Supplemental Text

(*) Supplemental Text 1

A more specific means by which FX GSK3 activity might be elevated is suggested by up-regulation of monoamine activity in the FX fly model (Zhang et al., 2005) as well as alterations in markers of monoamine synthesis in the FX mouse (Gruss and Braun, 2004). Dopamine elevations in the FX brain may contribute to hyperarousal, and possibly even cognitive symptoms, as elaborated by Zhang et. al. (2005). The phenotypes studied here, especially locomotor behavior in the open field, would also be likely impacted by elevated dopamine levels. Of particular note, amphetamines, known to interfere with dopamine uptake, produce an elevation of GSK3β Ser-9 phosphorylation in wild-type mice (via D2R inhibition of Akt) (Beaulieu et al., 2004). Therefore, elevated dopaminergic signaling could be one cause of elevated GSK3 activity in the FX brain. It has also been demonstrated that DHPG-induced mGluR group I signaling can produce enhanced dopamine release (Pintor et al., 2000) and that dopamine is required in the nucleus accumbens for mGluR stimulated locomotion (Meeker et al., 1998). Muscarinic inputs can have similar effects to glutamate on dopaminergic neurons (Grace, 2002), consistent with recent findings that muscarinic acetylcholine receptor-mediated LTD is altered in the FX mouse hippocampus (Volk et al., 2007). Therefore, a number of neurotransmitter signaling alterations described in the FX brain could exert a stimulatory effect on GSK3, albeit in a more indirect and circuit-based manner than localized to single-cell-based linear mGluR group I biochemical pathways.

Another possibly related means by which GSK3 has been found to be activated in neuronal cells is via Gα12 and Gα13 signaling, with the latter being Rho-mediated (Sayas et al.,

2002). There is evidence that Rac1/Rho activity has common effects coordinated with that of FMRP by an intermediary CYFIP protein in mammals and flies (Bardoni and Mandel, 2002; Schenck et al., 2003). FMRP also interacts with a Rac1 effector, p21-activated kinase (PAK), a dominant negative transgene of which has produced a substantial reversion of the FX mouse phenotype (Hayashi et al., 2007). Rho-mediated signaling leads to reorganization of the actin cytoskeleton, in particular causing neurite retraction in some assays; one of the most prominent effects of FXS on neurons is on altered synaptic spine morphology (Comery et al., 1997; Hinton et al., 1991). Furthermore, several known X-linked mental retardation genes are linked to Rho/Rac GTPase signaling (Bardoni and Mandel, 2002). One of the neurotransmitters that acts through Gα12/13 receptors is the monoamine serotonin, which as noted above may be disregulated in FXS.

(**) Supplemental Text 2

The lack of tolerance to lithium in FX mice is in accordance with its long history of use without the development of tolerance in psychiatric patients. In contrast, tolerance was to the other GSK3 inhibitors was evident in FX mice. This may be due to pharmacokinetic changes with repeated administration or to their ATP-competitive mechanism of inhibition of GSK3, but there is little *in vivo* data on which to base speculation about possible mechanisms.

(***) Supplemental Text 3

In addition to CREB, GSK3 phosphorylates translation factor eIF2B. eIF2B inhibition by GSK3 phosphorylation leads to a reduction in Met-tRNA loading onto the 40S ribosomal

subunits, and thereby reduced global protein synthesis (Kimball, 1999; Quevedo et al., 2000). Therefore, elevated GSK3 activity would be expected to generally diminish global protein translation, though translation of some mRNAs are increased upon phosphorylation of the eIF2B target eIF2α (Kimball, 1999). Elevated GSK3 activity in FX mouse brains could be an attempt to compensate for FMRP loss and the associated increase in translation of mRNAs usually bound by it. If so, a reactive increase in GSK3 activity may produce a significant component of the FXS pathology. However, given the finding that protein synthesis inhibitors reduce the proclivity for prolonged, epileptogenic discharges in FX hippocampal slices (Chuang et al., 2005), it might also be expected that GSK3 activity inhibitors would generally elevate and thereby possibly exacerbate FX seizure incidence, and the imbalance in protein translation created in the absence of FMRP. In this light, the clinical benefits of GSK3 inhibition might stem more from more direct effects on proteins misexpressed in the absence of FMRP such as MAP1B. MAP1B, a substrate for GSK3, may have direct relevance to the changes in neuronal spine morphology observed in FXS. The results presented here predict that mode I phosphorylation of MAP1B would be elevated in the FX mouse brain. Therefore, the benefit of reduced phosphorylation of MAP1B or similar targets in the FX brain might outweigh any global elevation in protein synthesis caused by GSK3 inhibitors in FX mice.

(****) Supplemental Text 4

Also potentially consistent with the relevance of glycogen regulation to the results presented here, chemically induced seizures in mouse strains have a decreased onset with increased brain glycogen content (Bernard-Helary et al., 2000). Any elevations of brain glycogen

levels by 30 minutes after inhibition of GSK3 in the current studies may have similarly decreased audiogenic seizures susceptibility.

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Supplemental Table

Table S1. Chronic lithium administration is effective against FX audiogenic seizures.

Strain	Li chow duration	AGS mouse numbers	AGS observed (%)	% nl chow AGS	av. Li level mmol/L	t-test Li level AGS+v.-	weight (g.) av.	% nl chow weight	t-test weight. AGS+v.-
FVB-FX	9 d.	10/17- 7/17+	41	~45	2.0- 2.2+	p=0.55	13.3- 13.5+	62 65	p=0.93
FVB-FX	29 d.	5/8- 3/8+	38	~41	2.8- 2.9+	p=0.89	5.2- 4.5+	24 22	p=0.36
HYB-FX	7 d.	9/9- 0/9+	0		1.0 (± 0.1)	n.a.	13.2 (± 0.4)		n.a.
FVBwt	9 d.	17/17- 0/17+	0	~33	1.4 (± 0.1)	n.a.	11.1 (± 0.3)	55	n.a.
HYBwt	7 d.	7/7- 0/7+	0		0.58 (± 0.04)	n.a.	16.2 (± 0.8)	67	n.a.
HYBwt	29 d.	6/6- 0/6+	0		2.2 (± 0.1)	n.a.	5.4 (± 0.2)	22	n.a.

Table S1. Chronic lithium administration is effective against FX audiogenic seizures.

All lithium carbonate chow durations refer to the administration time prior to the test (d=days). There was no normal chow wash out period. Abbreviations: “AGS” is audiogenic seizure; “nl” is normal, and “av” is average. For numbers of mice, “-“ indicates the sub-group of treated mice which did not have seizures; “+” indicates the sub-group of mice which did. “% nl chow AGS” represents a comparison of the AGS levels after Li chow to those obtained with the same sex, age, and strain of mice eating normal chow, for which our historical averages have been approximately 92% for FVB-FX and 20% for FVB-wt (see also Figure 1). “t-test Li AGS + v. –“

shows the probability value for a t-test comparing the serum lithium levels of those mice having seizures with those that did not. Statistical significance level was p = 0.5 or less. "% nl chow weight" compares the average weight of mice fed lithium chow (under the conditions for the given row) with comparable mice fed normal (non-lithium containing) chow. (Body weight was used as a measure of reduced growth and body size throughout development; there was no weight loss observed.) "t-test weight AGS + v. –" compares the weights of those mice which seized on the lithium diet with those that did not. "(± standard error of the mean)" is presented in the table for lithium levels and weights, except for FVB-FX mice, for which the additional data are as follows: 2.0 ±0.3, 2.2.±0.2, 2.8±0.2, 2.9±0.2, 13.3±0.95, 13.5±0.8, 5.2±0.5, 4.5±0.2.

Supplemental Figure Legends

Figure S1. Survival increases with body weight for nursling FX mice given repeat ip administration of GSK3 inhibitor SB-216763.

(A) Linear regression of percentage survival of groups of 4 – 7 nursing mice given SB-216763 (10 mg/kg; ip) vs. starting body weight ("wt."). Five of the groups tested were F1 hybrid FX mice, three were FVB FX mice, and two were FVB wild-type mice. The wild-type mice represent one of each pair at 100% survival (16.5g and 19.1g average weights). (B) The same SB-216763 data plus two groups of FX FVB mice injected with 70 µl DMSO vehicle only, as well as two groups which received 10 mg/kg MPEP + 10 mg/kg SB-216763 simultaneously.

Figure S2. Intracerebroventricularly administered DMSO has no significant effect on audiogenic seizure sensitivity.

Groups of approximately 15 FX and wt male mice (FVB strain; from left to right, n = 15, 14, 16, and 15) were given icv DMSO in the volumes listed 30 minutes prior to AGS testing either once at 30 days of age ("1x"), or once daily beginning at 26 days of age for 5 days ("5x"). The percentages of mice exhibiting audiogenic seizures ("AGS") were compared by chi-square analysis.

Figure S3. Intraperitoneal administration of GSK3 inhibitor SB-216763 and mGluR5 antagonist MPEP: no statistically significant difference between SB + MPEP and MPEP alone.

Groups of 15 male FX mice on FVB/NJ or "HYB" (C57BL/6J x FVB/NJ) backgrounds were given ip injections of SB-216763 ("SB"), MPEP, or vehicle (DMSO) in the doses and volumes indicated in the legend. Statistical significance was assessed by one factor ANOVA followed by Tukey-Kramer HSD test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Supplemental Figures

Survival vs. Initial Body Weight

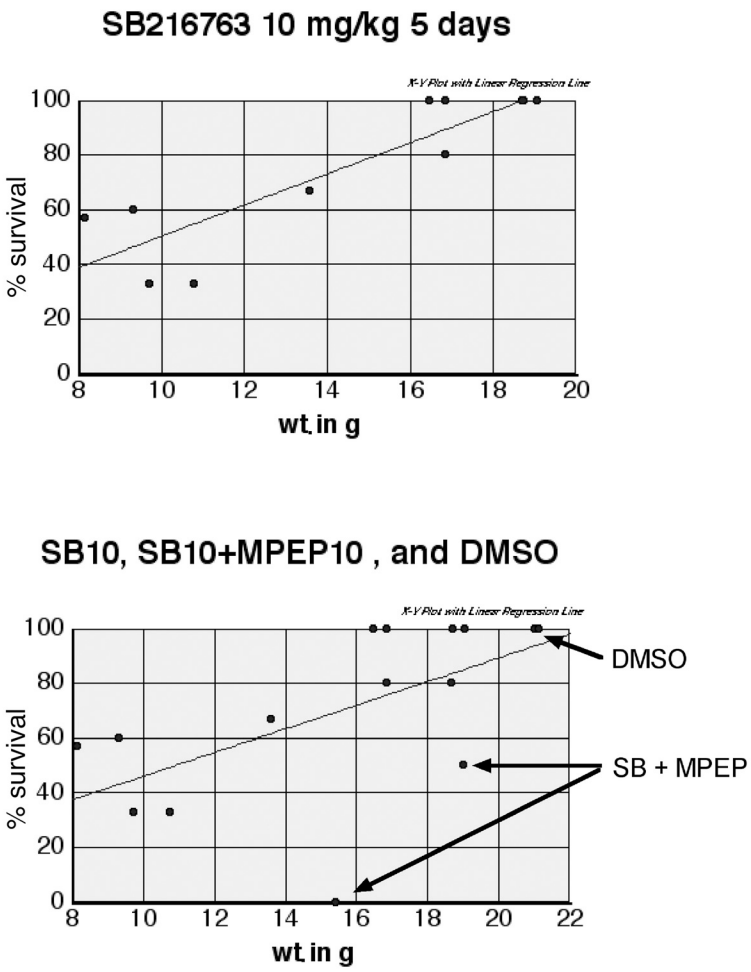

Figure S1

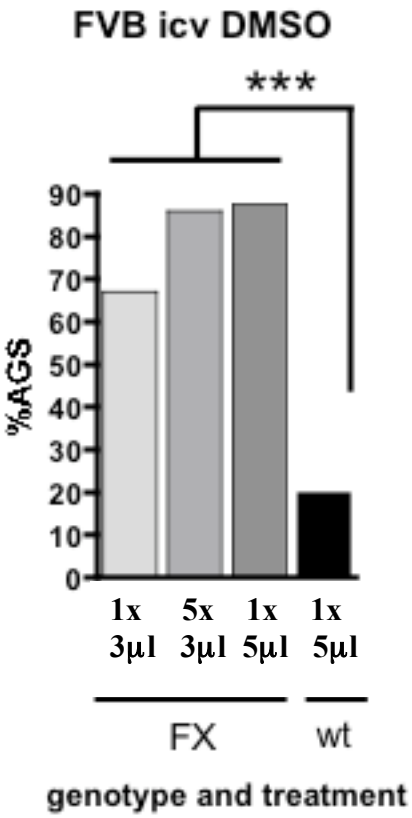
FVB icv DMSO
***
90
80
70
60
50
40
30
20
10
0
%AGS
1x 3µl
5x 3µl
1x 5µl
1x 5µl
FX
wt
genotype and treatment

Figure S2

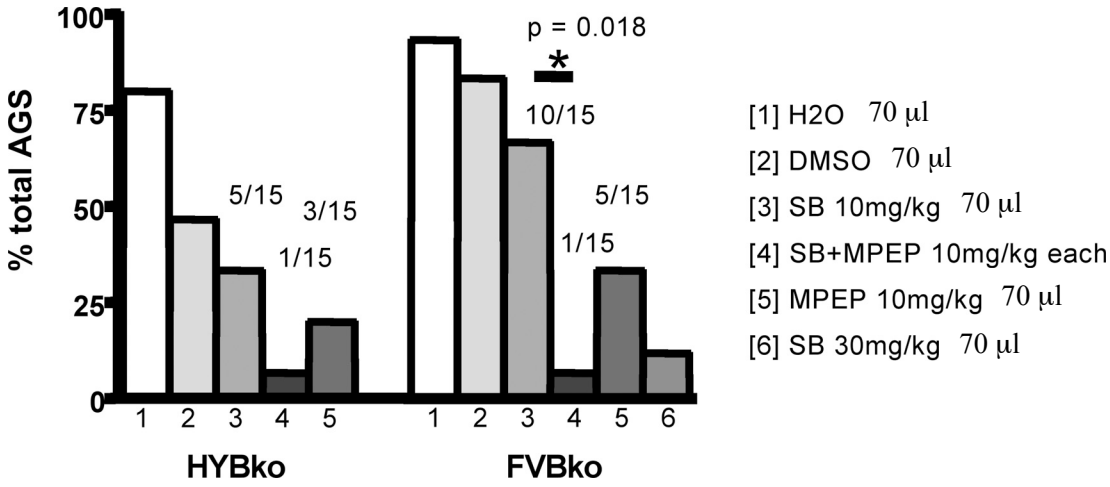
100
75
50
25
0
% total AGS
5/15
3/15
1/15
p = 0.018
*
10/15
5/15
1/15
1 2 3 4 5
1 2 3 4 5 6
HYBko
FVBko
[1] H2O 70 µl
[2] DMSO 70 µl
[3] SB 10mg/kg 70 µl
[4] SB+MPEP 10mg/kg each
[5] MPEP 10mg/kg 70 µl
[6] SB 30mg/kg 70 µl

Figure S3