild-type mice in two tests that measure depression-like immobility behavior in stressful conditions, the tail suspension test and the forced swim test [47]. In both the tail suspension test and the forced swim test there were no differences between *Fmr1* knockout and wild-type mice in immobility times (Fig. 7F and G, respectively), indicating that other behavioral changes were not due to a depressive-like phenotype in *Fmr1* knockout mice.

Based on previous findings that *Fmr1* knockout mice exhibit defects in passive avoidance behavior [5,7], we tested if this was ameliorated by chronic lithium administration. During the training session, untreated and lithium-treated wild-type and *Fmr1* knockout mice displayed the same latency to cross to the dark side of the chamber (Fig. 8A), whereupon they received a single foot-shock. Testing 24 h after conditioning revealed that *Fmr1* knockout mice exhibited a significant 30% decreased latency to cross to the foot-shock-paired side (Fig. 8B), and 87% of *Fmr1* knockout mice crossed into the dark chamber during the 9 min trial as opposed to 60% of wild-type mice (Fig. 8C). After chronic lithium treatment *Fmr1* knockout mice displayed a significant 44% increase in latency to enter the dark chamber (FX = 242 ± 41 s; FX + Li = 348 ± 53 s), and reduced the number of *Fmr1* knockout mice entering the chamber to 53% during the 9 min trial. Chronic lithium administration also increased the latency of wild-type mice to enter the dark chamber (WT = 346 ± 60 s; WT + Li = 481 ± 36 s), and only 27% crossed into the dark chamber during the trial. These results indicate that lithium ameliorates the passive avoidance deficit in *Fmr1* knockout mice and also enhances recall in the passive avoidance task in wild-type mice.

4. Discussion

Loss of FMRP expression in FXS causes mental retardation and autistic characteristics, but there are no known therapeutic treatments. Recent research raised the possibility that lithium may be therapeutically useful in FXS [11,12,35]. This is exciting because lithium is already used therapeutically as a mood stabilizer, likely due to its inhibition of GSK3 [48], so much is known about its pharmacokinetics, safety, and tolerability in humans [13]. Therefore, this study further investigated the potential involvement of GSK3 and the *in vivo* effects of lithium on *Fmr1* knockout mice phenotypes. The regulation of GSK3 by inhibitory serine-phosphorylation was impaired in the brains of C57BL/6 *Fmr1* knockout mice, supporting this as an underlying rationale for therapeutic application of lithium. Furthermore, administration of lithium or another potential therapy for FXS, MPEP, reversed the deficit in inhibitory serine-phosphorylation of GSK3 in *Fmr1* knockout mouse brains, and lithium ameliorated several behavioral deficits in C57BL/6 *Fmr1* knockout mice.

The therapeutic application of MPEP and other mGluR5 antagonists in FXS derives from the finding that mGluR5 receptor signaling is elevated in *Fmr1* knockout mice [23,49–51]. In the present study, we extended our previous findings [12] to show that MPEP administration to C57BL/6 *Fmr1* knockout mice caused a rapid increase in the serine-phosphorylation of GSK3 in the brain,

demonstrating that this action occurs in *Fmr1* knockout mice on both C57BL/6 and FVB backgrounds [12], although the response to MPEP was more robust in the C57BL/6 *Fmr1* knockout mice. Here we found that the MPEP effect on the serine-phosphorylation of GSK3 was rapid and transient, in accordance with the short in vivo half-life of MPEP [52]. Notably, the current study revealed for the first time that this effect of 30 mg/kg MPEP administration only occurred in *Fmr1* knockout mice, not wild-type mice, indicating a differential sensitivity to this dose of MPEP between the two groups of mice. Higher doses may be effective in wild-type mice, but a dose of 100 mg/kg MPEP was reported to cause sedation [24]. In contrast to the effect in *Fmr1* knockout mice, there was a tendency for MPEP to decrease serine-phosphorylation of GSK3 in the brains of wild-type mice, consistent with previous reports that activation of mGluR5 receptors inhibits GSK3 activity [26,53,54]. This difference between *Fmr1* knockout mice and wild-type mice suggests that the abnormally active mGluR5 signaling in *Fmr1* knockout mice leads to an aberrant activation of GSK3 that is blocked by antagonists of mGluR5. Interaction between mGluR5 and the postsynaptic scaffolding protein, Homer, are reduced in *Fmr1* knockout mice [55], which blocks mGluR5-stimulated Akt activation [56]. This blockade could reduce Akt-mediated serine-phosphorylation of GSK3 in *Fmr1* knockout mice, which could underlie decreased inhibitory serine-phosphorylation of GSK3 in *Fmr1* knockout mice. These results suggest that GSK3 may be an important component of the mGluR-linked impairments in FXS [32,49]. Since blocking mGluR5 receptors by MPEP administration increased serine-phosphorylation of GSK3 in several brain regions of *Fmr1* knockout mice, and directly increasing GSK3 serine-phosphorylation by lithium treatment ameliorated several behavioral abnormalities in these mice, this action of MPEP on GSK3 may contribute to its therapeutic effects in models of FXS.

Acute administration of lithium increased serine-phosphorylation of GSK3 in mouse brain, an indicator of lithium's inhibition of GSK3 [22]. Lithium inhibits GSK3 by a direct action [19,20], and this inhibitory effect is amplified by a subsequent increase in the inhibitory serine-phosphorylation of GSK3 [21]. Previous work showed that a 30 min lithium treatment increased phospho-Ser9-GSK3β in whole brain extracts from FVB *Fmr1* knockout mice [12]. In the current study, we assessed if acute lithium treatment increased inhibitory serine-phosphorylation of both isoforms of GSK3, GSK3α and GSK3β, tested the time course of this action, and tested if differences in brain regions were evident in the effect of lithium on GSK3 serine-phosphorylation. These experiments showed that acute lithium-induced increases both phospho-Ser21-GSK3α and phospho-Ser9-GSK3β, that these increases occurred to variable extents in different brain regions, and that the increases were similar in *Fmr1* knockout and wild-type mice. These results demonstrated that although serine-phosphorylation of GSK3 is deficient under basal conditions in *Fmr1* knockout mice, the mechanism mediating this indirect inhibitory phosphorylation of GSK3 induced by lithium is intact in *Fmr1* knockout mice.

We then determined if chronic lithium treatment, which models its therapeutic use in humans, also counteracts deficient serine-phosphorylation of GSK3 in *Fmr1* knockout mice. Chronic lithium treatment robustly increased serine-phosphorylation of both GSK3 isoforms in *Fmr1* knockout and wild-type mice in all brain regions examined. Specifically in *Fmr1* knockout mice, chronic lithium treatment was able to rescue the reduced serine-phosphorylated GSK3, also extending to *Fmr1* knockout mice on a C57BL/6 background our previous finding of impaired inhibitory serine-phosphorylation of GSK3 in the brains of FVB *Fmr1* knockout mice [12]. This is important because, like many animal models of diseases [57,58], inconsistent phenotypes occur among different strains of *Fmr1* knockout mice [59]. The reduced serine-phosphorylation of GSK3 in the brain was more robust with mice on the C57BL/6 background than the FVB/NJ background. In the C57BL/6 *Fmr1* knockout mice the striatum and hippocampus displayed the largest reductions in both GSK3α and GSK3β serine-phosphorylation, which may be linked to the high level of mGluR5 receptor expression in these regions [60]. As previously discussed [12], several targets of GSK3 are associated with abnormalities in FXS, supporting the hypothesis that loss of FMRP impairs inhibitory control of GSK3, which may contribute to some characteristics of FXS.

Although BDNF levels are not reduced in *Fmr1* knockout mice, BDNF rescued LTP deficits of *Fmr1* knockout mice, suggesting that increasing BDNF levels in FXS may be therapeutic [37]. Chronic lithium treatment increased BDNF levels in wild-type mice similar to previous reports [38–40,61], but importantly also in *Fmr1* knockout mice. This action has been ascribed to inhibition of GSK3 by lithium resulting in increased expression of BDNF [40]. These findings suggest that part of the therapeutic effect of inhibiting GSK3 with lithium in FXS may stem from increased BDNF.

To test if impaired inhibition of GSK3 contributes to FXS-linked phenotypic behaviors, we examined if lithium administration, using a therapeutically relevant regimen, ameliorated behavioral deficits in *Fmr1* knockout mice. Hyperactive behavior in the open-field is a robust behavioral phenotype of *Fmr1* knockout mice [59] and acute lithium treatment was reported to reduce this phenotypic behavior in FVB *Fmr1* knockout mice [12]. C57BL/6 *Fmr1* knockout mice also were found to be hyperactive and chronic lithium administration reduced activity to a level equivalent to wild-type mice. Importantly, lithium did not have a significant effect on the activity of wild-type mice in the open-field, demonstrating a FXS-specific reduction in hyperactive behavior by lithium. Taken in conjunction with a report that overexpression of constitutively active S9A-GSK3β caused open-field hyperactivity [62], these findings suggest that increased GSK3 activity contributes to the hyperactive behavior of *Fmr1* knockout mice, which would explain the FXS-specific rescue of hyperactivity by lithium. *Fmr1* knockout mice traveled a greater distance in the center-square of the apparatus than wild-type mice and this was rescued by chronic lithium treatment. Taken with the previous findings of these effects of lithium in FVB *Fmr1* knockout mice [12], this is clearly a robust phenotype that is normalized by lithium administration.

Mixed results have been reported in elevated plus-maze behavior of *Fmr1* knockout mice. FVB *Fmr1* knockout mice spent more time than wild-type mice on the open arm of the elevated plus-maze [44], but the opposite result was reported for 3 week old FVB *Fmr1* knockout mice [45], and other studies reported no differences between either FVB or C57BL/6 *Fmr1* knockout and wild-type mice [42,63]. In the present study, C57BL/6 *Fmr1* knockout mice spent more time on the open arm of the elevated plus-maze than wild-type mice. This difference was partially corrected by chronic lithium treatment, which reduced the time spent on the open arm by *Fmr1* knockout mice to an intermediate level between untreated *Fmr1* knockout and wild-type mice, but lithium treatment had no effect in wild-type mice.

Fmr1 knockout mice exhibited a decreased latency to enter the dark chamber in the passive avoidance test, as previously reported [5,6]. Experimental differences may explain the contrasting results from other groups [4,23]. The current results suggest that *Fmr1* knockout mice retain some ability to recall the shock that they received in the dark chamber because they displayed delayed entrance into the dark chamber. However, memory retrieval may not be as robust as in wild-type mice, which would explain the observed decreased latency of *Fmr1* knockout mice. This correlates with the subtle memory deficits of *Fmr1* knockout mice described in several other tests of memory [59]. Chronic lithium treatment rescued the passive avoidance deficit in *Fmr1* knockout mice,

suggesting a contribution of hyperactive GSK3 in the impairment. Chronic lithium treatment caused equivalent increases in the latency to cross to the dark chamber in both wild-type and *Fmr1* knockout mice. Chronic lithium treatment was previously reported to increase memory in passive avoidance behavior in wild-type rats [64]. This data support the hypothesis that GSK3 regulates passive avoidance behavior and that lithium has beneficial effects in passive avoidance behavior.

In summary, this study showed that impaired inhibitory serine-phosphorylation of GSK3 is a robust phenotype in *Fmr1* knockout mice, and that administration of lithium rescues some of the behavioral phenotypes of *Fmr1* knockout mice. This extends previous indications in flies [11], mice [12], and a preliminary trial in FXS patients [35] that lithium ameliorates some characteristics of FXS. Importantly, the involvement of GSK3 appears to be linked to the prevalent mGluR hypothesis of FXS [49], because the mGluR5 antagonist MPEP increased inhibitory phosphorylation of GSK3 in *Fmr1* knockout mice, but not in wild-type mice, suggesting that the increased mGluR signaling in *Fmr1* knockout mice contributes to the deficit in inhibitory control of GSK3. The capacity of lithium to reduce abnormal behaviors in *Fmr1* knockout mice strengthens the hypothesis that lithium may provide therapeutic benefits in FXS. Thus, as suggested previously, both lithium and mGluR antagonists may be therapeutically useful in the treatment of FXS [11,12,32,33,35].

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