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Glycogen synthase kinase-3 inhibitors reverse deficits in long-term potentiation and cognition in Fragile X mice

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Abstract

Background—Identifying feasible therapeutic interventions is crucial for ameliorating the intellectual disability and other afflictions of Fragile X Syndrome (FXS), the most common inherited cause of intellectual disability and autism. Hippocampal glycogen synthase kinase-3 (GSK3) is hyperactive in the mouse model of FXS (FX mice), and hyperactive GSK3 promotes locomotor hyperactivity and audiogenic seizure susceptibility in FX mice, raising the possibility that specific GSK3 inhibitors may improve cognitive processes.

Methods—We tested if specific GSK3 inhibitors improve deficits in *N*-methyl-D-aspartate receptor (NMDAR)-dependent long term potentiation (LTP) at medial perforant path synapses onto dentate granule cells (MPP-DGC) and dentate gyrus-dependent cognitive behavioral tasks.

Results—GSK3 inhibitors completely rescued deficits in LTP at MPP-DGC synapses in FX mice. Furthermore, synaptosomes from the dentate gyrus of FX mice displayed decreased inhibitory serine-phosphorylation of GSK3β compared with wild-type littermates. The potential therapeutic utility of GSK3 inhibitors was further tested on dentate gyrus-dependent congnitive behaviors. *In vivo* administration of GSK3 inhibitors completely reversed impairments in several cognitive tasks in FX mice, including novel object detection, coordinate and categorical spatial processing, and temporal ordering for visual objects.

Conclusions—These findings establish that synaptic plasticity and cognitive deficits in FX mice can be improved by intervention with inhibitors of GSK3, which may prove therapeutically beneficial in FXS.

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Keywords

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Introduction

Currently there are no adequate therapies for the treatment of Fragile X Syndrome (FXS), the most prevalent form of inherited mental retardation and the most common cause of autism (1, 2). FXS results from suppressed *FMR1* gene expression and deficiency in fragile X mental retardation protein (FMRP). The predominant characteristic of FXS is intellectual disability, which can be accompanied by hyperactivity, attention deficit, anxiety, seizures, and behaviors characteristic of autism spectrum disorders (3–7). Although some symptoms can be alleviated by anticonvulsants, antidepressants, stimulants and antipsychotics (8), or newly developed drugs affecting glutamatergic (9) and GABAergic (10) neurotransmission, no approved agent improves the central feature of FXS, impaired cognition. Insight into this fundamental issue has been attained using mice with genetic deletion of the *Fmr1* gene to model FMRP deficits in FXS (5, 11, 12). Initial studies using *Fmr1* knockout (FX) mice surprisingly reported normal *N*-methyl-D-aspartate receptor (NMDAR)-dependent long-term potentiation (LTP) and depression (LTD) at hippocampal CA1 synapses (13, 14), forms of synaptic plasticity that underlie learning and memory, whereas metabotropic glutamate receptor dependent LTD (mGluR-LTD) was pathologically enhanced (14). This and additional evidence led to the mGluR theory of FXS (15), which proposes that enhanced mGluR signaling is a major cause of the pathological deficits in FXS. Decreasing mGluR5 function corrects several behavioral and morphological phenotypes in developing and adult FX mice (16). Additionally, chronic, but not acute, inhibition of mGluR5 reverses cognitive deficits in young adult FX mice (17). However, recent studies investigating the dentate gyrus, a subregion of the hippocampal formation important for pattern separation, discovered NMDAR hypofunction and deficits in NMDAR-dependent LTP at medial perforant path synapses onto dentate granule cells (MPP-DGC) (18, 19). Interestingly, these deficits were accompanied by deficits in context discrimination, a behavior requiring normal function of the dentate gyrus (18, 20). These findings represent a clear link between synaptic dysfunction and learning deficits in FX mice and provide a synaptic and behavioral paradigm to investigate potential treatments.

The clinically used mood stabilizer lithium is a promising therapeutic agent for FXS because it reverses several behavioral phenotypes, attenuates enhanced mGluR-LTD in FX mice (21–23), and improves a cognitive task in FXS patients (24). Because lithium has a low therapeutic index and can cause side effects at serum concentrations modestly above the therapeutic level, identifying its target in FX mice could lead to the design of more specific and efficacious treatments for FXS. Lithium was first identified as a potential treatment for FXS by the seminal finding that lithium treatment was effective in a Drosophila model of FXS, which may have been due to inhibition of inositol monophosphatase (25). Other evidence shows that glycogen synthase kinase-3 (GSK3) is a target inhibited by lithium (26). The two isoforms of the Ser/Thr kinase GSK3 are primarily regulated by inhibitory phosphorylation at Ser21 in GSK3α and Ser9 in GSK3β which is often mediated by Akt (27). GSK3 has been implicated in the pathology of FXS because it is hyperactive in hippocampus, striatum, and cerebral cortex in FX mice and GSK3 inhibition decreases locomotor hyperactivity and audiogenic seizure susceptibility in FX mice (21, 28). Futhermore, LTP induction requires inhibition of GSK3β through increased Ser-9-phosphorylation, while GSK3β activity is required for LTD induction (29). Therefore, GSK3 is a bidirectional regulator of NMDAR-dependent synaptic plasticity. Additionally, artificially increasing GSK3 activity in adult wild-type (WT) mice impairs LTP (30, 31), a

finding consistent with the concept that pathologically increased GSK3 activity in FX mice could be causally related to deficits in LTP at MPP-DGC synapses. Thus, inhibition of GSK3 is likely an important component of the benefits of lithium in FX mice, raising the possibility that GSK3 may be a target for the development of new treatments for FXS. Here we tested the hypothesis that pharmacological inhibition of GSK3 rescues deficits in LTP at MPP-DGC synapses and dentate gyrus-dependent learning tasks. We report that lithium and selective GSK3 inhibitors, but not mGluR5 inhibition, reverse both the LTP deficit and cognitive impairments in FX mice, establishing GSK3 as an independent target for therapeutic development to treat FXS.

Methods and Materials

Antibodies were obtained from Cell Signaling Technology (Beverly, MA), LiCl and picrotoxin from Sigma (St. Louis, MO), Chir99021 (CT99021) from Biovision (Milpitas, CA) and 2-methyl-6-(phenylethynyl)pyridine (MPEP) from Tocris Bioscience (Ellisville, MO). Thiadiazolidindione-8 (TDZD-8) and N′-dodecanoyl-1-ethyl-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carbohydrazide (VP0.7) were prepared in the Martinez laboratory (35,36) and administered in 5% Tween80, 5% DMSO in saline.

Mice were housed in light and temperature controlled rooms and treated in accordance with National Institutes of Health, the University of Miami, and the University of Alabama at Birmingham Institutional Animal Care and Use Committee regulations. Crude synaptosomes were isolated from hippocampal subfields through a series of centrifugation steps and protein levels were determined using standard procedures. (32). Assessments of visual object novelty detection, temporal ordering for visual objects, coordinate and categorical spatial processing were carried out by published methods (33). Supplementary Information contains further details.

Coronal slices (400uM) were prepared from dorsal hippocampus of WT and FX mice. Extracellular dendritic field potential recordings (fEPSPs) at either Schaffer collateral (CA3-CA1) or MPP-DGC synapses were generated by baseline stimulation (0.1 Hz, 200μs duration). LTP was induced using high-frequency stimulation (HFS, 100 Hz, 1 sec duration × 4, 60 sec interval). To isolate excitatory inputs, all recordings were carried out in the presence of 100 μM picrotoxin. All n values represent animal number. See Supplementary Information for further details.

Data are expressed as mean ± SEM. Student’s t test, one-way ANOVA followed by Bonferroni’s post-hoc multiple comparison, and Kruskall-Wallis with Dunn’s multiple comparison test were used as noted. Significance was taken as $p<0.05$.

Results

Selective LTP deficit at medial perforant path-dentate granule cell synapses is accompanied by decreased serine-phosphorylated GSK3β

Using acute brain slices and high frequency stimulation (HFS), the LTP magnitude at CA3-CA1 synapses in slices from FX mice is not different from WT mice (WT: 197±12% of baseline fEPSP slope vs FX: 215±23% of baseline fEPSP slope, $p>0.05$) (Fig. 1A). In contrast, there is a significant LTP deficit in FX mice at MPP-DGC synapses (WT: 183±14% vs FX: 130±8%, $p<0.05$) (Fig. 1B). These results confirm previous reports (14, 18), and importantly, demonstrate hippocampal region-specific deficits in NMDAR-dependent synaptic plasticity in FX mice.

In hippocampal homogenates from FX mice, inhibitory serine-phosphorylation of GSK3β (p-GSK3β) is decreased, indicating increased activity (21, 28). However, it is not known whether the decrease in p-GSK3β is hippocampal region specific. Because GSK3 negatively regulates NMDAR-dependent LTP (29–31), the decrease in p-GSK3 should occur in DG but not CA1, if pathologically increased GSK3 activity is causing the specific deficit in LTP at MPP-DGC synapses. Western blot analysis of synaptosomal fractions revealed that p-GSK3β levels are unchanged in CA1 (135±8% of control, $p>0.05$) but decreased in DG (53±2% of control, $p<0.05$) (Fig. 1C and D) from FX mice compared to WT mice, whereas total GSK3β levels were equivalent. These results raise the possibility that hyperactive GSK3 in DG contributes to the deficit in LTP at MPP-DGC synapses.

Pharmacological blockade of GSK3 rescues the deficit in LTP at MPP-DGC synapses

Reduced p-GSK3β levels coupled with the LTP deficit specifically in DG suggest that pharmacological GSK3β inhibition might reverse the synaptic dysfunction. Because lithium rescues some behavioral phenotypes in FX mice and it inhibits GSK3 (21, 22), we first investigated whether bath application of LiCl (20 mM) rescues the LTP deficit. Indeed, LiCl, applied 30 min prior to HFS, significantly increases the LTP magnitude at MPP-DGC synapses in slices from FX mice (FX: 130±8% vs FX+Li: 162±15%, $p<0.05$) (Fig. 2B) without affecting the LTP magnitude at WT synapses (WT: 183±14% vs WT+Li: 183±12%, $p>0.05$) (Fig. 2A). In fact, the LTP magnitude is equivalent in WT and FX+Li treated slices (WT: 183±14% vs FX+Li: 162±15%, $p>0.05$) (Fig. 2C and D), indicating a complete rescue of the LTP deficit.

In order to confirm that lithium is rescuing LTP specifically through inhibition of GSK3 rather than off-target effects, we utilized CT99021, a highly selective GSK3 inhibitor (34). Slices were perfused with either vehicle or CT99021 (2 μM) for at least 30 min prior to HFS. Similar to LiCl treatment, CT99021 completely rescued the deficit in LTP in FX slices but had no significant effect on WT slices (WT: 146±5% vs WT+CT99021: 164±12%, $p>0.05$) (FX: 115±7% vs FX+CT99021: 146±10%, $p<0.05$) (WT: 146±5% vs FX +CT99021: 146±10%, $p>0.05$) (Fig. 2E-H). Collectively, these results conclusively demonstrate that inhibition of GSK3 reverses the deficit in LTP at MPP-DGC synapses.

Pharmacological blockade of GSK3 reverses the deficit in steady-state depolarization in FX slices during HFS

Because LTP induction requires sufficient depolarization to relieve the voltage-dependent Mg^{2+} block from NMDARs, we next investigated whether the deficit in LTP and subsequent rescue by GSK3 inhibition was due to alterations in the depolarization during HFS. We measured steady-state depolarization during the fourth round of HFS in WT and FX slices and found a significant decrease in FX slices compared to WT slices, which was reversed by LiCl (Fig. 3A and B) and CT99021 (Fig. 3C and D). These findings raise the possibility that GSK3 inhibition rescues the LTP deficit by increasing neurotransmitter release. However, we found no significant differences in the paired pulse ratio, an indirect measure of presynaptic neurotransmitter release probability (35), between WT and FX slices under any condition or interstimulus interval (Fig. S1 A and B), indicating that enhanced neurotransmitter release may not be the mechanism underlying rescued LTP.

Cognitive deficits in FX mice are rescued by in vivo GSK3 inhibition

The rescue of the LTP deficit at MPP-DGC synapses suggests that GSK3 inhibition should also rescue deficits in dentate gyrus-dependent learning in FX mice previously reported (18, 36–38). Thus a visual object novelty detection task, which requires the dentate gyrus (39–41) and assesses the ability to discriminate between a familiar and novel object, was used to evaluate the potential benefits of GSK3 inhibition on learning deficits in FX mice. WT mice

spent significantly more time exploring the novel versus familiar object (20±3 sec vs 4±1 sec, $p<0.01$), demonstrating learning (Fig. 4A). In contrast, FX mice spent equivalent amounts of time exploring the novel versus familiar object (8±2 sec vs 7±1 sec), indicating that FX mice are unable to learn the task. The exploration ratio (calculated by dividing the difference between the time spent with the novel object versus the familiar object divided by total time exploring) was significantly different between WT and FX mice (exploration ratio WT: 0.64±0.09; FX: −0.07±0.06, $p<0.05$) (Fig. 4B).

Due to the non-selective effects of LiCl and the limited solubility and CNS penetration of CT99021, GSK3 inhibition *in vivo* was achieved using two other selective GSK3 inhibitors with CNS bioavailability, TDZD-8 (5 mg/kg; ip), a highly selective ATP non-competitive inhibitor (42), and VP0.7 (5 mg/kg; ip), an allosteric (not competitive with ATP or substrate) selective GSK3 inhibitor (43). The GSK3 inhibitors did not alter the performance of WT mice, which spent more time investigating the novel versus familiar object (TDZD-8: 16±3 sec vs 3±1 sec, $p<0.01$; VPO.7: 21±2 sec vs 6±1 sec, $p<0.01$) (Fig. 4A). However, FX mice treated with TDZD-8 or VPO.7 spent significantly more time exploring the novel versus familiar object (TDZD-8: 20±2 sec vs 2±1 sec, $p<0.01$; VPO.7: 19±2 sec vs 3±1 sec, $p<0.01$), indicating that under conditions of GSK3 inhibition FX mice are capable of learning the task. Furthermore, the exploration ratio was significantly increased in FX mice treated with TDZD-8 or VP0.7, but had no effect in WT mice (FX exploration ratio: TDZD-8: 0.79±0.03; $p<0.05$; VP0.7: 0.72±0.04; $p<0.05$) (WT exploration ratio: TDZD-8: 0.69±0.05; VP0.7: 0.56±0.03) (Fig. 4B), indicating that GSK3 inhibition completely reverses the learning deficit in FX mice.

Next, we assessed whether FX mice displayed deficits in pattern separation using coordinate and categorical tasks, which require the dentate gyrus (33, 40, 44). In the coordinate spatial learning task, the distance between two identical objects is altered between the habituation and testing periods. Pattern separation is indicated when significantly more time is spent exploring objects during the 5 min testing period after repositioning the objects compared to the last 5 min of the habituation phase. WT mice displayed increased object exploration time during testing compared to the last 5 min of the habituation phase (WT exploration ratio: 0.61±0.05) (Fig. 4C), indicating successful pattern separation. In contrast, FX mice spent significantly less time than WT exploring the objects during the test period, indicating impaired behavior in this task (FX exploration ratio: 0.31±0.05, $p<0.05$). While neither TDZD-8 nor VP0.7 altered behavior of WT mice (WT exploration ratio TDZD-8: 0.60±0.05; VP0.7: 0.65±0.07), both drugs reversed the deficit in FX mice, as they spent significantly more time exploring the objects during testing compared to habituation (FX exploration ratio TDZD-8: 0.62±0.06, $p<0.05$; VP0.7: 0.66±0.08, $p<0.05$). The categorical spatial learning task involves interchanging the positions of two identical objects following the habituation phase, while maintaining the same distance between them. FX mice spent significantly less time than WT mice exploring the objects after they had been transposed (FX exploration ratio: 0.36±0.03; WT exploration ratio: 0.66±0.05, $p<0.05$) (Fig 4D), again revealing impaired spatial pattern separation in FX mice. Administration of GSK3 inhibitors did not alter the amount of time WT mice spent exploring the objects after they were transposed (WT exploration ratio: TDZD-8: 0.70±0.05; VP0.7: 0.80±0.03), but significantly increased the exploration times of FX mice (FX exploration ratio: TDZD-8: 0.63±0.06, $p<0.05$; VP0.7: 0.75 ± 0.04, $p<0.05$), demonstrating a reversal of the deficit. Thus, the results of the coordinate and categorical spatial learning tests demonstrate impaired function of the dentate gyrus in FX mice that is normalized by the administration of GSK3 inhibitors.

Finally, we assessed whether FX mice have deficits in temporal ordering of visual objects, a dorsal and ventral hippocampal CA1-dependent task in which rodents spend less time exploring the object most recently presented during a previous habituation period (33, 45–

49). In this task, we exposed mice to a series of 3 pairs of objects and then measured the time spent with the initial object when it was reintroduced along with the most recent object. Successful temporal ordering is evident when more time is spent exploring the initial object. WT mice displayed successful temporal ordering because more time was spent exploring the initial object (13±1 sec vs 7±2 sec, $p<0.05$), whereas FX mice spent significantly less time exploring the initial object presented (7±1 sec vs 16±2 sec, $p<0.01$) (Fig. 4E). Thus, the object exploration ratio (calculated by dividing the difference between the time spent with the initial object (object 5) versus the more recent object (object 7) by total time exploring), differed significantly between FX and WT mice, revealing a temporal order deficit (WT exploration ratio: 0.32±0.10; FX exploration ratio: −0.41±0.05, $p<0.05$) (Fig. 4F). FX mice treated with either GSK3 inhibitor, TDZD-8 or VP0.7, spent significantly more time exploring the first object compared to the most recent object presented (TDZD-8: 12±2 sec vs 4±2 sec, $p<0.01$; VP0.7: 9±3 sec vs 3±1 sec, $p<0.01$). Similarly, WT mice treated with TDZD-8 or VP0.7 spent significantly more time exploring the first versus the recent object (TDZD-8: 10±1 sec vs 5±1 sec, $p<0.01$; VP0.7: 11±1 sec vs 3±1 sec, $p<0.01$). Thus, administration of TDZD-8 or VP0.7 significantly increased the exploration ratio in FX mice (FX exploration ratio: TDZD-8: 0.47±0.07, $p<0.05$; VP0.7: 0.56±0.07; $p<0.05$), (Figure 4F), eliminating the impairment in temporal ordering. TDZD-8 or VP0.7 treatment also tended to improve the behavior of wild-type mice in this task (WT exploration ratio: TDZD-8: 0.37±0.08; VP0.7: 0.60±0.06) (Figure 4F). These results demonstrate that temporal ordering of visual objects is impaired in FX mice and that this deficit is corrected by inhibition of GSK3.

Synaptic and cognitive deficits in FX mice are not rescued by mGluR5 inhibition

Because inhibition of mGluR reverses many of the synaptic and behavioral phenotypes in FX mice (17, 50), we investigated whether MPEP, an mGluR5 antagonist, would also reverse the dentate gyrus associated deficits in synaptic plasticity and learning and memory. We show using bath application of MPEP (100 uM) that mGluR5 inhibition fails to rescue LTP magnitude in FX slices (WT: 143±8%; WT+MPEP 127±9%, $p>0.05$) (FX: 116±11%; FX+MPEP: 101±7%, $p>0.05$) (WT:143±8%; FX+MPEP: 101±7%, $p<0.05$) (Fig. 5A–C). Treatment of FX mice with MPEP did not affect the impairments in novel object detection (FX: 6±3 sec vs 8±3 sec vs FX+MPEP: 13±3 sec vs 15±2 sec) (Fig. 5D) (FX exploration ratio: −0.48±0.23; MPEP: −0.06±0.13) (Fig. 5E), coordinate spatial processing (FX exploration ratio: 0.31±0.09; MPEP: 0.18±0.03) (Fig. 5F), and categorical spatial processing (FX exploration ratio: 0.35±0.04; MPEP: 0.43±0.07) (Fig. 5G). Administration of MPEP did not alter the performance of WT mice in any of these tasks. Thus, impaired LTP in the dentate gyrus and cognitive deficits in FX mice are not rescued by acute inhibition of mGluR5.

Discussion

Here we report a significant decrease in inhibitory serine p-GSK3 levels specifically in synaptosomes from the dentate gyrus of FX mice that is associated with a deficit in NMDAR-dependent LTP at MPP-DGC synapses and severe impairments in behavioral tasks dependent upon normal function of the dentate gyrus. Pharmacological inhibition of GSK3, but not an inhibitor of mGluR5, completely reverses both synaptic and behavioral deficits, strongly suggesting that GSK3 should be considered as a therapeutic target for the treatment of cognitive dysfunction in FXS.

Intellectual disability is the predominant trait of FXS, but it is not improved by currently available medications. Advances in treatment strategies for cognitive impairment that is characteristic of FXS were initially slowed by the difficulty in identifying deficits in FX mice, as FX mice behave normally or display only mild impairments in several cortical- and

hippocampal-dependent cognitive tasks, such as the Morris water maze, radial arm maze, operant conditioning paradigms and contextual and conditioned fear memory (11, 51–59), rather than the severe impairments that would be expected in a valid FXS model. However, deficits in passive avoidance behavior have been observed, and more recently, robust deficits were identified in tasks that are heavily dependent upon normal function of the dentate gyrus, specifically, novel object recognition and context discrimination, which assesses pattern separation (18, 36–38). Here we extend these findings by demonstrating significant deficits, not only in novel object detection, but also in coordinate and categorical spatial processing tasks. Deficits in pattern separation are associated with NMDAR hypofunction and deficits in LTP at MPP-DGC synapses in FX mice previously reported (18) and because LTP is a cellular correlate of learning and memory (60), this deficit in LTP likely contributes to the impairment in dentate gyrus-dependent cognitive tasks in FX mice evaluated here.

GSK3 inhibitors have been proposed as potential therapeutic candidates for treating FXS, based to a large extent on the multiple beneficial effects resulting from administration of the GSK3 inhibitor lithium to FX mice (61). Lithium treatment of FX mice reverses locomotor hyperactivity, audiogenic seizure hypersensitivity, increased spine density, enhanced mGluR-mediated LTD, reactive astrocytes, macroorchidism, excess protein synthesis, and social behavior deficits (21–23, 28, 62–64). Furthermore, lithium improved deficient passive avoidance behavior, the only reported test of lithium's effect on cognitive impairments in FX mice. These actions of lithium are likely due to inhibition of GSK3 because GSK3 inhibitors other than lithium also have been reported to control locomotor hyperactivity, susceptibility to audiogenic seizures, trace conditioning, delayed non-matching-to-place radial arm maze and neurogenesis in FX mice (28, 65). Thus it appears that GSK3 inhibitors, including lithium, are able to normalize many abnormal characteristics of FX mice and thus are potential therapeutic interventions for FXS. Importantly, lithium treatment also proved beneficial in a cognitive task in FXS patients (24), suggesting that cognitive deficits in FX mice may be ameliorated by administration of specific inhibitors of GSK3, leading us to directly test this hypothesis. We found that both the impaired MPP-DGC LTP and impaired novel object detection and coordinate and categorical spatial processing in FX mice were repaired by administration of GSK3 inhibitors, suggesting that GSK3 inhibition reversed these cognitive deficits by normalization of LTP. These findings are in accordance with reports that hyperactive GSK3 impairs LTP (30, 31), and that GSK3 is hyperactive in the hippocampus of FX mice (21, 28), specifically in synapstosomes from the dentate gyrus, reported here.

Despite finding no difference in inhibitory serine p-GSK3 levels in CA1 synaptosomes or LTP magnitude at CA3-CA1 synapses between WT and FX mice, we observed a deficit in temporal ordering of objects, a visual task dependent upon area CA1 (48), which is surprisingly reversed by GSK3 inhibition (13, 14). However, because normal function of the hippocampal trisynaptic circuit is required during behavioral tasks, deficits at MPP-DGC synapses are likely propagated to downstream CA3-CA1 synapses, impacting CA1 dependent behavior, even though LTP at CA3-CA1 synapses is normal in slices from FX mice. In support of this concept, GSK3 inhibition, which reverses LTP deficits at MPP-DGC synapses, also normalizes temporal ordering deficits indicating that systemic GSK3 inhibition improves the overall function of the hippocampal tri-synaptic circuit. Another possibility is that there are deficits in plasticity present at the temporoammonic pathway, the monosynaptic projection from entorhinal cortex directly onto distal dendrites of CA1, that contribute to deficits in temporal ordering of objects. Our results indicate that if such deficits exist and underlie termporal order learning, inhibition of GSK3 will also rescue deficits at temporoammonic synapses although this possibility has not been explored.

Because aberrant mGluR5 function is known to regulate several of the phenotypes of FX mice and acute administration of the mGluR5 antagonist MPEP to FX mice reverses deficits in several behaviors and mEPSC frequency in the amygdala (28, 58, 66–69), we tested if administration of MPEP also reversed deficits in LTP and cognition. Acute administration of MPEP was completely ineffective in reversing the impaired MPP-DGC LTP and cognitive impairments in FX mice, in accordance with a report that MPEP fails to rescue LTP deficits in amygdala (67). Collectively, these results indicate that although mGluR5 is a promising therapeutic target (17, 50), GSK3 inhibitors must also be considered for treatment of FXS. Additonally, because acute GSK3, but not acute mGluR5, inhibition reverses these deficits, mGluR5 is not directly driving the pathologically hyperactive GSK3 in FX mice. However, a recent study showed that chronic, but not acute, mGluR5 inhibition reversed novel object recognition deficits (70), so aberrant mGluR function in FX could indirectly modulate GSK3 activity.

To fully understand how hyperactive GSK3 leads to synaptic and cognitive impairments in FX, identification of downstream targets of GSK3 is needed. Both LTP and pattern separation require proper NMDAR function. Hyperactive GSK3 may diminish NMDAR transmission which can be reversed by GSK3 inhibition, an idea consistent with GSK3-dependent NMDAR internalization in cortical neurons (71). Alternatively, inhibition of GSK3 may rescue LTP and cognition through effector targets downstream of NMDAR activation. GSK3 regulates AMPAR trafficking (72), however this mechanism is unlikely since there are no deficits in baseline AMPAR transmissison in FX mice. Other mechanisms upstream and downstream of GSK3 suspected to be involved in modulation of plasticity have been eloquently discussed (73). It will be of great interest in future studies to elucidate the precise mechanisms by which hyperactive GSK3 decreases plasticity and learning and memory, as these mechanisms represent novel targets for therapeutic development for the treatment of intellectual disability in FXS.

The cognitive assessments we studied in FX mice may model the nonverbal measures of intelligence used in FXS patients. Patients with FXS display impaired recognition memory, spatial memory, working memory and short-term memory (3, 74–76). FXS patients also have difficulty with inhibition and attentional control that is consistent with the memory deficits (77). The visual object novelty detection task and the temporal order memory task were used to assess recognition memory, working memory and short-term memory in FX mice. The coordinate metrical and categorical topological spatial learning tasks were used to assess spatial memory in FX mice. All of these cognitive abilities were impaired in FX mice and were corrected by administration of GSK3 inhibitors. Thus, abnormally active GSK3 in the hippocampus of FX mice (21, 22, 28, 78) appears to play an important role in these cognitive deficits, further supporting the possibility that GSK3 inhibitors may be beneficial for multiple aspects of FXS, including intellectual disability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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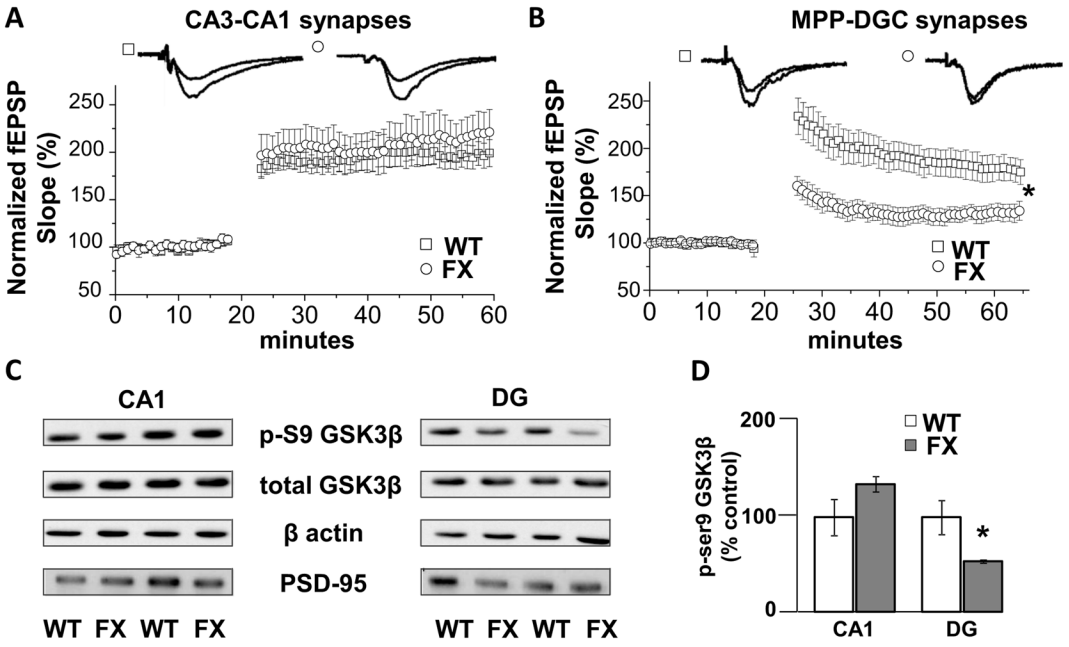

Figure 1.

Deficits in LTP at MPP-DGC synapses in FX mice are accompanied by decreased inhibitory serine-phosphorylation of GSK3β. (A) Summary plots of the magnitude of LTP induced by HFS at CA3-CA1 Schaffer collateral synapses in slices from WT (n=7) and FX (n=6) mice. (B) Summary plots of the magnitude of LTP induced by HFS at MPP-DGC synapses in slices from WT (n=6) and FX (n=9) mice. (C) Representative Western blots showing a reduction in phospho-serine9-GSK3β (p-S9 GSK3) protein in dentate gyrus (DG) but not in CA1 from FX versus WT mice. Total GSK3β protein levels are not different between groups. β-Actin and PSD-95 were used as cytosolic and synaptic protein loading controls. (D) Quantitation of the ratio of p-S9 GSK3β to total GSK3β normalized to values of WT mice (CA1, n=4, and DG, n=6, for each genotype). *p<0.05 (Student's t test).

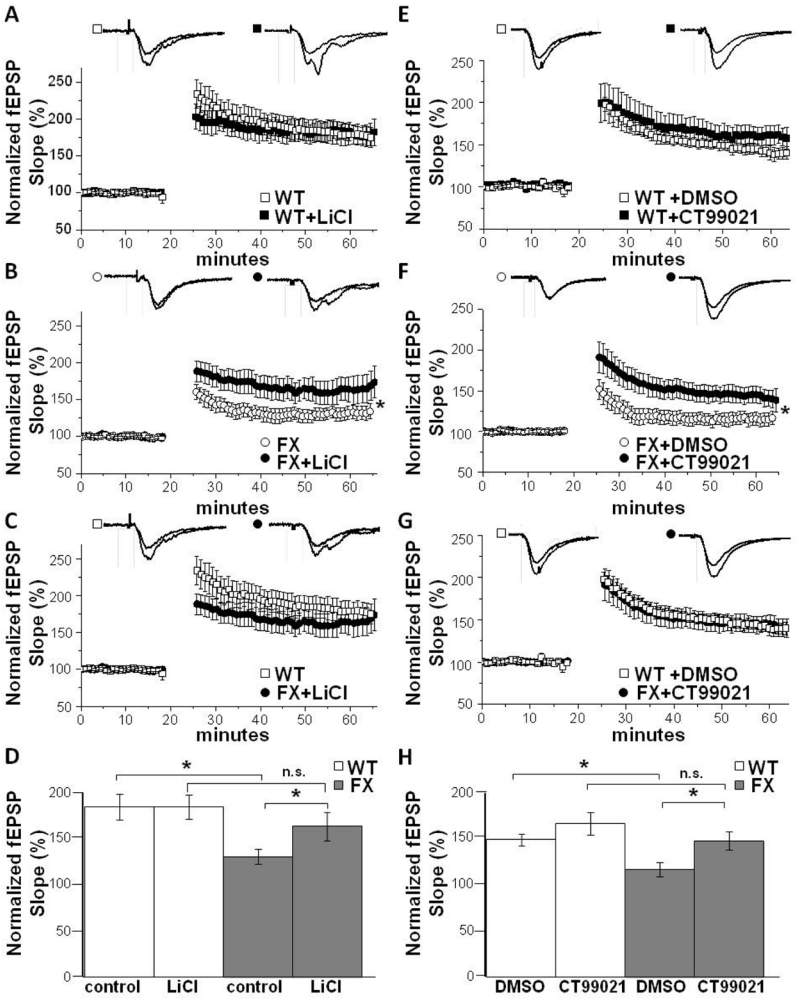

Figure 2.

Pharmacological blockade of GSK3 reverses the deficit in LTP at MPP-DGC synapses. Summary plots of the magnitude of LTP induced by HFS at MPP-DGC synapses in slices from (A) WT mice with (n=6) and without (n=6) bath application of LiCl (20 mM), and (B) FX mice with (n=6) and without (n=9) bath application of LiCl (20 mM). (C) Data from WT mice (from A) and FX mice (bath application of LiCl from B) replotted to compare the magnitudes of LTP.(D) Summary of data A–C. (E) WT mice with (n=8) and without (n=12) bath application of CT99021 (2 uM). (F) FX mice with (n=7) and without (n=7) bath application of CT99021. (G) WT (from E) and FX (bath application of CT99021 from F) replotted to compare the magnitudes of LTP. *p<0.05 (Student's t test). (H) Summary of data E–F

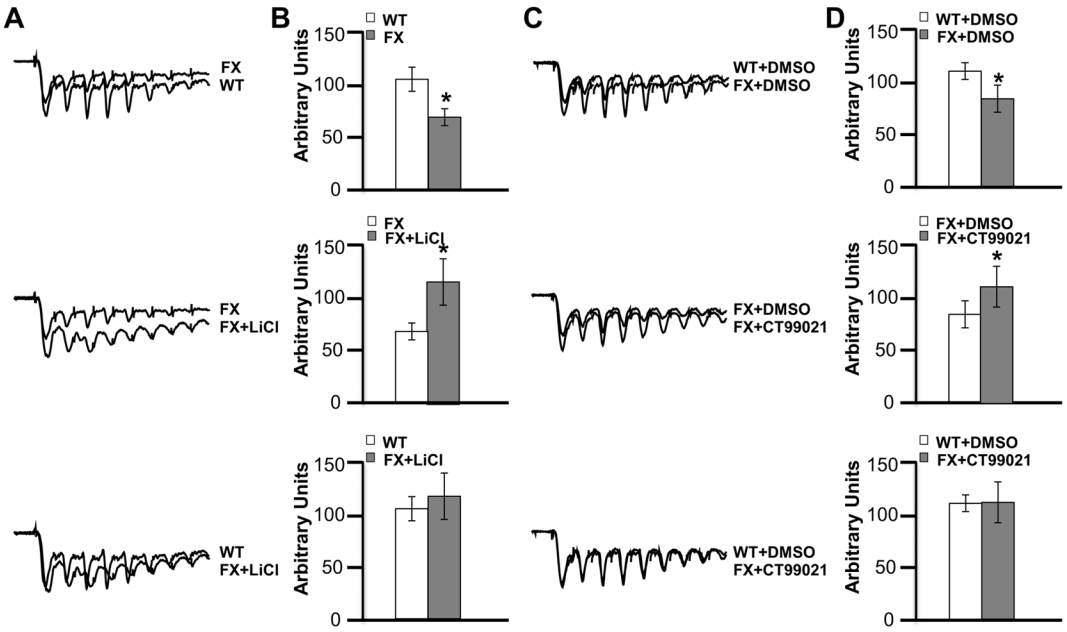

Figure 3.

Pharmacological blockade of GSK3 does not alter steady-state depolarization. (A) Averaged traces during the 4th tetanus from experiments in Fig 2A–C show reduced steady-state depolarization during tetanus in FX slices. (B) Pooled data from all LTP experiments in Fig 2 show LiCl treatment reverses the deficit in steady state depolarization in FX slices. (WT: 110±12 vs FX: 72±8) (FX: 71±8 vs FX+Li: 123±23) (WT: 110±12 vs FX+Li: 123±23) *p<0.05 (Student's t test). (C) Averaged traces during the 4th tetanus from experiments in Fig 2D–E show reduced steady-state depolarization during tetanus in FX slices. (D) Pooled data from experiments in Fig 2 show CT99021 treatment reverses the deficit in steady state depolarization in FX slices. (WT: 116±8 vs FX: 71±8) (FX: 88±13 vs FX+CT99021: 117±20)(WT: 116±8 vs FX+CT99021: 117±20) *p<0.05 (Student's t test)

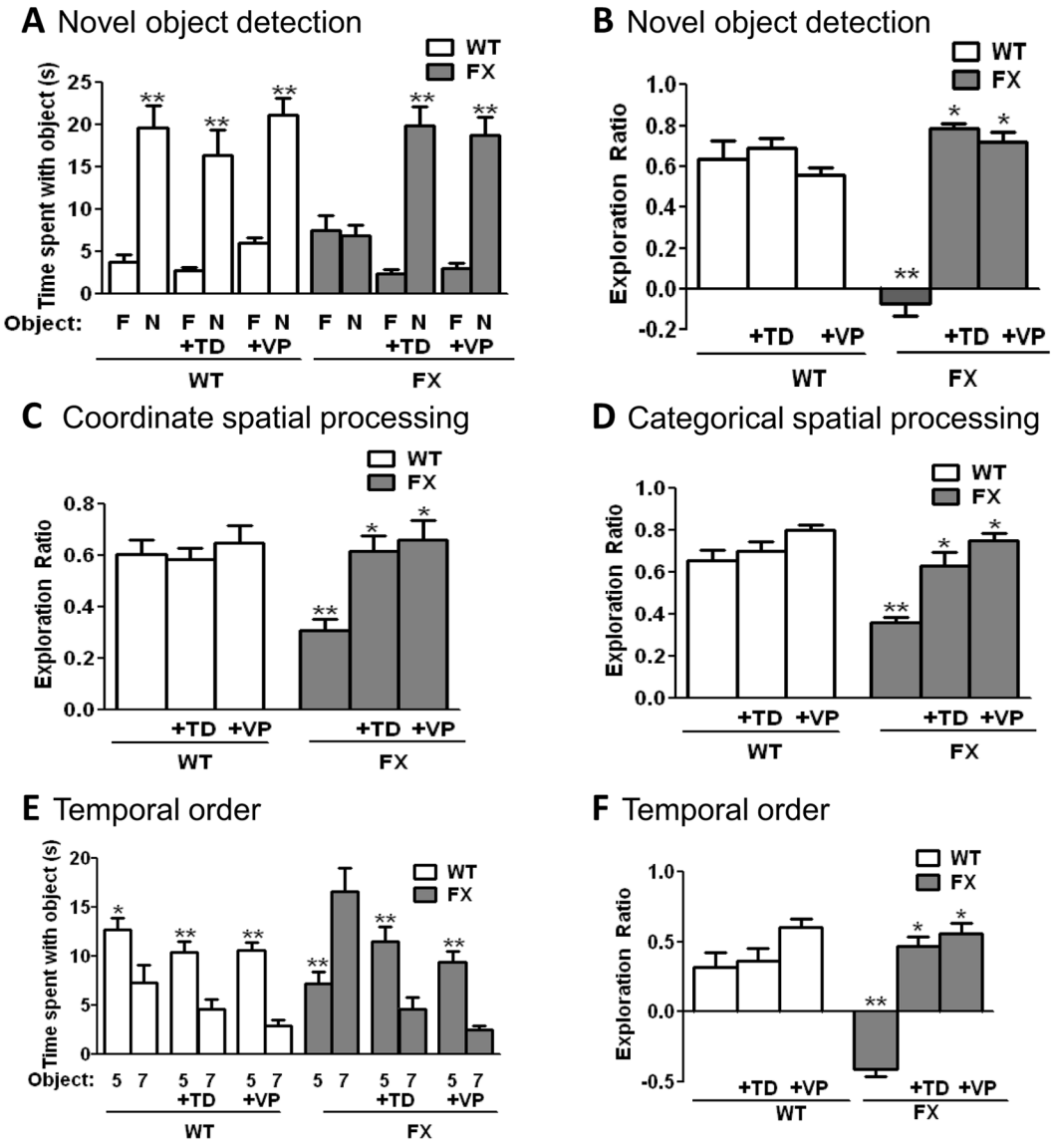

Figure 4. Inhibition of GSK3 ameliorates cognitive impairments in FX mice

FX and WT mice were treated with 5 mg/kg of TDZD-8 (TD) or VP0.7 (VP) 1 hr prior to cognitive assessments. (A,B) Performance in the novelty detection for visual objects task. (A) Times spent exploring the novel (N) and familiar (F) object. **p<0.01 compared to time spent with familiar object (Student's t test). (B) Exploration ratio. (C) Exploration ratio in the coordinate spatial processing task. (D) Exploration ratio in the categorical spatial processing task. (E,F) Performance in the temporal order for visual objects task. (E) Times spent exploring Object 5 and Object 7 (most recently explored). **p<0.01, *p<0.05 compared to time spent with Object 7 (Student's t test). (F) Exploration ratio. B, C, D and F: **p<0.05 compared to untreated wild-type mice; *p<0.05 compared to same genotype without treatment (Kruskall-Wallis [genotype x treatment] with Dunn's multiple comparison test). n=10–20 mice per group.

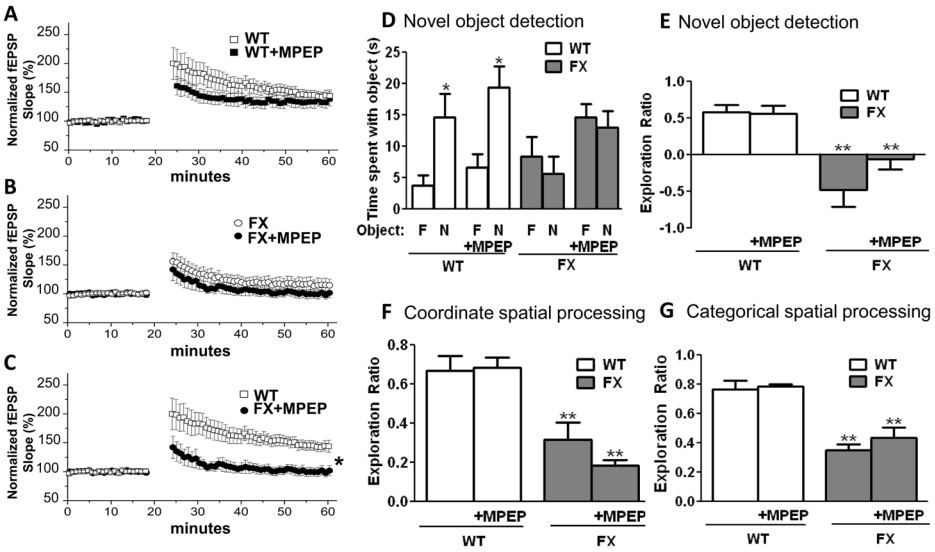

Figure 5.

Synaptic and cognitive deficits in FX mice are not altered by mGluR inhibition. Summary plots of the magnitude of LTP induced by HFS at MPP-DGC synapses in slices from (A) WT mice with (n=5) and without (n=5) bath application of MPEP (100 uM), and (B) FX mice with (n=7) and without (n=6) bath application of MPEP. (C) Data