

Lithium ameliorates phenotypic deficits in a mouse model of fragile X syndrome



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Abstract

As our understanding of the underlying defects in fragile X syndrome (FXS) increases so does the potential for development of treatments aimed at modulating the defects and ameliorating the constellation of symptoms seen in patients. Symptoms of FXS include cognitive disability, hyperactivity, autistic behaviour, seizures and learning deficits. Lithium is a drug used clinically to treat bipolar disorder, and it has been used to treat mood dysregulation in individuals with FXS. We examined whether dietary lithium would alter behavioural and morphological abnormalities in *fmr1* knockout (KO) mice. We studied wild-type (WT) and KO mice untreated (control chow) or treated with lithium (0.3% lithium-carbonate-containing chow) commenced at weaning and maintained throughout the experiment. At age 8–12 wk, mice were subjected to the following behavioural tests: open field, social interaction, elevated plus maze, elevated zero maze and passive avoidance. At 13 wk, brains were prepared for Golgi staining and analysis of dendritic spine morphology in medial prefrontal cortex. We found that compared to untreated WT, untreated KO mice were hyperactive and had reduced anxiety, impaired social interactions, and deficits on a learning test. Dendritic spines in medial prefrontal cortex were longer and increased in number. Lithium treatment ameliorated the hyperactivity and reversed impaired social interaction and deficits on the learning test. Lithium treatment also partially normalized general anxiety levels and dendritic spine morphology. Our findings and those from other laboratories on the efficacy of lithium treatment in animal models support further studies in patients with FXS.

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Introduction

Silencing of the *FMR1* gene and the consequent loss of its protein product, the fragile X mental retardation protein (FMRP), results in fragile X syndrome (FXS), the most common inherited form of intellectual disability (Hagerman, 2002). FXS is characterized by physical phenotypes such as distinct facial features and macroorchidism and behavioural features including hyperactivity, cognitive disability, learning deficits, autism, hyperarousal, and seizures (Hagerman, 2002). A distinctive neuroanatomical defect seen in FXS is abnormal dendritic spine morphology that has

been identified in autopsy specimens of FXS patients (Hinton *et al.* 1991).

Lithium is a well known mood stabilizer used clinically to treat bipolar disease. In rodents, lithium treatment alters many behavioural attributes such as aggression (Prasad & Sheard, 1982), depression (Bersudsky *et al.* 2007), amphetamine-induced hyperlocomotion (Gould *et al.* 2007) and reserpine-induced hypolocomotion (Borison *et al.* 1978). Chronic lithium also enhances learning (Nocjar *et al.* 2007) and spatial memory in rats (Tsaltas *et al.* 2007; Vasconcellos *et al.* 2003). Electrophysiologically, chronic lithium increases long-term potentiation (LTP) in hippocampal neurons (Shim *et al.* 2007) and alleviates stress-induced LTP impairment (Lim *et al.* 2005). Lithium also exerts neuroprotective effects (Rowe & Chuang, 2004). The effects of lithium are mediated by several known mechanisms such as down-regulation of the phospholipase C signalling pathway (Berridge

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et al. 1989), reduction of NMDA receptor-initiated signalling (Basselín *et al.* 2006) and inhibition of glycogen synthase kinase-3 (GSK-3) (Jope & Roh, 2006; Klein & Melton, 1996). The latter is considered by many to be the primary mediator of lithium's biological effects.

In a *Drosophila* model of FXS, lithium reverses learning deficits and improves viability (Chang *et al.* 2008; McBride *et al.* 2005). In the knockout (KO) mouse model, lithium reduces the incidence of audiogenic seizures and normalizes an elevation in GSK-3 β activity (Min *et al.* 2009). Clinically, lithium alone or in combination with antipsychotic drugs, has been used successfully to stabilize mood dysregulation and bipolar disorder in individuals with FXS (Al-Semaan *et al.* 1999). A recent pilot add-on trial demonstrated that lithium improves behaviour, adaptive skills, and verbal memory in patients with FXS (Berry-Kravis *et al.* 2008). These findings suggest that lithium may provide some functional benefits in FXS. We undertook a study of chronic lithium treatment in KO mice to systematically examine its effects on known fragile X phenotypes. We measured effects on behaviour, dendritic spine morphology, and GSK-3 β activity.

Methods

Animals and treatments

Generation of male KO and wild-type (WT) mice by FVB/129P-*fmr1*^{tm1Cgr}/J breeding pairs (heterozygous females and hemizygous males), genotyping by PCR amplification of tail DNA, and animal housing were as described previously (Qin *et al.* 2002). All procedures were performed in accordance with the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals and an animal study protocol approved by the National Institute of Mental Health Animal Care and Use Committee.

Four groups of mice (22–26 per group) were studied: (1) WT fed control diet (WT-C); (2) KO fed control diet (KO-C); (3) WT fed lithium-supplemented diet (WT-Li); (4) KO fed lithium-supplemented diet (KO-Li). The lithium-supplemented diet was NIH-31 to which 0.3% (w/w) lithium carbonate had been added (Harlan Teklad, USA). Feeding commenced at weaning (21 d) and was provided *ad libitum* for the duration of the study. Mice fed lithium-supplemented chow were given drinking water with 1.5% (w/v) sodium chloride to counteract potential toxicity of lithium.

From age 8–11 wk, mice were subjected to a battery of behavioural tests with 1-wk intervals between

tests: open field, social interaction, elevated plus maze (EPM) and passive avoidance. In a subset of animals, an elevated zero maze (EZM) was inserted between the EPM and passive avoidance test; accordingly, the interval for these tests was reduced to 3–4 d. All behavioural tests were performed between 10:00 and 15:00 hours in low light (60 lx). At age 12–13 wk, brains were processed for either biochemical analysis or Golgi staining; testicles were dissected and weighed. Blood was sampled at the time of decapitation and lithium concentrations were determined (Medtox Laboratories, USA).

Open field test

At age 8 wk, mice were subjected to open field testing for evaluation of locomotor activity and general anxiety. Activity was recorded at 5-min intervals for 30 min by means of a computer-operated tracking system (Coulbourn Instruments, USA). Total distance moved, distance moved in the margins (within 6.25 cm of walls), and number of entries into the centre (>6.25 cm from walls) were measured.

Social interaction test

At age 9 wk, mice were tested for social interaction behaviour in an automated three-chambered social approach apparatus (Nadler *et al.* 2004), following the procedure reported previously (Liu & Smith, 2009). Briefly, the test had three consecutive phases. (a) Habituation: With doorways open, the test mouse was placed in the centre chamber and allowed to freely explore for 5 min. The amount of time spent in each chamber was recorded. Mice that spent ≥ 3 min in any one chamber were eliminated. (b) Social approach: The test mouse was confined to the centre chamber with doors closed, and an unfamiliar mouse (stranger 1) was placed inside a wire cup in one of the side chambers. (Chambers 1 and 2 contained inverted wire cups placed in similar locations within each chamber; Fig. 2*a*.) The doors were opened, and the subject was allowed to explore freely for 5 min. In addition to the amount of time spent in each chamber, time spent sniffing the stranger mouse or the empty cup, and the entries into each chamber were scored. (c) Preference for social novelty: The subject was confined to the centre chamber with doors closed. With stranger 1 remaining in its original wire cup, a new unfamiliar mouse (stranger 2) was placed inside a cup in the opposite chamber, which had been empty during the social approach phase. Doors were reopened and the subject was allowed to explore for 5 min. Measures were taken as described above.

Elevated plus maze

At age 10 wk, mice were tested for general anxiety in an EPM as described previously (Liu & Smith, 2009). Briefly, the mouse was placed in the centre of the apparatus facing an open arm. The time spent in the closed and open arms and the numbers of entries into closed and open arms were recorded for 5 min. We defined an arm entry as the mouse having his head and forepaws in the arm.

Elevated zero maze

We tested 48 mice for general anxiety in the EZM (Med Associates Inc., USA) as described previously (Liu & Smith, 2009). Briefly, mice were placed in the centre of a closed quadrant and allowed to explore freely for 5 min; behaviour was recorded by video camera. Time spent and number of head dips in and number of entries into each quadrant were evaluated. Mice that fell off the maze were not included.

Passive avoidance

At age 11 wk, mice were subjected to the passive avoidance test as previously described (Qin *et al.* 2002). Briefly, mice underwent one trial training and one test session 24 h later. On training day the mouse was placed in the lighted chamber of a two-chambered apparatus (Coulbourn Instruments, USA). After 10 s the door to the dark chamber was raised. Once the mouse entered the dark chamber, the door automatically closed and an electric shock (0.3 mA for 1 s) was administered. The mouse was removed from the apparatus after 10 s, returned to its home cage, and 24 h later latency to enter the dark compartment of the apparatus was recorded up to 300 s.

Golgi staining and morphological analysis of dendritic spines

At age 12 wk, 24 mice ($n=6$ per group) were perfused with saline and brains were subjected to Golgi-staining (Rapid GolgiStain™ kit, FD NeuroTechnologies, USA). Coronal sections 100 μm in thickness were prepared. Pyramidal neurons (seven each for apical and basal dendrites) in layer III of medial prefrontal cortex (MPC) (infralimbic and prelimbic) were selected for quantification. Dendritic branches directly originating from cell soma were classified as primary dendrites, and those originating from primary dendrites were classified as secondary dendrites. In this study, primary basal and secondary apical dendrites originating from the apical trunk located 25–50 μm from the soma were selected for analysis. Spine length and density

were analysed in 50- μm segments starting 25 μm from the origin of a branch. Only one segment from each neuron was analysed.

Western blotting

At age 12 wk, 24 mice ($n=6$ per group) were decapitated, brains were removed quickly and homogenized in 3% (w/v) ice-cold T-PER tissue protein extraction reagent with 1% Halt protease inhibitor cocktail (Thermo Scientific, USA) and 1% phosphatase inhibitor cocktails (Sigma-Aldrich, USA). Homogenates were centrifuged (12 000 g, 4 °C, 15 min) and supernatant fractions were used for Western blot analyses. Protein concentrations were determined by BCA protein assay kit. Two equivalent protein samples (20 μg) were subjected to electrophoresis on 10% NuPAGE Bis-Tris gels. Proteins were transferred electrophoretically to nitrocellulose membranes, and membranes were incubated with either GSK-3 β rabbit monoclonal antibody (1:20 000, Cell Signaling Technology, USA) or phosphorylated GSK-3 β (Ser9) rabbit monoclonal antibody (1:2000, Cell Signaling Technology). β -Actin rabbit polyclonal antibody (1:2000, Cell Signaling Technology) was used as a loading control. Specific reactions were detected by the Western Breeze Chemiluminescent kit (Invitrogen, USA).

Statistical analyses

Data were expressed as mean \pm s.e.m. Open field and social interaction results were analysed by repeated-measures (rm)-ANOVA. All other results were analysed by two-way ANOVA with genotype and treatment as factors. Statistically significant interactions were further probed with *post-hoc* Bonferroni *t* tests. Spine-length distributions were compared by two-way Kruskal–Wallis test followed by Kolmogorov–Smirnov tests. The criterion for statistical significance was $p < 0.05$.

Results

Body weight

We compared body weights in a subset of animals at age 11 wk (Supplementary Fig. S1, available online). The genotype \times treatment interaction, was not statistically significant, but main effects of both genotype ($F_{1,29}=14.79$, $p < 0.001$) and treatment ($F_{1,29}=6.88$, $p < 0.05$) were. Body weights of KO mice were 9% higher than WT, and weights of lithium-treated mice were about 6% higher than control mice. The

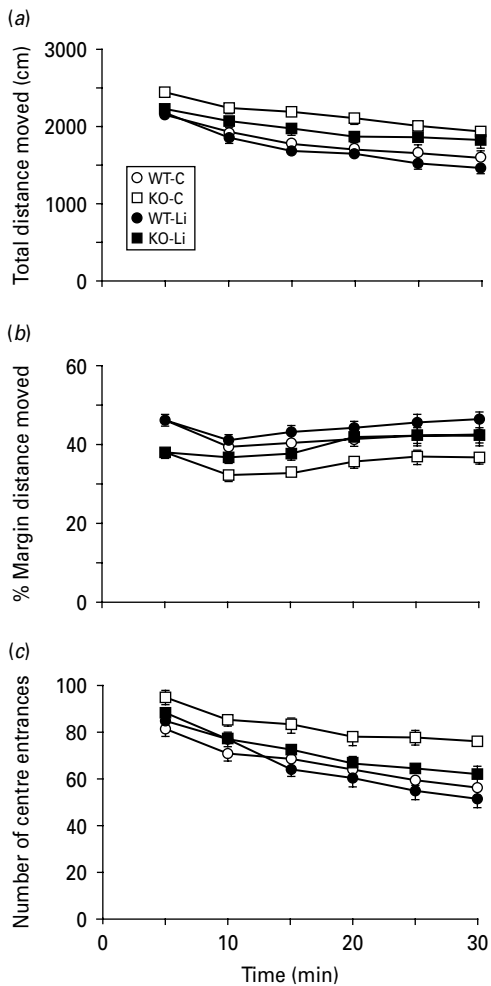


Fig. 1. Open-field activity in WT-C ($n=23$), KO-C ($n=22$), WT-Li ($n=26$), and KO-Li ($n=22$). Each point represents the mean \pm S.E.M. for each 5-min epoch. Data were analysed by means of rm-ANOVA with genotype (WT, KO), treatment (untreated, lithium-treated) and epoch as factors and repeated measures on epoch. (a) Total distance moved in the horizontal plane. The epoch \times genotype \times treatment ($F_{4,351}=1.52$, n.s.) and epoch \times treatment ($F_{4,351}=0.30$, n.s.) interactions were not statistically significant, but epoch \times genotype interaction ($F_{4,351}=2.67$, $p\leq 0.05$) was statistically significant. The genotype \times treatment interaction ($F_{1,89}=0.81$, n.s.) was not statistically significant, but main effects of both genotype ($F_{1,89}=25.60$, $p\leq 0.001$) and treatment ($F_{1,89}=5.17$, $p<0.05$) were. (b) Percent distance moved in the margins of the field. The epoch \times genotype \times treatment ($F_{4,383}=0.171$, n.s.), epoch \times treatment ($F_{4,383}=1.56$, n.s.), epoch \times genotype ($F_{4,383}=1.19$, n.s.), and genotype \times treatment ($F_{1,89}=0.67$, n.s.) interactions were not statistically significant, but main effects of both genotype ($F_{1,89}=21.55$, $p\leq 0.001$) and treatment ($F_{1,89}=7.87$, $p\leq 0.01$) were statistically significant. (c) Number of entries into the centre of the field. The epoch \times genotype \times treatment ($F_{5,433}=0.373$, n.s.) and

concentrations of lithium in whole blood from WT and KO mice were 0.38 ± 0.03 ($n=4$) and 0.38 ± 0.02 mequiv/l ($n=4$), respectively. No toxicity of the lithium treatment was observed.

Testes weight

Testes were dissected from mice aged 12–13 wk and weighed. The genotype \times treatment interaction was not statistically significant. The testes weight of KO mice was significantly higher than WT ($F_{1,90}=186.1$, $p<0.001$), but the effect of lithium treatment ($F_{1,90}=3.87$, $p=0.052$) was small (<5% decrease) (Supplementary Fig. S2, available online).

Open field activity

In all four groups the total distance travelled per epoch gradually decreased during the 30-min session (Fig. 1a). Data were analysed by rm-ANOVA with genotype, treatment and epoch as factors and repeated measures on epoch. The three-way interaction was not statistically significant, and neither were the epoch \times treatment and genotype \times treatment interactions, but the epoch \times genotype interaction ($F_{4,351}=2.67$, $p<0.05$) and the main effects of all three factors were statistically significant. Overall, the time \times distance curves of KO mice were higher than those of WT mice ($p<0.01$ all epochs) indicating hyperactivity of KO mice. Regardless of genotype and epoch, lithium treatment decreased locomotor activity of mice ($p<0.05$). The curve of KO-Li mice was below the curve of KO-C mice, suggesting partial reversal of hyperactivity in KO mice by lithium treatment.

As an indicator of the level of general anxiety, we analysed the distance travelled in the margins of the field as a percent of the total distance moved (Fig. 1b). There were no statistically significant interactions, but we did find statistically significant main effects of treatment ($F_{1,89}=7.81$, $p<0.01$), genotype ($F_{1,89}=21.5$, $p<0.001$) and epoch ($F_{5,445}=7.29$, $p<0.001$). KO mice travelled less in the margins than WT mice regardless of treatment, suggesting that KO mice have less anxiety than WT mice. Regardless of genotype, mice with lithium treatment moved more in the margins than control mice, implying that lithium treatment increased anxiety levels.

epoch \times genotype ($F_{5,433}=1.90$, n.s.) interactions were not statistically significant, but epoch \times treatment interaction ($F_{5,433}=2.68$, $p\leq 0.05$) was statistically significant. The genotype \times treatment interaction ($F_{1,89}=4.23$, $p\leq 0.05$) was also statistically significant.

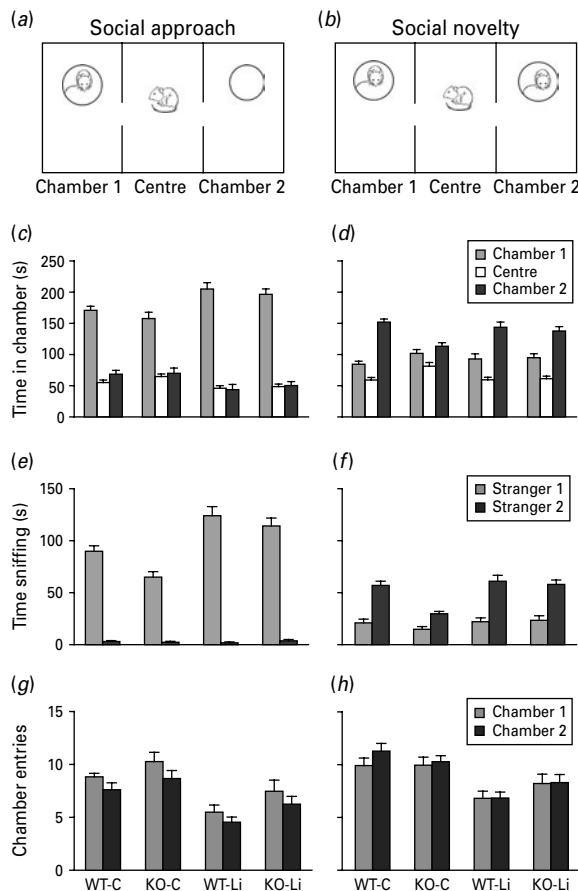


Fig. 2. Behaviour of WT-C ($n=21$), KO-C ($n=22$), WT-Li ($n=25$), and KO-Li ($n=23$) mice on a test of social interaction. Bars (mean \pm S.E.M.) represent the time spent in each chamber (*c, d*), the time spent sniffing the wire cage in each chamber (*e, f*), or the number of chamber entries (*g, h*). Results were analysed by rm-ANOVA with genotype (WT, KO), treatment (untreated, lithium-treated), condition (social approach, social novelty), and chamber (chamber 1, chamber 2) as factors and repeated measures on condition and chamber. For the time spent in a chamber the chamber \times condition \times genotype \times treatment interaction was not statistically significant ($F_{1,87}=1.78$, n.s.), but the chamber \times condition \times treatment ($F_{1,87}=12.02$, $p \leq 0.001$) and chamber \times condition \times genotype ($F_{1,87}=5.63$, $P \leq 0.05$) interactions were both statistically significant. The genotype \times treatment interaction ($F_{1,87}=3.12$, $p=0.08$) approached statistical significance and there were statistically significant main effects of both genotype ($F_{1,87}=6.76$, $p \leq 0.05$) and treatment ($F_{1,87}=9.20$, $p \leq 0.01$). For the time spent sniffing, the chamber \times condition \times genotype \times treatment interaction ($F_{1,87}=2.47$, n.s.) did not reach statistical significance, but chamber \times condition \times treatment ($F_{1,87}=32.41$, $p \leq 0.001$) and chamber \times condition \times genotype ($F_{1,87}=10.92$, $p \leq 0.001$) interactions were both statistically significant. The genotype \times treatment interaction ($F_{1,87}=6.25$, $p \leq 0.05$) was also statistically

Another indicator of the level of general anxiety is the number of entries into the centre of the field (Fig. 1c). The three-way interaction was not statistically significant, and neither was the epoch \times genotype interaction, but the epoch \times treatment interaction ($F_{5,433}=2.68$, $p < 0.05$) was, indicating that the shape of the curve was influenced by lithium treatment but not by genotype. There was also a statistically significant genotype \times treatment interaction ($F_{1,89}=4.23$, $p < 0.05$). *Post-hoc* tests revealed that the total number of centre entries was higher in KO-C than WT-C mice ($p < 0.001$), suggesting less anxiety in KO mice. Compared to KO-C, KO-Li mice entered the centre less ($p < 0.01$) and behaved more like WT mice, implying alleviation of this behavioural deficit.

Social interaction

During the habituation phase, two WT-C and one WT-Li mice remained in one side chamber for more than 3 min and were eliminated from further study.

The social approach phase began with the introduction of a novel mouse (stranger 1) under the cup in chamber 1 (Fig. 2a). The social novelty phase began with the introduction of a second novel mouse (stranger 2) under the cup in chamber 2 with stranger 1 remaining in its original wire cup (Fig. 2b). The times spent in the two chambers (Fig. 2c, d) were analysed by rm-ANOVA with chamber, condition, genotype and treatment as factors with repeated measures on chamber and condition. Although there was no statistically significant four-way interaction, chamber \times condition \times treatment ($F_{1,87}=12.02$, $p < 0.001$) and chamber \times condition \times genotype ($F_{1,87}=5.63$, $p < 0.05$) interactions were both statistically significant. *Post-hoc* analyses indicated that during the social approach phase, mice of all groups spent more time in chamber 1 than chamber 2 (empty chamber) ($p < 0.001$), and the preference for chamber 1 was enhanced by lithium treatment in both genotypes ($p < 0.001$). During the social novelty phase all mice showed a switch in

significant. For the number of chamber entries, the chamber \times condition \times genotype \times treatment interaction was not statistically significant ($F_{1,87}=0.37$, n.s.). The only statistically significant interaction was the condition \times chamber ($F_{1,87}=24.94$, $p \leq 0.001$). The main effect of treatment ($p < 0.001$) was statistically significant. Regardless of genotype or treatment the number of entries into both chambers (chamber 1, $p < 0.05$; chamber 2, $p < 0.001$) was higher in social novelty *vs.* social approach, and in social approach entries into chamber 1 were higher than entries into chamber 2 ($p < 0.001$).

preference for chamber 2 ($p < 0.01$). Compared to WT, KO mice spent significantly less time in chamber 2 ($p < 0.01$), and KO-C mice spent about the same amount of time in chamber 2 as in chamber 1. Analysis of the between-subjects effects indicates that the genotype \times treatment interaction approached statistical significance ($F_{1,87} = 3.12$, $p = 0.081$). Main effects of both genotype ($F_{1,87} = 6.75$, $p < 0.05$) and treatment ($F_{1,87} = 9.2$, $p < 0.01$) were statistically significant. These results show clear effects of both genotype and treatment, and a tendency for the effect of treatment with lithium to be greater in KO mice.

The measure of time spent sniffing the wire cups in chambers 1 and 2 (Fig. 2*e, f*) is considered to be an index of more direct social interest (Nadler *et al.* 2004). There was no statistically significant four-way interaction, but chamber \times condition \times treatment ($F_{1,87} = 32.4$, $p < 0.001$) and chamber \times condition \times genotype ($F_{1,87} = 10.9$, $p < 0.001$) interactions were both statistically significant. Further analysis revealed that during the social approach phase, mice of all groups spent more time sniffing stranger 1 than the empty cup in chamber 2 ($p < 0.001$). KO mice spent significantly less time sniffing stranger 1 than WT mice ($p < 0.01$), and, in both genotypes, lithium treatment increased the time spent sniffing stranger 1 ($p < 0.001$). During the social novelty phase, all animals showed a clear switch in preference for sniffing stranger 2 ($p < 0.001$). Compared to WT, KO mice spent significantly less time sniffing stranger 2 ($p < 0.001$), while there was no difference between these two genotypes for time sniffing stranger 1. In KO mice, lithium treatment appeared to normalize sniffing behaviour. The treatment \times genotype interaction was statistically significant ($F_{1,87} = 6.25$, $p < 0.05$). *Post-hoc* tests revealed a statistically significant difference in sniffing time between WT-C and KO-C mice regardless of chamber ($p < 0.001$). Further, sniffing time in both WT and KO mice was increased by lithium treatment ($p < 0.01$).

The number of entries into each chamber (Fig. 2*g, h*) can be a measure of activity as well as social interest. There were no statistically significant four-way or three-way interactions, but the chamber \times condition ($F_{1,87} = 24.94$, $p < 0.001$) interaction was statistically significant. *Post-hoc* tests indicated that both genotypes regardless of treatment made more entries into chamber 1 *vs.* chamber 2 during social approach ($p < 0.001$) and into both chamber 1 ($p < 0.05$) and chamber 2 ($p < 0.001$) during social novelty *vs.* social approach. There was also a statistically significant main effect of treatment ($F_{1,87} = 24.1$, $p < 0.001$) indicating that overall numbers of entries into both chambers decreased with lithium treatment.

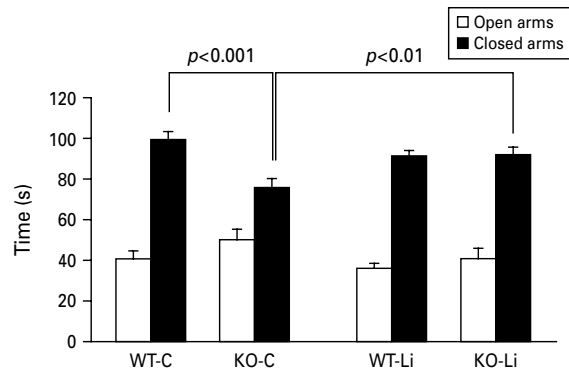


Fig. 3. Behaviour of WT-C ($n = 23$), KO-C ($n = 22$), WT-Li ($n = 26$), and KO-Li ($n = 23$) mice on the elevated plus maze (EPM). Bars indicate mean \pm S.E.M. Time spent in open or closed arms in the EPM were analysed by rm-ANOVA with genotype (WT, KO), treatment (untreated, lithium-treated), and arm (open, closed) as factors and repeated measures on arm. The arm \times genotype \times treatment interaction ($F_{1,90} = 4.72$, $p \leq 0.05$) was statistically significant. *Post-hoc* Bonferroni *t* tests were used to compare WT and KO mice as indicated in the figure.

Overall our results indicate that KO-C mice exhibit deficits in social interaction which are corrected by lithium treatment. Lithium also appeared to influence some aspects of behaviour in WT mice.

Elevated plus maze

To evaluate general anxiety, times spent in open and closed arms were analysed by rm-ANOVA with genotype, treatment, and arm as factors and repeated measures on arm (Fig. 3). We found a statistically significant arm \times genotype \times treatment interaction ($F_{1,90} = 4.72$, $p < 0.05$). Differences among groups in time spent in the open arms were not statistically significant by *post-hoc* tests but time spent in the closed arm was statistically significantly lower in KO-C than WT-C mice ($p < 0.001$), indicating lower anxiety in KO mice. This difference was ameliorated by lithium treatment ($p < 0.01$). The lack of effect on time spent in the open arms may be a function of the ambiguity of the open centre which was not counted as part of the open arm but was nevertheless open.

Elevated zero maze

In a subset of mice we also measured general anxiety by the EZM (Fig. 4). One WT-Li mouse was eliminated from further analysis after it fell off the platform. Time spent (Fig. 4*a*) and head dips (Fig. 4*b*) in the open quadrants were analysed by two-way ANOVA. For

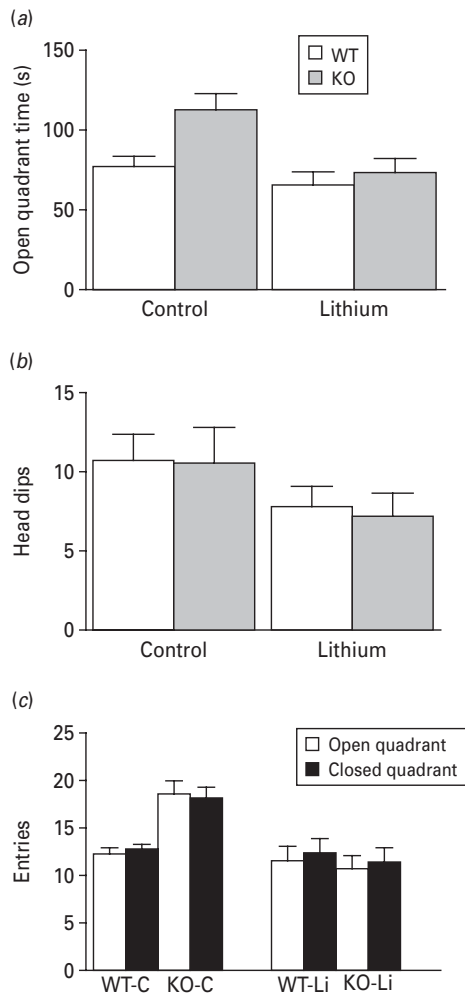


Fig. 4. Behaviour of WT-C ($n=14$), KO-C ($n=11$), WT-Li ($n=13$), and KO-Li ($n=12$) mice on the elevated zero maze. Bars indicate mean \pm S.E.M. Results of time spent in open quadrants and number of head dips were analysed by means of two-way ANOVA with genotype (WT, KO) and treatment (untreated, lithium-treated) as factors. The numbers of entries into open and closed quadrants were analysed by means of rm-ANOVA with genotype, treatment, and quadrant (open, closed) as factors with repeated measures on quadrant. (a) Time spent in the open quadrants. The genotype \times treatment ($F_{1,46}=2.80$, $p=0.101$) interaction approached statistical significance, and main effects of both genotype ($F_{1,46}=6.93$, $p\leq 0.05$) and treatment ($F_{1,46}=9.14$, $p\leq 0.01$) were statistically significant. (b) Number of head dips. Neither the genotype \times treatment interaction ($F_{1,46}=0.32$, n.s.) nor the main effect of genotype ($F_{1,46}=0.59$, n.s.) and treatment ($F_{1,46}=1.44$, n.s.) were statistically significant. (c) Number of entries into open and closed quadrants. Both genotype \times treatment \times quadrant ($F_{1,46}=2.38$, $p=0.130$) and genotype \times quadrant ($F_{1,46}=2.60$, $p=0.114$) interactions approached statistical significance, and the treatment \times quadrant interaction ($F_{1,46}=3.70$, $p\leq 0.01$) was statistically significant.

time spent in the open quadrants the genotype \times treatment interaction was not statistically significant, but main effects of genotype ($F_{1,46}=6.93$, $p<0.05$) and treatment ($F_{1,46}=9.17$, $p<0.01$) were both statistically significant. KO mice spent more time in the open quadrants than WT mice, indicating reduced anxiety in KO mice and lithium treatment decreased the time spent in open quadrants suggesting a normalizing effect of lithium on anxiety. For the number of head dips (Fig. 4b) neither the interaction nor the main effects achieved statistical significance. We also recorded the number of entries into the open and closed quadrants of the zero maze (Fig. 4c). These data were analysed with rm-ANOVA with genotype, treatment and quadrant as factors and repeated measures on quadrant. The genotype \times treatment \times quadrant, and quadrant \times genotype interactions were not statistically significant, but the quadrant \times treatment ($F_{1,46}=7.61$, $p<0.01$) and genotype \times treatment ($F_{1,46}=7.68$, $p<0.01$) interactions were. *Post-hoc* tests showed that regardless of quadrant, the total number of entries was higher in KO-C than WT-C mice ($p<0.001$), reflecting the hyperactivity of KO mice. Lithium treatment significantly decreased the activity of KO mice ($p<0.001$).

Passive avoidance

In the results of the passive avoidance test the genotype \times treatment interaction did not reach statistical significance, but main effects of both genotype ($F_{1,60}=5.29$, $p<0.05$) and treatment ($F_{1,60}=4.76$, $p<0.05$) were statistically significant. The latency to enter the dark compartment was lower in KO mice, and latency was increased by lithium treatment (Fig. 5).

Dendritic spine morphology

Dendritic spines on apical and basal (Fig. 6a) dendrites of layer III pyramidal neurons in MPC were analysed. Cumulative frequency distributions of spine length showed that spines on both apical (Fig. 6b) and basal (Fig. 6c) dendrites were longer in KO-C than WT-C mice, and lithium treatment normalized the length of spines in KO mice. Two-way Kruskal–Wallis tests showed statistically significant treatment \times genotype interactions for both apical ($H=10.6$, $p<0.01$) and basal ($H=18.6$, $p<0.001$) dendrites. Pairs of cumulative frequencies were further probed by Kolmogorov–Smirnov tests; results indicate that for apical dendrites there were statistically significant differences between WT-C and KO-C ($p<0.001$), WT-C and WT-Li ($p<0.05$), and KO-C and KO-Li ($p<0.001$) mice. For basal dendrites there were statistically significant

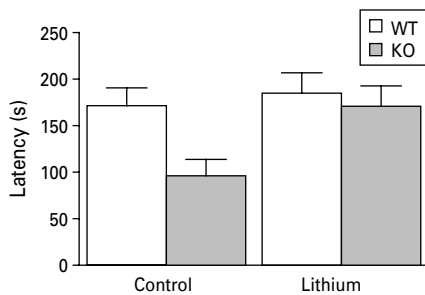


Fig. 5. Behaviour of WT-C ($n=23$), KO-C ($n=22$), WT-Li ($n=26$), and KO-Li ($n=23$) mice on the passive avoidance test. Bars represent the mean \pm S.E.M. latency to enter the dark chamber 24 h after a single training session in which mice received a foot-shock (0.3 mA for 1 s) upon entering the dark chamber. Results were analysed by a two-way ANOVA with genotype (WT, KO) and treatment (untreated, lithium-treated) as factors. The genotype \times treatment interaction was not quite statistically significant ($F_{1,90}=2.38$, $p=0.126$), but main effects of both genotype ($F_{1,90}=5.11$, $p\leq 0.05$) and treatment ($F_{1,90}=4.96$, $p\leq 0.05$) were statistically significant.

differences between WT-C and KO-C ($p<0.001$) and KO-C and KO-Li ($p<0.001$) mice, but differences between WT-C and WT-Li mice were not statistically significant.

We also analysed the density of spines along both apical and basal dendrites. For apical dendrites (Fig. 6d), there was no statistically significant interaction, but main effects of genotype ($F_{1,164}=5.64$, $p<0.05$) and treatment ($F_{1,164}=7.69$, $p<0.01$) were both statistically significant. Overall, the density of apical spines was higher in KO mice and was reduced by lithium treatment. However, in basal dendrites (Fig. 6e), only the main effect of genotype was statistically significant ($F_{1,164}=14.9$, $p<0.001$). Spine density on basal dendrites was higher in KO mice, but this effect was not significantly influenced by lithium.

Activity of GSK-3 β

The levels of total and Ser9-phosphorylated GSK-3 β in whole brain were studied (Fig. 7a) and results were expressed as percent of WT-C. For phosphorylated GSK-3 β (Fig. 7b) a statistically significant genotype \times treatment interaction ($F_{1,20}=5.83$, $p<0.05$) was found. *Post-hoc* tests revealed that the level of phosphorylated GSK-3 β of KO mice was markedly increased following lithium treatment ($p<0.01$), suggesting that the up-regulated GSK-3 β activity is reduced by lithium. The levels of total GSK-3 β were similar in all four groups (Fig. 7c).

Discussion

The ameliorative effects of chronic lithium treatment on neuropathology and a wide range of behavioural measures in the *fmr1* KO mouse indicate that lithium is a promising candidate drug for treatment of FXS. Lithium treatment initiated at the time of weaning reversed behavioural phenotypes of KO mice including hyperactivity, reduced anxiety, reduced social interaction, and a deficit in a test of learning/memory. Lithium treatment also mitigated abnormal spine length and density in MPC. One of the novel findings of our study is the wide range of effects of lithium treatment suggesting that lithium treatment may affect underlying chemical pathology.

For most of the measures studied, effects of lithium treatment were selective for KO mice, but in some cases lithium effects were seen in both genotypes. With respect to effects on body and testes weights and spine density on apical dendrites in MPC there were clear effects of lithium treatment in both WT and KO mice. In the cases of open field behaviour (horizontal distance and % distance moved in the margins) effects appeared to be greater in the KO *vs.* WT mice, but the three-way interactions were not statistically significant.

Animals in our study were male, closely matched for age, group-housed ($n=3-4$ per cage), and studied at about the same time of day. We controlled for genetic drift by using a breeding scheme that produces WT and KO littermates. FVB/129P-*fmr1*^{tm1Cgr}/J mice are prone to retinal degeneration which occurs at about age 21 d, so it is probable that at the time of study our mice had reduced visual acuity. It is unlikely that poor visual acuity would have affected the results of this study because both WT and KO mice were similarly affected. Moreover, non-visual sensory cues such as olfaction and somatosensory input are probably more important in mice.

We used robust behavioural phenotypes to assess the effects of lithium. Hyperactivity in the open field has been observed in KO mice on several different genetic backgrounds (Bakker *et al.* 1994; de Diego-Otero *et al.* 2009; Nielsen *et al.* 2002; Peier *et al.* 2000; Qin *et al.* 2002, 2005b; Spencer *et al.* 2005). In the present study chronic lithium treatment partially normalized hyperactivity in KO mice. Activity was also assessed in the social interaction and EZM tests as the number of chamber and quadrant entries, respectively. By design these two tests produce increased arousal and are less neutral than the open field test. In the EZM, KO-C mice made more entries into both open and closed quadrants than WT-C mice and this

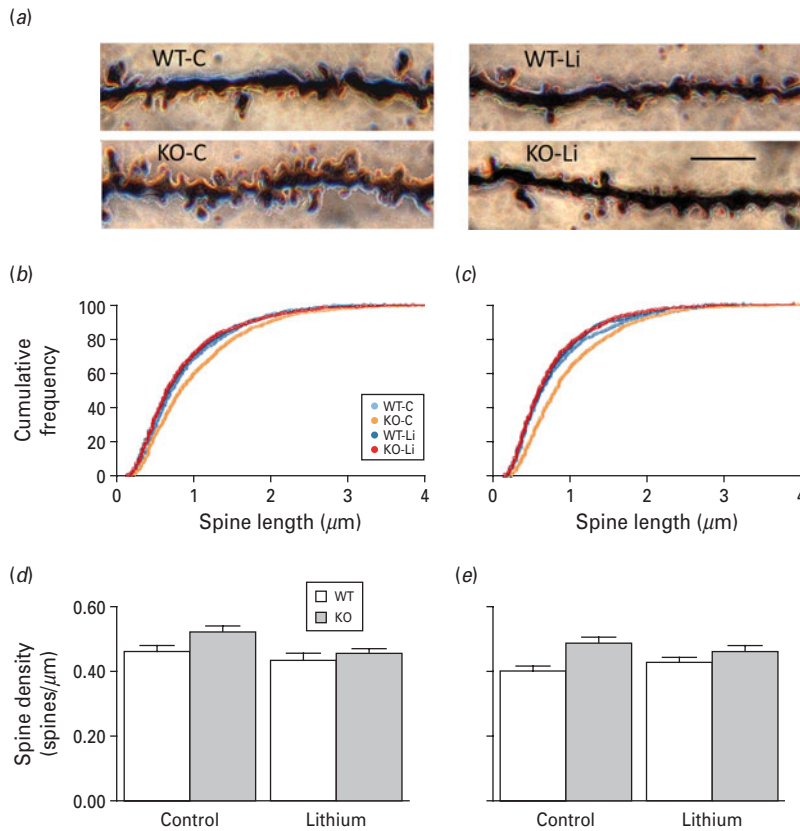


Fig. 6. Dendritic spines on apical and basal dendrites of layer III pyramidal neurons in medial prefrontal cortex. (a) Golgi-stained sections of spines on basal dendrites. Scale bar represents 5 μm . Cumulative frequency distributions of dendritic spine lengths on apical (b) and basal (c) dendrites. Statistical analysis of all four cumulative frequency distributions of spine length on both apical and basal dendrites indicate statistically significant differences for both apical and basal dendrites (two-way Kruskal–Wallis test; apical: $H = 10.6$, $p \leq 0.01$; basal: $H = 18.6$, $p \leq 0.001$). Pairs of cumulative frequencies were further probed by means of Kolmogorov–Smirnov tests; results indicate that for apical dendrites there were statistically significant differences between WT-C and KO-C ($p \leq 0.001$), WT-C and WT-Li ($p \leq 0.05$), and KO-C and KO-Li ($p \leq 0.001$) mice. For basal dendrites there were statistically significant differences between WT-C and KO-C ($p \leq 0.001$) and KO-C and KO-Li ($p \leq 0.001$) mice. The density of spines along both apical (d) and basal (e) dendrites were analysed by means of two-way ANOVA with genotype (WT, KO) and treatment (untreated, lithium-treated) as factors. Values are the mean \pm S.E.M. of 42 dendrites per group. For apical dendrites, the genotype \times treatment interaction ($F_{1,164} = 1.33$, n.s.) was not statistically significant, but main effects of genotype ($F_{1,164} = 5.64$, $p \leq 0.05$) and treatment ($F_{1,164} = 7.69$, $p \leq 0.01$) were both statistically significant. In basal dendrites the genotype \times treatment interaction ($F_{1,164} = 2.73$, $p = 0.10$) was not quite statistically significant. The main effect of treatment ($F_{1,164} = 0.01$, n.s.) was not statistically significant, but the main effect of genotype was ($F_{1,164} = 14.94$, $p \leq 0.001$).

‘hyperactivity’ appeared to be normalized with lithium treatment. In the social interaction test both WT and KO mice regardless of treatment made more entries into both chambers during social novelty *vs.* social approach. The latter demonstrates the highly activating nature of the social novelty condition. Because the effect was seen in all four groups it is unlikely to be a reflection of some attribute of the KO mice.

Reduced anxiety is another generally agreed upon phenotype (Bakker *et al.* 1994; de Diego-Otero *et al.*

2009; Liu & Smith, 2009; Peier *et al.* 2000; Qin *et al.* 2002, 2005b; Qin & Smith, 2008; Yuskaitis *et al.* 2010). We evaluated generalized anxiety by means of three different tests: (1) behaviour in the open field, (2) EPM, and (3) EZM. The EZM is a modification of the EPM; it removes any ambiguity in interpretation of time spent in the central square, and it allows uninterrupted exploration. By the criteria of all three tests, KO mice appeared to be less anxious than WT mice. In open field (centre entries) and EPM the generalized anxiety levels were normalized in KO mice by lithium

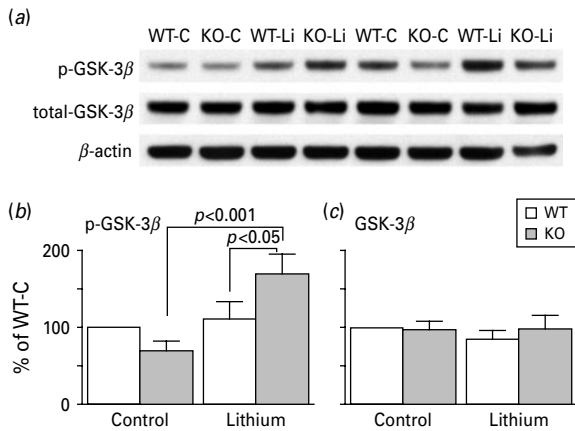


Fig 7. Western blot analysis of phosphorylated-GSK-3 β (Ser9) and total GSK-3 β levels in extracts prepared from whole mouse brain. Representative Western blots (a) and summary data from six mice per group for p-GSK-3 β (b) and total GSK-3 β (c). Optical densities were normalized to β -actin and values were expressed as percent of WT-C. The genotype \times treatment interaction ($F_{1,20}=5.83$, $p \leq 0.05$) was statistically significant for p-GSK-3 β levels, and results of *post-hoc* Bonferroni *t* tests are shown in the figure. For total GSK-3 β levels the genotype \times treatment interaction ($F_{1,20}=0.42$, n.s.) was not statistically significant and neither were the main effects of genotype ($F_{1,20}=0.24$, n.s.) nor treatment ($F_{1,20}=0.39$, n.s.).

treatment. Results of the EZM were not quite as clear cut probably due to the smaller number of mice studied. Hyperactivity of KO-C mice also may have been a factor. However, despite a higher number of open and closed quadrant entries, KO-C mice spent on average 38% of the time in the open quadrants while WT-C mice spent 26%. Hyperactivity of KO mice was quelled by lithium treatment and open quadrant time was reduced to an average of 25% *vs.* 22% in WT-Li mice. The effect of lithium treatment on general anxiety in WT mice was small compared to its effect in KO mice.

Social anxiety is a major component of autism and a frequent symptom found in FXS (Hagerman, 2002). We used a three-chambered apparatus designed specifically to assess social behaviour in mice (Nadler *et al.* 2004). Quantification of social interaction is in terms of time spent with a novel mouse and preference for a novel *vs.* familiar mouse. We confirm here our previously reported finding that social approach and response to social novelty are both diminished in KO mice (Liu & Smith, 2009). Reduced levels of social approach are in accord with some studies (Mineur

et al. 2006), but differ from others reporting normal or increased interest of KO mice in an unfamiliar partner (McNaughton *et al.* 2008; Spencer *et al.* 2005). Differences in age of the animals tested, characteristics of stranger mice used (see Supplementary text, available online), time of day of tests, and housing conditions probably explain the disparate results. We found that reduced social interaction is a robust phenotype in KO mice, and is reversed by chronic lithium treatment. Results of time sniffing were stronger than results of time in chamber, probably due to the fact that sniffing is a more direct index of social interest in mice. Mice are primarily olfactory animals and as such use olfaction to learn about objects and other animals in their environment. Thus, it is possible that the increased exploration of the novel social object may be involved in an effort to learn. The present novel finding suggests that lithium treatment, in addition to ameliorating hyperactivity and normalizing general anxiety levels, may also address autistic features of FXS.

Cognitive impairment and low intelligence quotients are characteristic of patients with FXS, but measures of these phenotypes have been difficult to demonstrate in the mouse model (Bernardet & Crusio, 2006). Tests of spatial learning and memory such as the Morris water maze have not shown remarkable effects in KO mice (Bakker *et al.* 1994; Peier *et al.* 2000; Yan *et al.* 2004). We, and others, have found that the passive avoidance test may be a good marker of learning/memory impairment, albeit non-spatial learning, in KO mice (Qin *et al.* 2002, 2005b; Yuskaitis *et al.* 2010). This is an aversively motivated task that likely involves the dorsal striatum (White & McDonald, 2002). In some aspects the passive avoidance test is similar to the lever press escape/avoidance task which is also impaired in KO mice (Brennan *et al.* 2006). In the present study, performance of both genotypes improved with lithium treatment but on average the improvement was much greater in KO mice (78% *vs.* 8% increase in latency in KO and WT mice, respectively). Whereas the genotype \times treatment interaction did not reach statistical significance the magnitude of the difference in improvement suggests that chronic lithium treatment improves performance. These results may represent an improvement in learning/memory. Another possible interpretation is that the shorter latency in KO-C mice is a reflection of their hyperactivity and impulsivity (Moon *et al.* 2006), and that improvement with lithium treatment is due to reduced activity and improved inhibitory control. These findings are of particular interest in light of the reversal by lithium treatment of abnormal dendritic

spines in MPC in KO mice because of the role of MPC in behavioural control (Birrell & Brown, 2000; Muir *et al.* 1996).

Complementing our findings of the effects of lithium treatment on behavioural phenotypes in KO mice are our findings on changes in neuronal morphology imposed by lithium treatment. Abnormality of dendritic spine morphology in neocortical pyramidal cells is the most prominent neuropathological finding in FXS (Hinton *et al.* 1991). Spines are higher in density and have long, thin and tortuous shapes reminiscent of immature spines on developing neurons. Similar morphological changes have been reported in KO mice (Comery *et al.* 1997). In the present study we show that spine pathology also occurs in MPC and that it is normalized by chronic lithium treatment. MPC plays an important role in social cognition (Nachev, 2006). Thus, it is plausible that abnormal dendritic spine morphology in this region may be linked to the autistic-like behaviour. A recent study found that the dendritic spine densities on cortical projection neurons were increased in autism spectrum disorders (Hutsler & Zhang, 2010). MPC is also critically involved in executive function and management of impulsive and compulsive responses. Attentional dysfunction, impulsivity and resistance to change are all features of FXS and have also been found in the *fmr1* KO mouse (Moon *et al.* 2006). The ability of lithium treatment to decrease hyperactivity and to improve performance on the passive avoidance and social interaction tests may be linked to its ameliorative effects on spine morphology in MPC.

Efficacy of lithium treatment has been demonstrated in two animal models of FXS. In the *dfmr1* mutant (McBride *et al.* 2005), treatment with lithium reversed deficient courtship behaviour and mushroom body defects and restored memory deficits in an experience-dependent behaviour modification task. In another study in which the sensitivity of the *dfmr1* mutant to excess glutamate in food was used as a drug screen, lithium rescued lethal effects of elevated glutamate (Chang *et al.* 2008). The effects of lithium treatment have also been studied in the *fmr1* KO mouse. Both acute and chronic treatment with lithium reduced the susceptibility to audiogenic seizures (Min *et al.* 2009). It is noteworthy that a 2-month pilot, open-label treatment trial of lithium in 15 subjects with FXS indicated a trend towards behavioural improvement (Berry-Kravis *et al.* 2008).

In the present study, we measured the lithium concentration in whole blood. This concentration is equivalent to a plasma lithium level that is at the low end of the therapeutic range of plasma lithium for

treatment of bipolar disorder in human subjects (0.4–1.2 mequiv/l) (Aydemir *et al.* 2007; Shimizu *et al.* 1979). Importantly, in our study lithium activity was well below the toxic range, and we saw no evidence of lithium toxicity in treated mice. The therapeutic range in the mouse probably depends on the therapeutic target, and one target of lithium is GSK-3 β (Jope & Roh, 2006; Klein & Melton, 1996). The decreased level of Ser9-phosphorylated GSK-3 β suggests an up-regulation of GSK-3 β activity in untreated KO mice that was reversed by lithium treatment. These findings are in agreement with those of Min *et al.* (2009) and Yuskaitis *et al.* (2010). Evidence that GSK-3 is the therapeutic target of lithium in the KO mouse is supported by the finding that lithium, as well as selective inhibitors of GSK-3, reduced susceptibility to audiogenic seizures and modified open field behaviour (Min *et al.* 2009). GSK-3 is a highly regulated enzyme with a regulatory role in a large number of processes (Grimes & Jope, 2001) including transcription factors that control gene expression, such as AP-1, β -catenin, CREB, NF- κ B; cell signalling cascades such as cAMP-dependent protein kinase, eIF2B, glycogen synthase; and cell architecture through phosphorylation of structural proteins such as MAP1B, MAP2, and Tau. Lithium increases cell survival by inducing neurotrophins, such as BDNF which in turn stimulates activity in the phosphatidylinositol 3-kinase (PI3-K)/Akt and the mitogen-activated protein kinase (MAPK) pathways.

The protein lacking in FXS patients, FMRP, is a negative regulator of translation. In *fmr1* KO mice, the lack of FMRP results in elevated *in-vivo* regional rates of cerebral protein synthesis (Qin *et al.* 2005a) which may underlie the range of phenotypes in this disorder. We posit that chronic lithium treatment acting through a decrease in GSK-3 β activity modulates this excessive translation and, in doing so, ameliorates many of the symptoms of FXS. A study of the effect of lithium treatment on cerebral protein synthesis rates is currently underway in our laboratory. Our results in the mouse model coupled with the results from other laboratories studying the mouse model (Min *et al.* 2009), the *Drosophila* model (McBride *et al.* 2005), and human subjects (Berry-Kravis *et al.* 2008) make a strong case for instituting a placebo-controlled trial of lithium in subjects with FXS.

Note

Supplementary material accompanies this paper on the Journal's website (<http://journals.cambridge.org/jnp>).

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Statement of Interest

None.

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