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**Evidence of reactive astrocytes but not peripheral immune system
activation in a mouse model of Fragile X Syndrome**

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Running title: Neuroinflammation in FXS mice

Abbreviations: ASDs, Autism Spectrum Disorders; FXS, Fragile X syndrome; *Fmr1*, *fragile X mental retardation 1*; FRMP, fragile X mental retardation protein; GFAP, glial fibrillary acidic protein; GSK3, glycogen synthase kinase-3; IFN γ , interferon- γ ; IL-6, interleukin-6; LPS, lipopolysaccharide; mGluR5, metabotropic glutamate receptor 5; TNF α , tumor necrosis factor- α .

Abstract

Fragile X syndrome (FXS) is the most common form of inherited mental retardation and is one of the few known genetic causes of autism. FXS results from the loss of *Fmr1* gene function, thus *Fmr1* knockout mice provide a model to study impairments associated with FXS and autism and to test potential therapeutic interventions. The inhibitory serine-phosphorylation of glycogen synthase kinase-3 (GSK3) is lower in brain regions of *Fmr1* knockout mice than wild-type mice and the GSK3 inhibitor lithium rescues several behavioral impairments in *Fmr1* knockout mice. Therefore, we examined if the serine-phosphorylation of GSK3 in *Fmr1* knockout mice also was altered outside the brain and if administration of lithium ameliorated the macroorchidism phenotype. Additionally, since GSK3 regulates numerous functions of the immune system and immune alterations have been associated with autism, we tested if immune function is altered in *Fmr1* knockout mice. The inhibitory serine-phosphorylation of GSK3 was significantly lower in the testis and liver of *Fmr1* knockout mice than wild-type mice, and chronic lithium treatment reduced macroorchidism in *Fmr1* knockout mice. No alterations in peripheral immune function were identified in *Fmr1* knockout mice. However, examination of glia, the immune cells of the brain, revealed reactive astrocytes in several brain regions of *Fmr1* knockout mice and treatment with lithium reduced this in the striatum and cerebellum. These results provide further evidence of the involvement of dysregulated GSK3 in FXS, and demonstrate that lithium administration reduces macroorchidism and reactive astrocytes in *Fmr1* knockout mice.

Keywords: astrocytes; Fragile X Syndrome; glycogen synthase kinase-3; lithium; macroorchidism

1. Introduction

Fragile X syndrome (FXS) is caused by functional loss of the *fragile X mental retardation 1 (Fmr1)* gene on the X chromosome, resulting in lack of the gene product, fragile X mental retardation protein (FMRP), an RNA binding protein that regulates translation [1, 2]. FXS is the most common cause of inherited mental retardation and is the first identified autism-related gene because FXS patients have many characteristics commonly associated with Autism Spectrum Disorders (ASDs), such as developmental delays, communication impairments, and anxiety [2-9]. These conditions are modeled in *Fmr1* knockout mice [10] that display several phenotypes of FXS and ASDs [11-20]. Thus, *Fmr1* knockout mice provide an important animal model to study characteristics of FXS as well as autistic traits, and to test potential therapeutic interventions. Studies of pharmacological interventions in *Fmr1* knockout mice have identified therapeutic effects of antagonists of metabotropic glutamate receptor 5 (mGluR5) [1, 21] and of lithium [13, 14, 20], an inhibitor of glycogen synthase kinase-3 (GSK3) [22, 23].

FXS and autism are generally considered to be neuronal disorders because of the predominant behavioral and cognitive abnormalities. However, neuronal function can be modified by many types of cells, such as glia and immune cells, and there is substantial evidence that neuronal dysfunction can be caused by neuroinflammation [24, 25]. Notably, treatment with minocycline, a tetracycline antibiotic that exerts anti-inflammatory effects, rescued some FXS-related impairments in *Fmr1* knockout mice [26]. Neuroinflammation occurs in response to brain injury, degenerating cells, insults, or infection, as well as psychological stress, and is mediated by the immune resident cells in the brain, microglia and astrocytes, as well as by infiltration of peripheral immune cells [24, 25, 27]. Although a role for immune responses and associated inflammation in autism is controversial [28-30], there is some evidence of activated glia in autism

[28, 31-33] and altered plasma cytokines associated with FXS [34]. However, little is known about the immune system in *Fmr1* knockout mice.

GSK3 represents a potential link between FXS and inflammation. GSK3 is a partially constitutively active serine/threonine kinase that is predominantly controlled by inhibitory serine phosphorylation of its two isoforms, serine-9 in GSK3 β and serine-21 in GSK3 α [35-37]. Recently, we found that the inhibitory serine-phosphorylation of both GSK3 isoforms is decreased in several brain regions of *Fmr1* knockout mice compared with wild-type mice [13, 20]. GSK3 has many regulatory influences on the immune system [38], particularly promoting inflammation both in the periphery [39] and in glia [40, 41]. Additionally, administration of GSK3 inhibitors ameliorated a number of immune-mediated conditions in animal models, such as septic shock [39] reviewed in [42]). The present study extended the examination of GSK3 serine-phosphorylation to peripheral tissues, and tested if the hyperactive GSK3 in *Fmr1* knockout mice was associated with changes in the peripheral or central immune systems because GSK3 has widespread influences on immune function [38].

2. Materials and Methods

2.1. Animals and in vivo tests

This study used adult, male C57Bl/6J littermates, ~3 months of age, with or without a disruption of the *Fmr1* gene (originally kindly provided by Dr. W. Greenough, University of Illinois). The *Fmr1* knockout mice were generated by breeding male C57BL/6J hemizygous *Fmr1* knockout mice and female C57BL/6J heterozygous *Fmr1* knockout mice to generate male homozygous *Fmr1* knockout mice and wild-type littermates. Genotype was confirmed by PCR using the Jackson Laboratory protocol for genotyping *Fmr1* mice. Mice were given water and food *ad libitum*. Lithium was administered in pelleted food containing 0.2% lithium carbonate (Harlan-

Teklad) and mice were given 0.9% saline in addition to water to prevent hyponatremia. Protein-free *E. coli* (K235) LPS was prepared as described [39]. All mice were housed and treated in accordance with National Institutes of Health and the University of Alabama at Birmingham Institutional Animal Care and Use Committee guidelines.

2.2. Flow cytometry, T cell proliferation and ELISA assays

Cells obtained from lymph nodes or spleens were incubated with anti-CD16/32 (FcR block, eBioscience) to prevent non-specific staining and then stained for 30 min in the dark with anti-CD4-FITC, anti-CD8-APC, and anti-CD25-PE or anti-CD45-FITC and anti-CD11b-Alexa647 (eBioscience). Stained cells were analyzed using a FACSCalibur and data was analyzed using CellQuest software (BD Biosciences). The Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega) was used to measure cell proliferation according to the manufacturer's instructions. Primary microglia from *Fmr1* knockout (FX) or wild-type (WT) mice were prepared and cultured as described previously [40]. Primary microglia were treated with 100 ng/mL LPS for 6 h followed by collection of the media and measurements of cytokines. *Fmr1* knockout (FX) or wild-type (WT) mice were treated with LPS (10 mg/kg; i.p.) or vehicle (control) and serum was collected after 4 h. Tumor necrosis factor- α (TNF α), interleukin-6 (IL-6) and interferon- γ (IFN γ) were measured by enzyme-linked immunosorbent assays (ELISA) according to manufacturer's instructions (eBioscience).

2.3. Tissue preparation and immunoblotting

Mice were decapitated, the brains rapidly frozen, and dissected brain regions were homogenized in ice-cold lysis buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 5 μ g/ml pepstatin, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium vanadate, 50 mM sodium fluoride, and 100 nM okadaic acid. The lysates were centrifuged at 20,000xg 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 (10-20 μg) were resolved in SDS-polyacrylamide gels, and transferred to nitrocellulose. Blots were probed with antibodies to phospho-Ser9-GSK3 β , phospho-Ser21-GSK3 α (Cell Signaling Technology, Beverly, MA), total GSK3 α/β , GFAP (Millipore, Bedford, MA) and β -actin as a loading control (Sigma, St Louis, MO). Immunoblots were developed using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA), followed by detection with enhanced chemiluminescence, and quantitation by densitometry. To compare differences between *Fmr1* knockout and wild-type mice, values are shown as the percents of densities of immunoblots of wild-type samples analyzed on the same gels.

3. Results

3.1. GSK3 phosphorylation in testis and liver of *Fmr1* knockout mice

We previously reported that the inhibitory serine-phosphorylation of both isoforms of GSK3 is lower in several brain regions of *Fmr1* knockout mice than in wild-type mice [13, 20]. We extended this assessment to test if phosphorylation of GSK3 is also altered in testis because although FMRP is expressed in all tissues, its highest levels of expression are in neurons and testis [43], and *Fmr1* knockout mice display a macroorchidism phenotype. The serine-phosphorylation of GSK3 α was significantly $\sim 40\%$ lower in the testis of *Fmr1* knockout mice than wild-type mice (Fig 1A). The serine-phosphorylation of GSK3 β also tended to be $\sim 40\%$ lower, but this did not reach statistical significance because its very low level compared with GSK3 α , as reported previously [44], resulted in greater variability in the wild-type mice (Fig 1B).

There was no difference in the total level of GSK3 α or GSK3 β , indicating a deficit in serine-phosphorylation, not expression, of GSK3 in testis of *Fmr1* knockout mice. As reported previously [10], testicular weight was greater in *Fmr1* knockout mice than in wild-type mice (Fig. 1C). Administration of the GSK3 inhibitor lithium for 4 weeks modestly, but significantly, reduced testicular weight in *Fmr1* knockout mice treated between the ages of 12 to 16 weeks, but had no effect in wild-type mice. The partial correction of macroorchidism in *Fmr1* knockout mice by lithium administration suggests that GSK3 contributes to this phenotype.

To further examine tissue distribution of reduced serine-phosphorylation of GSK3 in *Fmr1* knockout mice, livers were examined. As in the testis, phospho-Ser21-GSK3 α was significantly lower in the liver of *Fmr1* knockout mice (~25% reduction) than in wild-type mice, with no difference in total levels of GSK3 α (Fig 1D). In contrast, there was no difference in phospho-Ser9-GSK3 β , or total GSK3 β , in the liver of *Fmr1* knockout and wild-type mice (Fig 1E). These results indicate that the lack of FMRP contributes to impaired inhibitory serine-phosphorylation of GSK3 in tissues besides the brain, suggesting that FMRP contributes to maintaining inhibitory control of GSK3.

3.2. Peripheral immune system of *Fmr1* knockout mice

We evaluated several measures of basal immune system functions in *Fmr1* knockout mice because GSK3 has widespread regulatory effects on the immune system [38] that may be altered by the impaired inhibitory serine-phosphorylation of GSK3 in central and peripheral tissues of *Fmr1* knockout mice and because immune activation has been hypothesized to contribute to autism spectrum disorders [28-30]. The immune system comprises early responders, such as macrophages and dendritic cells, and a second line of defense that includes T cells. Macrophages are characterized by the expression of the surface markers CD45 and CD11b upon activation. There were no difference in the expression of these two markers in spleen and draining lymph

node cells between *Fmr1* knockout mice and wild-type mice (Figure 2A). Similarly, no differences were detected between *Fmr1* knockout and wild-type mice in the percentage of helper CD4⁺, cytotoxic CD8⁺, or regulatory CD25⁺ T cell populations (Figure 2B), or in the expression of the surface markers CD4, CD8 or CD25 in spleen or draining lymph node cells (Figure 2C). Consistent with this, proliferation of T cells from spleen and draining lymph nodes in response to stimulation with CD3 or a combination of CD3 and CD28 was not different between *Fmr1* knockout and wild-type mice (Figure 3). Taken together, these results indicate that there are not major alterations in these components of the immune system in *Fmr1* knockout mice.

3.3. Cytokine response to LPS in *Fmr1* knockout mice

To test if the production of cytokines is altered in *Fmr1* knockout mice, ELISA measurements were used to determine the serum levels of the pro-inflammatory cytokines TNF α and IFN γ . Basal serum levels of TNF α and IFN γ were low, and there were no differences between *Fmr1* knockout and wild-type mice (Figure 4). In response to in vivo administration of the bacterial endotoxin, lipopolysaccharide (LPS), there were large increases in the serum levels of TNF α and IFN γ , and the increases were equivalent in *Fmr1* knockout and wild-type mice (Figure 4). However, examination of the time course of the responses of these and other cytokines to LPS administration would provide a more complete analysis.

3.4. FMRP expression in glia

Much of the research on *Fmr1* knockout mice has focused on neuronal function. However, FMRP is also expressed in astrocytes [45], which we confirmed using primary astrocytes cultures prepared from 1 day old wild-type mice, although the level of FMRP was lower than in primary hippocampal neurons (Figure 5A). However, mouse primary microglia and primary neural precursor cells express FMRP at levels comparable to that of neurons (Figure 5A). The microglial immunoblot was validated by staining FMRP in BV-2 microglial cells (Figure 5B).

Since microglia express FMRP and are considered the macrophages of the brain, we analyzed the production of the cytokines IL-6 and TNF α by primary microglia prepared from *Fmr1* knockout or wild-type mice. Basal levels of IL-6 and TNF α were low in microglia from both *Fmr1* knockout and wild-type mice (data not shown). Treatment with 100 ng/mL LPS for 6 h resulted in activation of microglia and large increases in IL-6 and TNF α production, however no differences were detected in microglia lacking FMRP (Figure 5C). Thus, despite microglial expression of FMRP, acute activation of microglia and the production of these two cytokines were not altered in cells deficient in FMRP.

3.5. GFAP is increased in *Fmr1* knockout mouse brain and reduced by lithium

Increased GFAP expression is a hallmark characteristic of reactive astrogliosis, a sign of inflammation in the brain, and increased astrocyte reactivity has been reported in postmortem examination of brains from patients with autism [33, 46]. Immunoblots revealed that GFAP expression was significantly up-regulated in the striatum, hippocampus, and cerebral cortex of *Fmr1* knockout mice compared to wild-type mice (Figure 6A). Since GSK3 promotes inflammatory reactions [38] and administration of GSK3 inhibitors, such as lithium, rescue several behavioral deficits of *Fmr1* knockout mice [13, 20], we tested if lithium treatment reversed the abnormally high levels of GFAP in *Fmr1* knockout mice. Chronic lithium treatment significantly decreased GFAP levels in the striatum and cerebellum of *Fmr1* knockout mice (Figure 6B) and also reduced GFAP levels in several brain regions in wild-type mice (Figure 6C). This extends a previous report that lithium reduces LPS-induced up-regulation of GFAP expression [40]. Taken together, this is the first evidence of reactive astrocytes in *Fmr1* knockout mice and demonstrates that GFAP expression is reduced following lithium administration.

4. Discussion

Fmr1 knockout mice provide a model to study phenotypes associated with FXS and autism and to examine responses to potential therapeutic interventions [3-5, 8, 9]. Previously, we found lower inhibitory serine-phosphorylation of GSK3 in several brain regions of *Fmr1* knockout mice than wild-type mice, and this was implicated as a contributory factor in several behavioral impairments because they were ameliorated by administration of the GSK3 inhibitor lithium [13, 14, 20]. In the present study, we tested if the loss of FMRP resulted in changes in serine-phosphorylation of GSK3 in peripheral cells, as well as the brain, or altered the peripheral or central immune systems. The results show that serine-phosphorylation of GSK3 α was lower in testis and liver of *Fmr1* knockout mice than wild-type mice, demonstrating that loss of FMRP can regulate GSK3 phosphorylation in peripheral tissues as well as in the brain. Furthermore, a functional effect of the hyperactive GSK3 α in testis was implicated by the finding that administration of the GSK3 inhibitor lithium reduced macroorchidism in *Fmr1* knockout mice. Although alterations in the peripheral immune system were not found, *Fmr1* knockout mice displayed significantly increased astrogliosis compared to wild-type mice, and this was alleviated by lithium administration, suggesting that GSK3 contributes to astrocyte activation in *Fmr1* knockout mice.

Changes in the inhibitory serine-phosphorylation are important because this is the major mechanism for controlling the activity of GSK3, and since GSK3 phosphorylates over 50 substrates, impairment of this control mechanism can influence many cellular functions [35, 36, 47]. The lower inhibitory serine-phosphorylation of GSK3 in brain regions of *Fmr1* knockout mice compared with wild-type mice [13, 20] could result from altered neuronal activity regulating GSK3 in *Fmr1* knockout brains, or it could indicate a fundamental role for FMRP in regulating intracellular signaling leading to serine-phosphorylation of GSK3 that is lacking in *Fmr1*

knockout mice. Evidence of the latter mechanism, particularly for GSK3 α , is indicated by the lower phospho-Ser21-GSK3 α in testis and liver in *Fmr1* knockout mice than wild-type mice. The possibility that GSK3 α and GSK3 β are differentially influenced by FMRP in testis and liver is consistent with growing evidence of different functions for the two GSK3 isoforms [48]. The reduced inhibitory serine-phosphorylation of testis GSK3 in *Fmr1* mice, indicative of increased GSK3 activity, appears to have a contributory role in the macroorchidism that is characteristic of these mice and of patients with FXS because chronic lithium treatment to inhibit GSK3 significantly reduced testicular weight in *Fmr1* knockout mice but not wild-type mice. Although the decreased testicular weight in *Fmr1* knockout mice after lithium treatment was modest, it was achieved within 4 weeks of treatment, raising the possibility that longer treatment would be even more effective. This adds a morphological feature to the previously described behavioral characteristics of *Fmr1* knockout mice that are partially normalized by lithium treatment.

GSK3 has many regulatory influences on immune function, particularly promoting inflammation [38], and alterations in immune system function have been suggested to contribute to the onset and/or progression of ASDs [28-30]. Although few animal models of ASDs exist, *Fmr1* knockout mice exhibit some phenotypes characteristic of ASDs [11-20]. Therefore, we examined if *Fmr1* knockout mice display signs of immune dysfunction or inflammation. T-cells play a central role in coordinating immune functions and mediate signaling between the innate and adaptive immune systems. No alterations were observed in the unchallenged peripheral immune system, in LPS-stimulated production of TNF α or IFN γ , in T-cell numbers or expression of cell surface markers in *Fmr1* knockout mice. Thus, these immune functions appear normal in *Fmr1* knockout mice, although it remains to be determined if other components of the peripheral immune system are altered.

In contrast to the apparently normal function of the peripheral immune system, the central immune system was clearly affected by loss of FMRP, as a significant increase in the astrogliosis marker GFAP was evident in the brains of *Fmr1* knockout mice. GFAP is the most widely used marker of astrocyte activation [49] and its up-regulation in *Fmr1* knockout mouse brain is indicative of a chronic stress response. The regional distribution of increased GFAP in *Fmr1* knockout mice (striatum, hippocampus, cerebral cortex, but not cerebellum) matched the regional distribution of reduced inhibitory serine-phosphorylation of GSK3 in *Fmr1* knockout mice on the C57Bl/6 background (striatum, hippocampus, cerebral cortex, but not cerebellum) that we reported previously [20]. This raised the possibility that the two may be related, especially considering that GSK3 promotes activation of the STAT3 transcription factor that induces GFAP expression [50]. However, although inhibition of GSK3 with lithium treatment occurred in all four brain regions [20], lithium administration reduced GFAP levels only in the striatum and cerebellum of *Fmr1* knockout mice (Figure 7B), indicating that other factors besides hyperactive GSK3 promote increased GFAP expression in *Fmr1* knockout mice. Further studies of the regulation of astrocyte activation and GFAP expression in FMRP-knockout astrocytes should reveal the causes of astrogliosis in *Fmr1* knockout mice. Since astrocytes are now well-known to regulate neuronal function, it is possible that activated astrocytes contribute to impaired neuronal activities in FXS. This is consistent with the recent finding that astrocytes from *Fmr1* knockout mice cultured with wild-type hippocampal neurons caused abnormal neuronal morphologies, and wild-type astrocytes ameliorated morphological impairments in cultured FMRP-knockout neurons [51]. Although the causes and effects of reactive astrocytes in the brains of *Fmr1* knockout mice remain questions to be addressed in further studies, the observed reduction of GFAP expression by lithium treatment may contribute to its amelioration of behavioral abnormalities in these mice.

Loss of FMRP expression in FXS causes mental retardation and characteristics of autism, but there are no satisfactory therapeutic treatments. Recent research raised the possibility that lithium may be therapeutically useful in FXS [13, 20, 52, 53]. Lithium is already used therapeutically as a mood stabilizer, likely due to its inhibition of GSK3, so much is known about lithium's pharmacokinetics, safety, and tolerability in humans [54]. Our current results show that lithium rescues macroorchidism of *Fmr1* knockout mice, which adds to the previous findings of the beneficial effects of lithium on behavioral deficits of *Fmr1* knockout mice [13, 20]. Furthermore, lithium treatment also reduced GFAP levels, a marker of reactive astrogliosis, in *Fmr1* knockout mice, an action that may contribute to its therapeutic actions on abnormal behaviors. These results demonstrate that lithium does not only ameliorates neuronal-based behaviors in *Fmr1* knockout mice, but also attenuates abnormalities in testis and astrocytes, lending further support to the proposal that lithium may be beneficial in patients with FXS and ASDs.

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5. References

- [1] M.F. Bear, K.M. Huber, S.T. Warren, The mGluR theory of fragile X mental retardation, *Trends Neurosci* 27 (2004) 370-377.
- [2] S.D. Koukoui, A. Chaudhuri, Neuroanatomical, molecular genetic, and behavioral correlates of fragile X syndrome, *Brain Res Rev* 53 (2007) 27-38.
- [3] M.K. Belmonte, T. Bourgeron, Fragile X syndrome and autism at the intersection of genetic and neural networks, *Nat Neurosci* 9 (2006) 1221-1225.
- [4] R.J. Hagerman, M.Y. Ono, P.J. Hagerman, Recent advances in fragile X: a model for autism and neurodegeneration, *Curr Opin Psychiatry* 18 (2005) 490-496.
- [5] D.D. Hatton, J. Sideris, M. Skinner, J. Mankowski, D.B. Bailey, Jr., J. Roberts, P. Mirrett, Autistic behavior in children with fragile X syndrome: prevalence, stability, and the impact of FMRP, *Am J Med Genet A* 140A (2006) 1804-1813.
- [6] A.S. Kau, E. Tierney, I. Bukelis, M.H. Stump, W.R. Kates, W.H. Trescher, W.E. Kaufmann, Social behavior profile in young males with fragile X syndrome: characteristics and specificity, *Am J Med Genet A* 126A (2004) 9-17.
- [7] D.Z. Loesch, Q.M. Bui, C. Dissanayake, S. Clifford, E. Gould, D. Bulhak-Paterson, F. Tassone, A.K. Taylor, D. Hessler, R. Hagerman, R.M. Huggins, Molecular and cognitive predictors of the continuum of autistic behaviours in fragile X, *Neurosci Biobehav Rev* 31 (2007) 315-326.
- [8] S.S. Moy, J.J. Nadler, Advances in behavioral genetics: mouse models of autism, *Mol Psychiatry* 13 (2008) 4-26.
- [9] K. Sullivan, D. Hatton, J. Hammer, J. Sideris, S. Hooper, P. Ornstein, D. Bailey, Jr., ADHD symptoms in children with FXS, *Am J Med Genet A* 140 (2006) 2275-2288.

- [10] T.D.-B.F.X. Consortium, *Fmr1* knockout mice: a model to study fragile X mental retardation, *Cell* 78 (1994) 23-33.
- [11] Z.H. Liu, C.B. Smith, Dissociation of social and nonsocial anxiety in a mouse model of fragile X syndrome, *Neurosci Lett* 454 (2009) 62-66.
- [12] C.H. McNaughton, J. Moon, M.S. Strawderman, K.N. Maclean, J. Evans, B.J. Strupp, Evidence for social anxiety and impaired social cognition in a mouse model of fragile X syndrome, *Behav Neurosci* 122 (2008) 293-300.
- [13] W.W. Min, C.J. Yuskaitis, Q. Yan, C. Sikorski, S. Chen, R.S. Jope, R.P. Bauchwitz, Elevated glycogen synthase kinase-3 activity in Fragile X mice: key metabolic regulator with evidence for treatment potential, *Neuropharmacology* 56 (2009) 463-472.
- [14] M.A. Mines, C.J. Yuskaitis, M.K. King, E. Beurel, R.S. Jope, GSK3 influences social preference and anxiety-related behaviors during social interaction in a mouse model of fragile X syndrome and autism, *PLoS One* 5 (2010) e9706.
- [15] Y.S. Mineur, L.X. Huynh, W.E. Crusio, Social behavior deficits in the *Fmr1* mutant mouse, *Behav Brain Res* 168 (2006) 172-175.
- [16] Y.S. Mineur, F. Sluyter, S. de Wit, B.A. Oostra, W.E. Crusio, Behavioral and neuroanatomical characterization of the *Fmr1* knockout mouse, *Hippocampus* 12 (2002) 39-46.
- [17] S.S. Moy, J.J. Nadler, N.B. Young, R.J. Nonneman, A.W. Grossman, D.L. Murphy, A.J. D'Ercole, J.N. Crawley, T.R. Magnuson, J.M. Lauder, Social approach in genetically engineered mouse lines relevant to autism, *Genes Brain Behav* 8 (2009) 129-142.
- [18] C.M. Spencer, O. Alekseyenko, E. Serysheva, L.A. Yuva-Paylor, R. Paylor, Altered anxiety-related and social behaviors in the *Fmr1* knockout mouse model of fragile X syndrome, *Genes Brain Behav* 4 (2005) 420-430.

- [19] C.M. Spencer, D.F. Graham, L.A. Yuva-Paylor, D.L. Nelson, R. Paylor, Social behavior in Fmr1 knockout mice carrying a human FMR1 transgene, *Behav Neurosci* 122 (2008) 710-715.
- [20] C.J. Yuskaitis, M.A. Mines, M.K. King, J.D. Sweatt, C.A. Miller, R.S. Jope, Lithium ameliorates altered glycogen synthase kinase-3 and behavior in a mouse model of fragile X syndrome, *Biochem Pharmacol* 79 (2010) 632-646.
- [21] G. Dolen, E. Osterweil, B.S. Rao, G.B. Smith, B.D. Auerbach, S. Chattarji, M.F. Bear, Correction of fragile X syndrome in mice, *Neuron* 56 (2007) 955-962.
- [22] P.S. Klein, D.A. Melton, A molecular mechanism for the effect of lithium on development, *Proc Natl Acad Sci U S A* 93 (1996) 8455-8459.
- [23] V. Stambolic, L. Ruel, J.R. Woodgett, Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells, *Curr Biol* 6 (1996) 1664-1668.
- [24] M. Di Filippo, P. Sarchielli, B. Picconi, P. Calabresi, Neuroinflammation and synaptic plasticity: theoretical basis for a novel, immune-centred, therapeutic approach to neurological disorders, *Trends Pharmacol Sci* 29 (2008) 402-412.
- [25] S. Lehnardt, Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury, *Glia* 58 (2010) 253-263.
- [26] T.V. Bilousova, L. Dansie, M. Ngo, J. Aye, J.R. Charles, D.W. Ethell, I.M. Ethell, Minocycline promotes dendritic spine maturation and improves behavioural performance in the fragile X mouse model, *J Med Genet* 46 (2009) 94-102.
- [27] A.H. Miller, V. Maletic, C.L. Raison, Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression, *Biol Psychiatry* 65 (2009) 732-741.
- [28] P. Ashwood, S. Wills, J. Van de Water, The immune response in autism: a new frontier for autism research, *J Leukoc Biol* 80 (2006) 1-15.

- [29] K. Garbett, P.J. Ebert, A. Mitchell, C. Lintas, B. Manzi, K. Mirnics, A.M. Persico, Immune transcriptome alterations in the temporal cortex of subjects with autism, *Neurobiol Dis* 30 (2008) 303-311.
- [30] P.H. Patterson, Immune involvement in schizophrenia and autism: etiology, pathology and animal models, *Behav Brain Res* 204 (2009) 313-321.
- [31] W.K. Dong, W.T. Greenough, Plasticity of nonneuronal brain tissue: roles in developmental disorders, *Ment Retard Dev Disabil Res Rev* 10 (2004) 85-90.
- [32] C.A. Pardo, D.L. Vargas, A.W. Zimmerman, Immunity, neuroglia and neuroinflammation in autism, *Int Rev Psychiatry* 17 (2005) 485-495.
- [33] D.L. Vargas, C. Nascimbene, C. Krishnan, A.W. Zimmerman, C.A. Pardo, Neuroglial activation and neuroinflammation in the brain of patients with autism, *Ann Neurol* 57 (2005) 67-81.
- [34] P. Ashwood, D.V. Nguyen, D. Hessel, R.J. Hagerman, F. Tassone, Plasma cytokine profiles in Fragile X subjects: Is there a role for cytokines in the pathogenesis?, *Brain Behav Immun* (2010) in press.
- [35] S. Frame, P. Cohen, GSK3 takes centre stage more than 20 years after its discovery, *Biochem J* 359 (2001) 1-16.
- [36] R.S. Jope, G.V. Johnson, The glamour and gloom of glycogen synthase kinase-3, *Trends Biochem Sci* 29 (2004) 95-102.
- [37] J.R. Woodgett, Molecular cloning and expression of glycogen synthase kinase-3/factor A, *Embo J* 9 (1990) 2431-2438.
- [38] E. Beurel, S.M. Michalek, R.S. Jope, Innate and adaptive immune responses regulated by glycogen synthase kinase-3 (GSK3), *Trends Immunol* 31 (2010) 24-31.

- [39] M. Martin, K. Rehani, R.S. Jope, S.M. Michalek, Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3, *Nat Immunol* 6 (2005) 777-784.
- [40] E. Beurel, R.S. Jope, Lipopolysaccharide-induced interleukin-6 production is controlled by glycogen synthase kinase-3 and STAT3 in the brain, *J Neuroinflammation* 6 (2009) 9.
- [41] C.J. Yuskaitis, R.S. Jope, Glycogen synthase kinase-3 regulates microglial migration, inflammation, and inflammation-induced neurotoxicity, *Cell Signal* 21 (2009) 264-273.
- [42] R.S. Jope, C.J. Yuskaitis, E. Beurel, Glycogen Synthase Kinase-3 (GSK3): Inflammation, Diseases, and Therapeutics, *Neurochem Res* 32 (2007) 577-595.
- [43] K. Singh, P. Gaur, S. Prasad, Fragile x mental retardation (Fmr-1) gene expression is down regulated in brain of mice during aging, *Mol Biol Rep* 34 (2007) 173-181.
- [44] H.B. Yao, P.C. Shaw, C.C. Wong, D.C. Wan, Expression of glycogen synthase kinase-3 isoforms in mouse tissues and their transcription in the brain, *J Chem Neuroanat* 23 (2002) 291-297.
- [45] L.K. Pacey, L.C. Doering, Developmental expression of FMRP in the astrocyte lineage: implications for fragile X syndrome, *Glia* 55 (2007) 1601-1609.
- [46] J.A. Laurence, S.H. Fatemi, Glial fibrillary acidic protein is elevated in superior frontal, parietal and cerebellar cortices of autistic subjects, *Cerebellum* 4 (2005) 206-210.
- [47] B.W. Doble, J.R. Woodgett, GSK-3: tricks of the trade for a multi-tasking kinase, *J Cell Sci* 116 (2003) 1175-1186.
- [48] T. Force, J.R. Woodgett, Unique and overlapping functions of GSK-3 isoforms in cell differentiation and proliferation and cardiovascular development, *J Biol Chem* 284 (2009) 9643-9647.

- [49] A. Messing, M. Brenner, GFAP: functional implications gleaned from studies of genetically engineered mice, *Glia* 43 (2003) 87-90.
- [50] E. Beurel, R.S. Jope, Differential regulation of STAT family members by glycogen synthase kinase-3, *J Biol Chem* 283 (2008) 21934-21944.
- [51] S. Jacobs, L.C. Doering, Astrocytes prevent abnormal neuronal development in the fragile x mouse, *J Neurosci* 30 (2010) 4508-4514.
- [52] E. Berry-Kravis, A. Sumis, C. Hervey, M. Nelson, S.W. Porges, N. Weng, I.J. Weiler, W.T. Greenough, Open-label treatment trial of lithium to target the underlying defect in fragile X syndrome, *J Dev Behav Pediatr* 29 (2008) 293-302.
- [53] S.M. McBride, C.H. Choi, Y. Wang, D. Liebelt, E. Braunstein, D. Ferreiro, A. Sehgal, K.K. Siwicki, T.C. Dockendorff, H.T. Nguyen, T.V. McDonald, T.A. Jongens, Pharmacological rescue of synaptic plasticity, courtship behavior, and mushroom body defects in a *Drosophila* model of fragile X syndrome, *Neuron* 45 (2005) 753-764.
- [54] R.S. Jope, Anti-bipolar therapy: mechanism of action of lithium, *Mol Psychiatry* 4 (1999) 117-128.

Figure Legends

Fig. 1. Reduced serine-phosphorylation of GSK3 α in the testis and liver of FX mice.

Immunoblots of protein extracts from testis (A-B) or liver (D-E) of *Fmr1* knockout (FX) or wild-type (WT) mice were probed with antibodies to (A, D) phospho-Ser21-GSK3 α or total GSK3 α , and (B, E) phospho-Ser9-GSK3 β or total GSK3 β . Immunoblots were quantified by densitometry and values shown are the percentage of wild-type values. means \pm SEM; n=5 mice per group; *p<0.05 compared to wild-type values (Student's t-test). (C) Testicular weights were measured from *Fmr1* knockout mice and wild-type mice with and without lithium treatment between the ages of 12 to 16 weeks of age and compared to untreated littermates. means \pm SEM; n = 10 mice per group; *p<0.05 compared to wild-type, **p<0.05 compared to untreated (one-way ANOVA).

Fig. 2. Peripheral immune system is intact in FX mice.

Flow cytometry was used to identify cells from spleen and draining lymph nodes of *Fmr1* knockout (FX) and wild-type (WT) mice. (A) Surface expression of CD45 and CD11b was evaluated by geographic mean intensity on cells from spleen and draining lymph nodes. (B) CD4⁺, CD8⁺, and CD4⁺CD25 T cells are presented as a percentage of total cells. (C) Surface expression of CD4, CD8, and CD25 was measured on the respective T cell populations as evaluated by geographic mean intensity. n = 5 mice per group.

Fig. 3. Loss of FMRP does not alter proliferation of lymphocytes.

Cells from (A) lymph nodes or (B) spleen of *Fmr1* knockout (FX) or wild-type (WT) mice were isolated and stimulated with anti-CD3 or anti-CD3 with anti-CD28 antibodies for 72 h. Proliferation was assessed using an MTS assay system as described in Section 2. Results shown

are means \pm SEM; n = 5 mice per group; *p < 0.05, Student's t-test as compared to untreated of the same genotype; **p < 0.05, Student's t-test as compared to anti-CD3 of the same genotype.

Fig. 4. LPS-induced production of TNF α and IFN γ is not altered in *Fmr1* knockout mice.

Fmr1 knockout (FX) or wild-type (WT) mice were treated with LPS (10 mg/kg; i.p.) or saline (control) and serum was collected after 4 h. (A) TNF α and (B) IFN γ levels was measured by ELISA. Results are means \pm SEM; n = 8 mice per group.

Fig. 5. FMRP expression and microglia response to LPS

(A) Protein lysates were prepared from cultured primary hippocampal neurons (Neu), primary astrocytes (Astro), primary microglia (MG), and primary neuronal precursor cells (NPCs) derived from brains of C57BL/6J wild-type mice. Immunoblots (10 μ g protein) were probed with anti-FMRP antibody. (B) Representative merged image of BV-2 microglia stained with anti-FMRP antibody visualized under fluorescence (red) and brightfield (20x magnification). (C) Cultured primary microglia from *Fmr1* knockout (FX) or wild-type (WT) mice were treated with 100 ng/mL LPS for 6 h followed by measurement of IL-6 and TNF α production by ELISA. Values from FX samples are presented as the percentage of wild-type values; means \pm SEM; n = 8 mice per group.

Fig. 6. GFAP expression is increased in *Fmr1* knockout mice and reduced by lithium treatment.

(A) Immunoblots of striatum (Str), hippocampus (Hip), cerebral cortex (Ctx), and cerebellum (Cblm) extracts from *Fmr1* knockout (FX) and wild-type (WT) mice were probed with anti-GFAP antibody. Immunoblots were quantified by densitometry and are presented as the percentage of wild-type values analyzed on the same gels. means \pm SEM; n=8 mice per group;

* $p < 0.05$ compared to wild-type values (Student's t-test). (B) *Fmr1* knockout mice and (C) wild-type mice were treated with lithium for 3-4 weeks and compared to untreated littermates. Homogenates of the striatum (Str), hippocampus (Hip), cerebral cortex (Ctx), and cerebellum (Cblm) were probed with anti-GFAP antibody. Immunoblots were quantified by densitometry and are presented as the percents of values from untreated wild-type mice. means \pm SEM; $n=10$ mice per group; * $p < 0.05$ compared with matched sample without lithium treatment.

Figure 1.

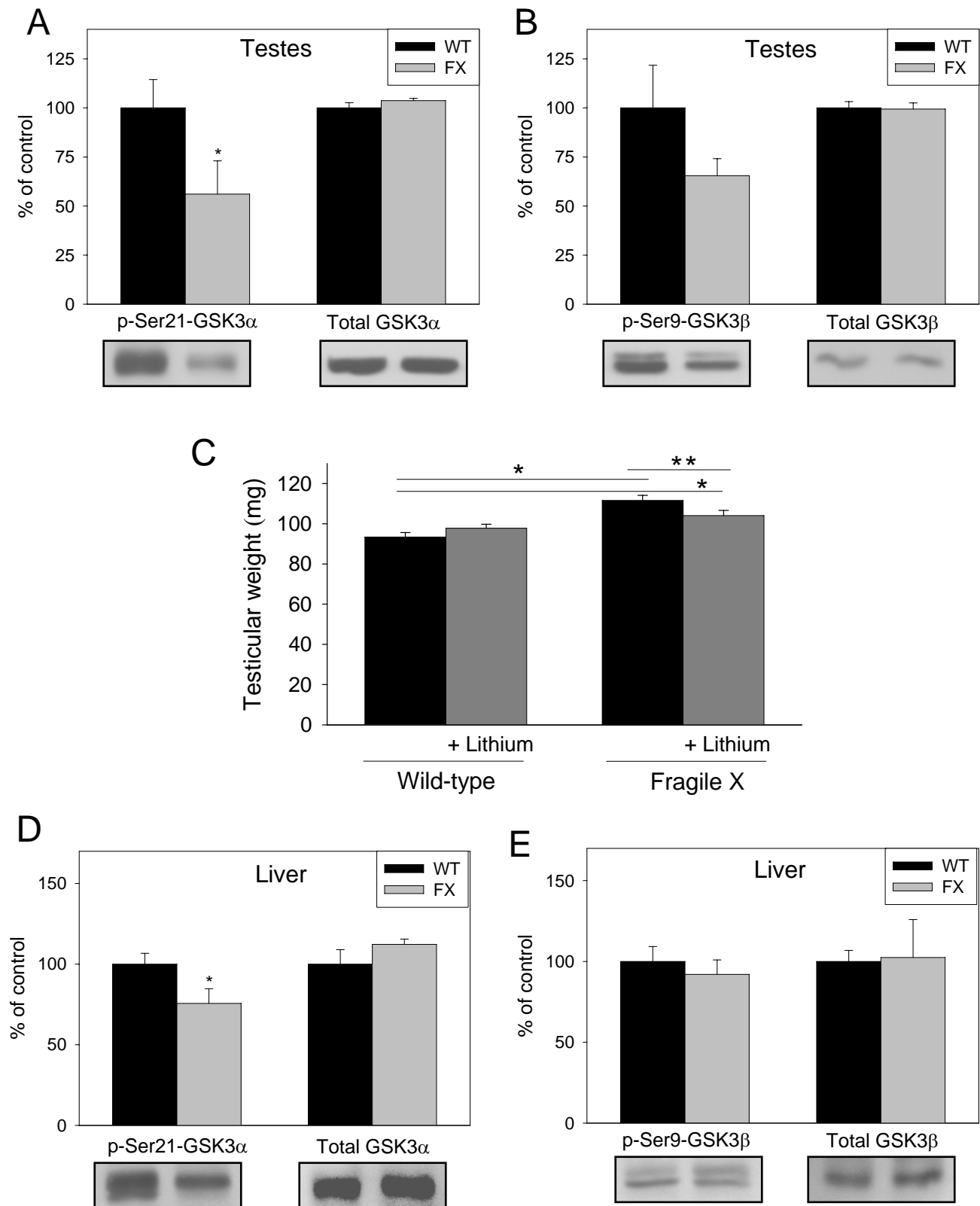


Figure 2.

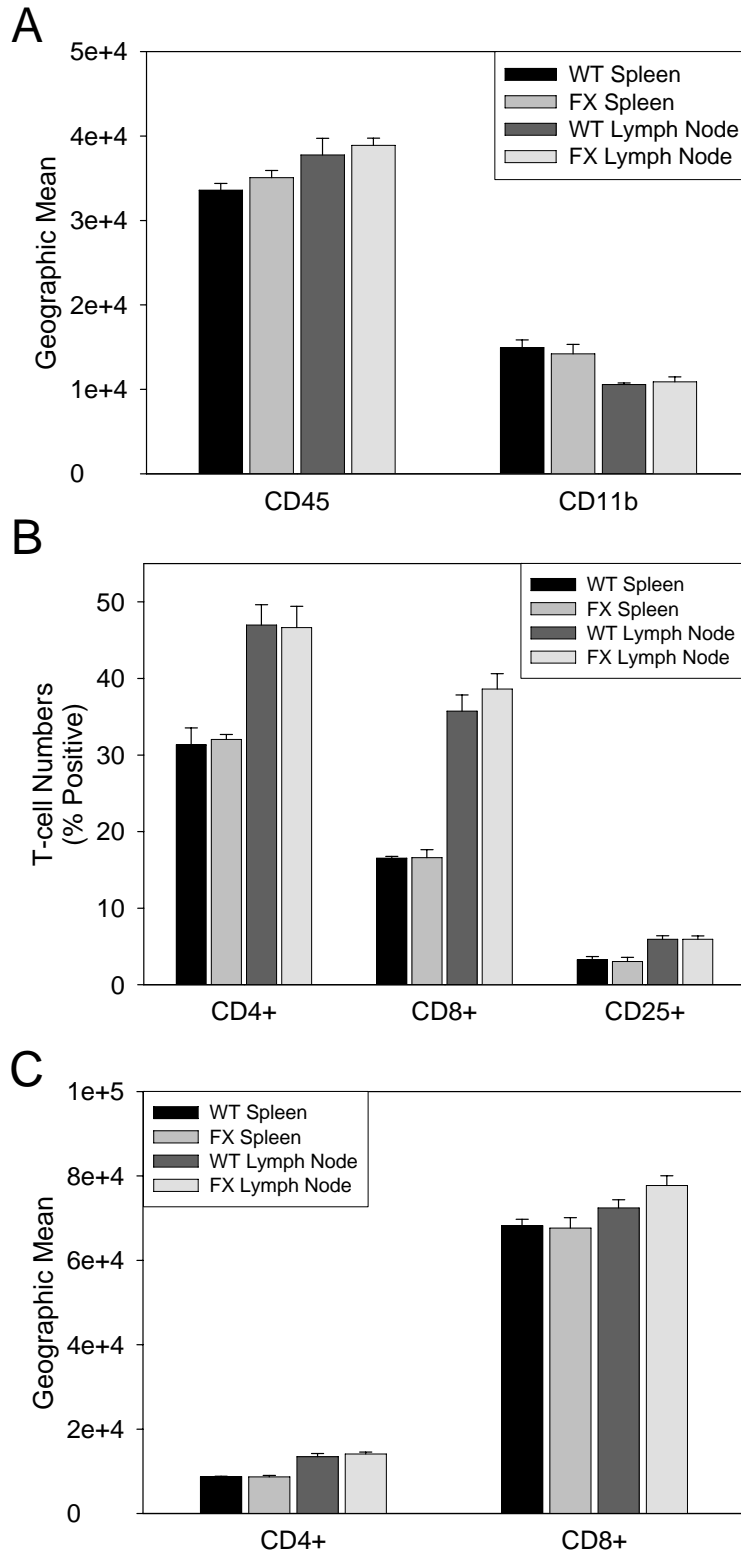


Figure 3.

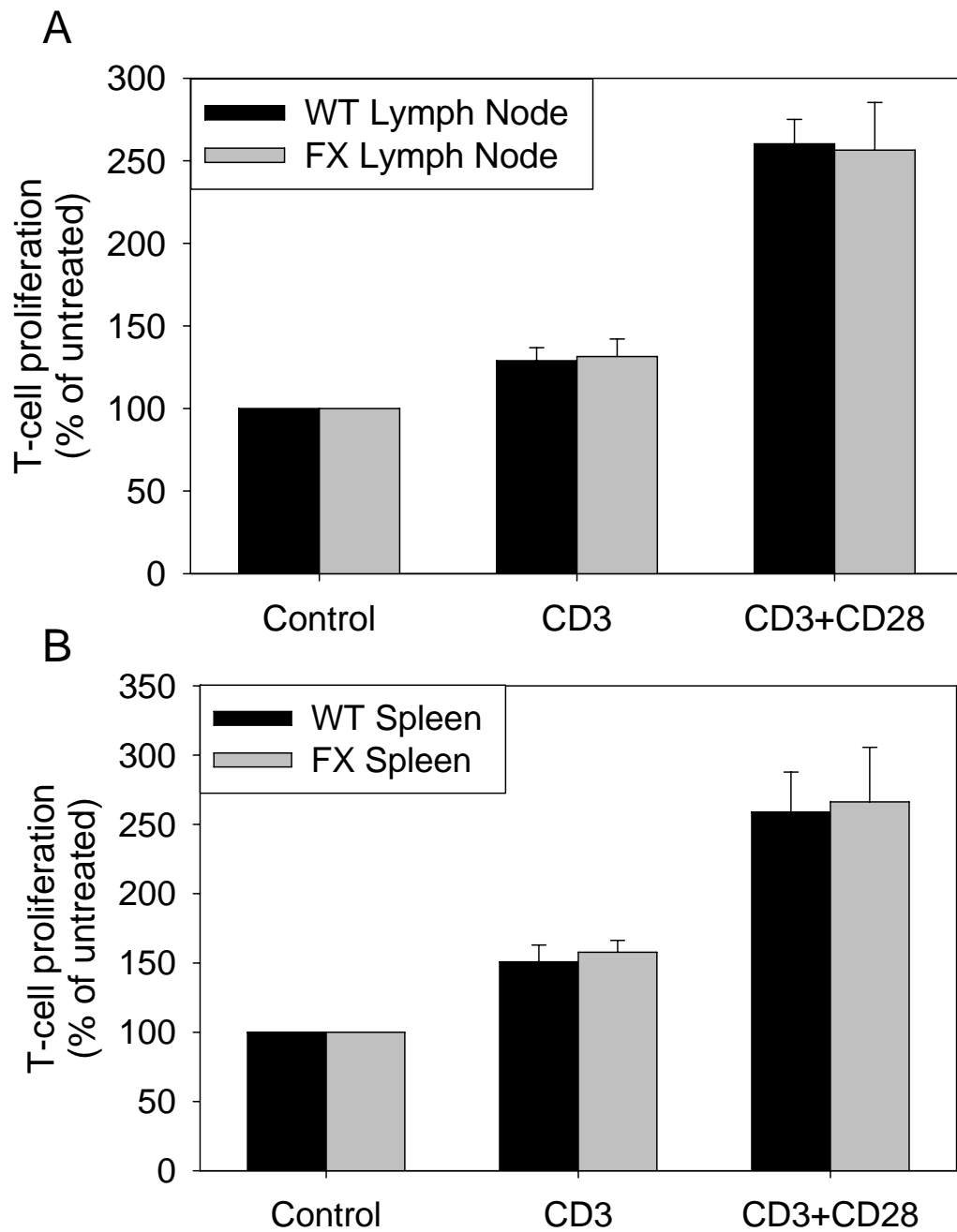


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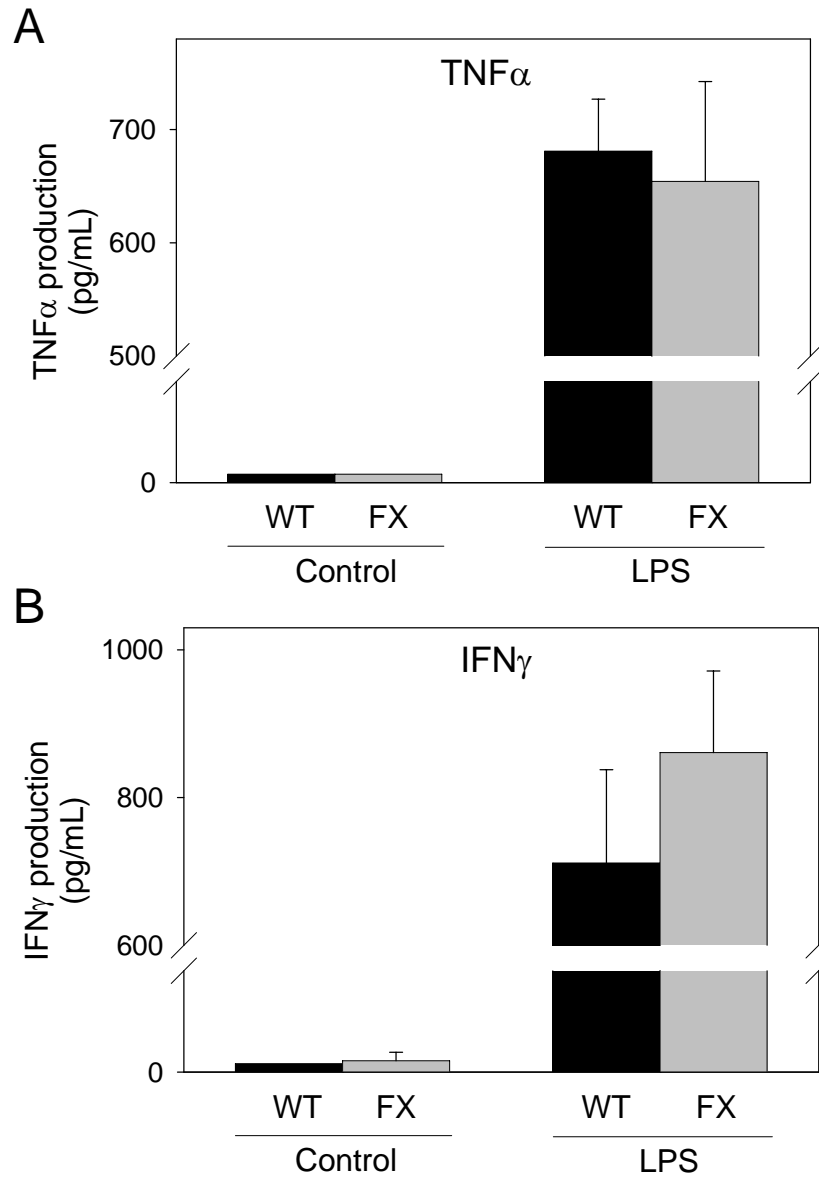


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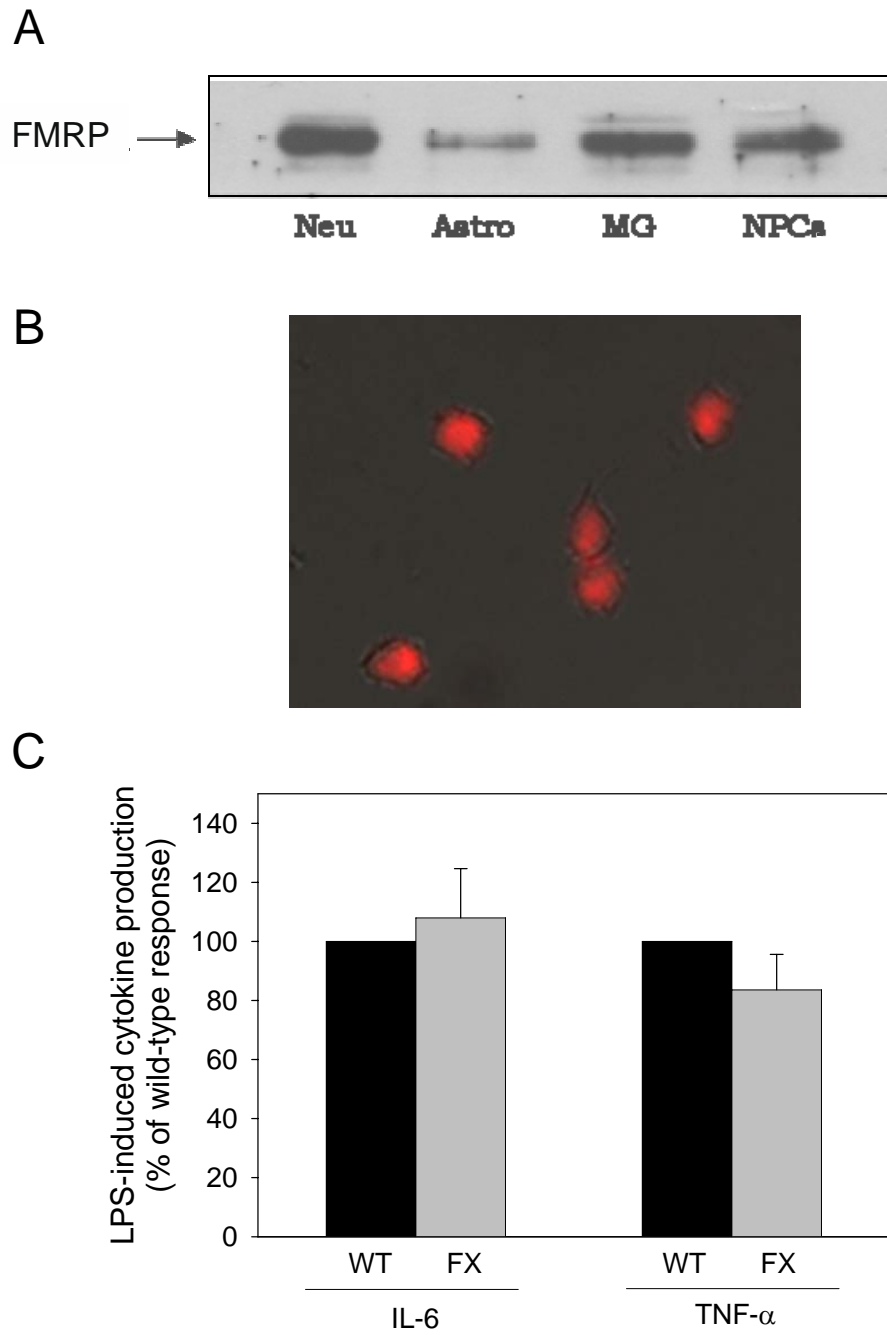


Figure 6.

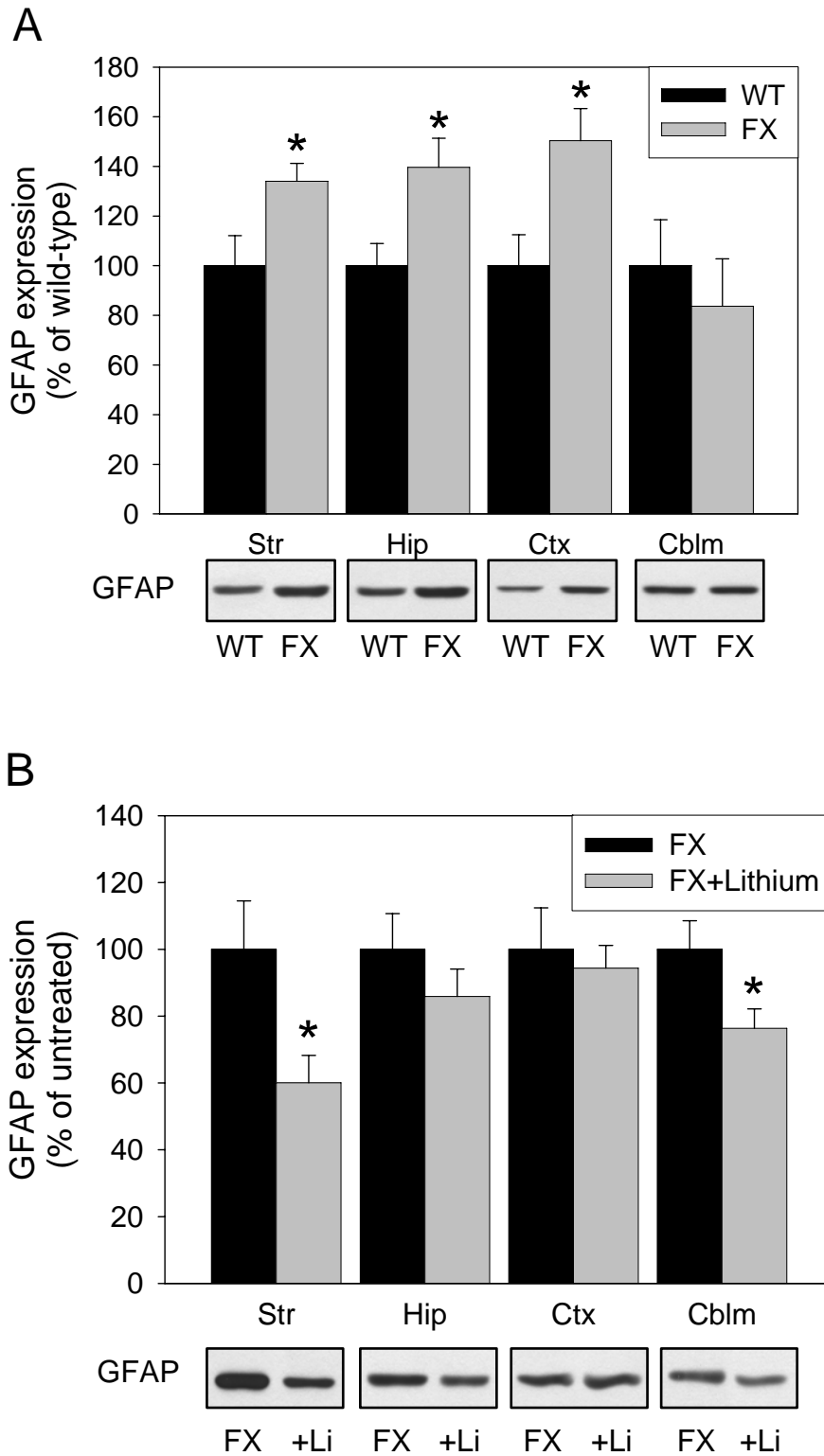


Figure 6.

