Glycogen Synthase Kinase-3 Inhibitors Reverse Deficits in Long-term Potentiation and Cognition in Fragile X Mice

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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–dependent long-term potentiation at medial perforant path synapses onto dentate granule cells and dentate gyrus–dependent cognitive behavioral tasks.

Results: GSK3 inhibitors completely rescued deficits in long-term potentiation at medial perforant path–dentate granule cells 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 cognitive 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.

Key Words: Cognition, fragile X syndrome, glycogen synthase kinase-3, learning, long-term potentiation (LTP), synaptic plasticity

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. 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 gamma-aminobutyric acid (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 fragile X mental retardation protein 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 cornu ammonis area 1 (CA1) synapses (13,14), forms of synaptic plasticity that underlie learning and memory, whereas metabotropic glutamate receptor–dependent (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 pathologic deficits in FXS. Decreasing mGluR5 function corrects several behavioral and morphologic 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 (DG), 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 DG (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

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Received May 7, 2013; revised Jul 30, 2013; accepted Aug 1, 2013.

0006-3223/$36.00
http://dx.doi.org/10.1016/j.biopsych.2013.08.003

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). Furthermore, LTP induction requires inhibition of GSK3β through increased Ser-9-phosphorylation, whereas 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 pharmacologic inhibition of GSK3 rescues deficits in LTP at MPP-DGC synapses and DG-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, Massachusetts), LiCl and picrotoxin from Sigma (St. Louis, Missouri), Chir99021 (CT99021) from Biovision (Milpitas, California) and 2-methyl-6-(phenylethynyl)pyridine (MPEP) from Tocris Bioscience (Ellisville, Missouri). 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% dimethyl sulfoxide 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, and coordinate and categorical spatial processing were carried out using published methods (33). Supplement 1 contains additional details.

Coronal slices (400 microns) 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 (.1 Hz, 200-μs duration). LTP was induced using high-frequency stimulation (HFS, 100 Hz, 1-second duration × 4, 60-second interval). To isolate excitatory inputs, all recordings were carried out in the presence of 100-μmol/L picrotoxin. All *n* values represent animal number. See Supplement 1 for additional details.

Data are expressed as mean ± SEM. Student *t* test, one-way analysis of variance followed by Bonferroni post hoc multiple comparison, and Kruskall-Wallis with Dunn's multiple comparison test were used as noted. Significance was taken as $p < .05$.

Results

Selective LTP Deficit at MPP-DGC Synapses Is Accompanied by Decreased Serine-Phosphorylated GSK3β

Using acute brain slices and HFS, the LTP magnitude at CA3-CA1 synapses in slices from FX mice is not different from

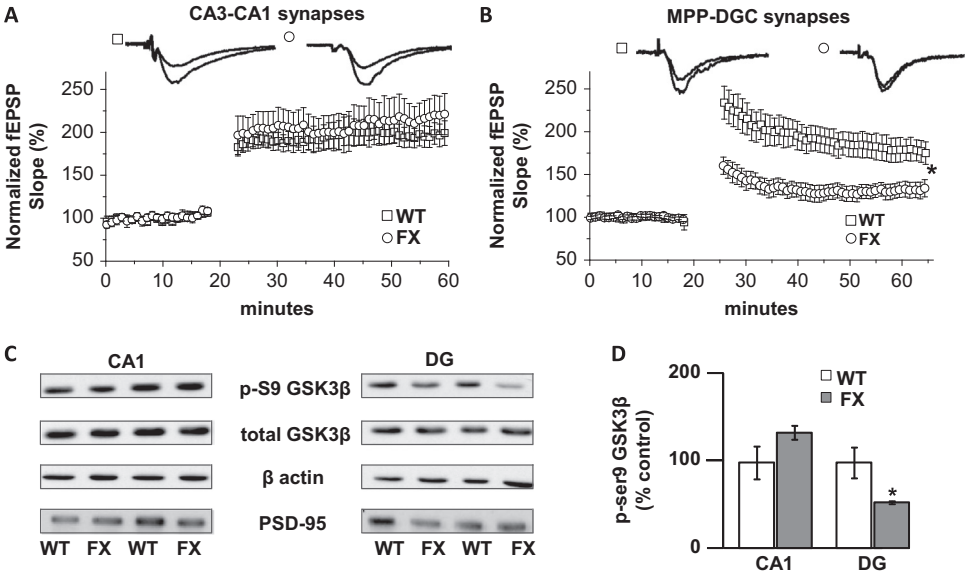

Figure 1. Deficits in long-term potentiation (LTP) at medial perforant path–dentate granule cells (MPP-DGC) synapses in fragile X syndrome (FX) mice are accompanied by decreased inhibitory serine-phosphorylation of glycogen synthase kinase-3 (GSK3)β. **(A)** Summary plots of the magnitude of LTP induced by high-frequency stimulation at cornu ammonis (CA)3-CA1 Schaffer collateral synapses in slices from wild-type (WT; $n = 7$) and FX ($n = 6$) mice. **(B)** Summary plots of the magnitude of LTP induced by high-frequency stimulation 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 GSK3β) 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 postsynaptic density protein 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). *$p < .05$ (Student *t* test). fEPSPs, extracellular dendritic field potential recordings.