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Review Article

The role of glycogen synthase kinase-3 signaling in neurodevelopment and fragile X syndrome

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Abstract: Fragile X syndrome (FXS), one of the most common genetic causes of autism, results from a loss of fragile X mental retardation protein (FMRP) expression. At the molecular level, abnormal neurodevelopment is thought to result from dysregulated protein synthesis of key neural synaptic proteins, however recent evidence suggests broader roles for this protein including glutamate signaling, memory, and regulation of the critical serine/threonine regulatory kinase, glycogen synthase kinase-3 (GSK-3). In this review, genetic and molecular features of FXS are detailed in the context of FXS neuropathology. Additionally, potential mechanisms by which FMRP silencing impacts GSK-3 and GSK-3-associated signaling pathways are discussed. As GSK-3 signaling represents a central regulatory node for critical neurodevelopmental pathways, understanding how FXS results from FMRP-mediated GSK-3 dysregulation may provide novel therapeutic targets for disease-modifying interventions for FXS and related ASDs.

Keywords: Glycogen synthase kinase, fragile X, neuroinflammation, trinucucleotide repeat, microglia, lithium, flavonoids

Introduction

Fragile X syndrome (FXS), originally known as Martin-Bell syndrome [1] was first characterized in 1943 [2] is the most common cause of inherited mental retardation and is the first identified autism-related genetic disorder. The primary symptom of FXS is intellectual disability, but patients also share characteristics commonly associated with autism spectrum disorders (ASDs), such as developmental delays, communication impairments, and anxiety [3-9]. The most severely affected FXS individuals additionally display dysmorphic features, and other neurological pathology, including seizures. In this review, genetic and molecular features of FXS are detailed in the context of FXS neuropathology. Finally, potential mechanisms by which FMRP silencing impacts GSK3 and GSK3-associated signaling pathways are discussed. As GSK3 signaling represents a central regulatory node for critical neurodevelopmental pathways, understanding how FXS results from FMRP-mediated GSK3 dysregulation may provide novel therapeutic targets for disease-modifying interventions for FXS and related ASDs.

Eitiology of FXS

The first evidence regarding the molecular origin of FXS was generated in 1969, at which time a non-typical constriction, or fragile site, was observed at the end of the X chromosome in several affected individuals [10, 11]. In 1991, the fragile site was mapped to a specific location in the genome [10]. The fragile X mental retardation 1 (Fmr1) gene on the X chromosome was found to yield a lack of the gene product, the fragile X mental retardation protein (FMRP), an RNA binding protein which regulates translation [3, 12]. This functional loss typically occurs when there is an expansion of the CGG trinucleotide repeat in the 5′ untranslated region (5′

UTR) of the fragile X mental retardation 1 (FMR1) gene [13]. Healthy individuals usually have between 5 to 54 repeats, but fully affected individuals have greater than 200 CGG repeats on what are known as "full mutation alleles" [14]. Permutation alleles (55-200 CGG repeats) of the FMR1 gene contribute to the FXS phenotype through genetic instability and can expand into the full mutation during the process of germline transmission [15, 16].

FMRP contains three RNA-binding domains and binds to a significant number of mRNAs. In *in vitro* studies, it has been found that dihydroxyphenylglycine-activated protein synthesis in synaptoneurosomes is reduced in a mouse model of FXS (the Fmr1 knockout mouse), which cannot produce full-length FMRP, suggesting that FMRP is involved in this process. FMRP is generated in synaptoneurosomes in response glutamate or metabotropic glutamate receptor (mGluR) agonists. Moreover, Fmr1 knockout mice demonstrate a substantial reduction in the ability to translate mRNA in response to activation in an experimental synaptoneurosome preparation as well as a reduction in the presence of postsynaptic polyribosomal aggregates *in vivo* [17].

Neuronal morphology and function in FXS

FXS patients and murine models of FXS demonstrate increased long-term depression (LTD) in hippocampal synapses [18]. FMRP functions to inhibit the synthesis of proteins that stabilize LTD. With functional loss of this protein, metabotropic glutamate receptor-5 (mGluR5) remains active and increases the synthesis of proteins associated with LTD. Increased activation of mGluR5 (and consequent increase in glutamate activity) has been implicated in audiogenic seizure activity associated with FXS. A study utilizing an mGluR antagonist and lithium to treat Fmr1 knockout mice found that the treatment alleviated mGluR-induced LTD [18]. Visualization of dendrites and dendritic spines can be performed using Golgi staining, which allows for quantitative evaluation [19] of developmental pruning of neural processes [20-22]. In humans, spine density on the dendritic apical shafts of cortical pyramidal cells increases within the first few months of life [23]. Autopsy tissue of normal human subjects ranging in age from fetal to adult revealed synapse density peaks between 3 months and 3.5 years, depending on the cortical region in question [24, 25]. Following this initial burst of synaptic development, synapses are selectively pruned, leaving synapse density measures at approximately 60% of their original peak numbers [26, 27], although somewhat smaller losses are observed when neuron density is taken into account [25]. Regardless of the biological basis for this developmental delay, dendritic spine dysgenesis frequently characterizes neuronal morphology in disorders associated with intellectual disability [28]. Studies utilizing samples from patients with FXS have suggested that dendritic spines do not assume a normal mature size and shape and that there are more dendritic spines per unit dendrite length in the patient samples compared to unaffected individuals. Similar findings on spine size and shape have come from studies of FXS model mice in which the development of the somatosensory cortical region contains barrel-like cell arrangements that process whisker sensory information [29]. This suggests that normal dendritic pruning is impaired in the knockout mice [17] and indicates that FMRP may be required for the normal processes of maturation and elimination to occur in cerebral cortical development [17].

Structural magnetic resonance imaging (MRI) has shown a reduction in the size of the posterior cerebellar vermis which may result in the enlargement of the fourth ventricle in males with FXS [30, 31]. Such gross morphological aberrations are not unique to the cerebellum, because the volume of hippocampus [32], caudate nucleus, and lateral ventricles [33, 34] also have all been noted to be enlarged in FXS patients. The generalizability of these observations is controversial as several of these differences in brain morphology have not been replicated in a study using physical measurements of autopsy material from one underpowered study (2 FXS patients) [35].

At the molecular level, the consequence of the aforementioned CGG trinucleotide expansion in the 5' untranslated region of the FMR1 gene leads to a hypermethylation of the promoter region of the DNA, thus silencing transcription of the gene and resulting in the absence of FMRP. The function of FMRP has not yet been fully elucidated, although it is found to be associated with polyribosomal complexes near synapses and contains mRNA-binding domains. This suggests that it may be involved in mRNA

transport or translation of proteins required for synaptic plasticity [36]. FMRP's role as an mRNA-binding protein is so critical for normal development that a point mutation in one of its RNA binding sites results in severe intellectual disability [37].

To test indirectly the role of FMRP in neuroplasticity, FMRP expression has been analyzed in rats after exposure to experimental paradigms known to induce synaptic plasticity. Regional increases in FMRP immunoreactivity were observed after training on a motor learning task or exposure to a complex environment [38]. It has also been shown that cortical levels of FMRP are elevated following sensory stimulation [39]. These observations suggest that the expression of FMRP is activity-dependent, and that the protein is involved in processes underlying synaptic plasticity [40]. Thus, it has been suggested that the loss of FMRP may lead to deficits in synaptic plasticity that could impair neuronal development [17].

Several other studies of FXS patients support that the syndrome is associated with dendritic spine dysgenesis, suggestive of abnormal neuronal development. In qualitative studies of Golgi-impregnated cortical neurons from human autopsy tissue, immature-appearing dendritic spine morphology has been described [41, 42]. Specifically, long, thin, tortuous spines with prominent heads and irregular dilations on apical dendrites of pyramidal cells in layers III and V of parieto-occipital neocortex and in the pyramidal layer of allocortex have been observed. Investigators noted that this spine morphology was reminiscent of that described in children and infants with other disorders associated with intellectual disability, such as Down syndrome and Patau syndrome [43]. Decreased synaptic contact area also was found, but no other major neuropathologies were observed. The lack of altered neuronal density in FXS patients, with the absence of significant cortical atrophy in the MRI research, indicates normal neurogenesis and cell migration and no prominent atrophy of processes.

The role of GSK-3 in neurodevelopment

Glycogen synthase kinase-3 (GSK-3) regulates a variety of developmental processes, such as neurogenesis, gliogenesis, cell migration, cell morphology, and axonogenesis through interaction with a variety of signaling pathways [44-47]. GSK-3 is a partially constitutively active serine/threonine kinase that is predominantly modulated by inhibitory serine phosphorylation of its two isoforms, serine-9 on GSK-3β and serine-21 on GSK-3α [48-50].

FMRP is known to play a critical role in adult hippocampal neurogenesis and regulates adult neural stem cell (NSC) fate by modulating the translation of glycogen synthase kinase-β (GSK-3β) [51]. One study examined the effects of GSK-3β inhibition on Fmr1 knockout mice [51]. GSK-3β inhibition increased hippocampal neurogenesis and improved performance in hippocampal-dependent learning tasks. It is possible that while overall neuronal density is not significantly altered in FXS patients, a decrease in hippocampal neurogenesis through loss of FMRP and the resultant dysregulation of GSK3 contributes to the pathogenesis of the disorder.

Underlying this are changes in the inhibitory serine-phosphorylation, which has a robust impact on GSK-3 activity, as this is the cardinal mechanism by which it is regulated. The phosphoinositide-3-OH kinase (PI3K)/Akt pathway is an essential pathway for neuronal and glial survival and is also one of the main regulatory pathways for serine-phosphorylation of GSK3 [47, 52]. However, GSK-3β can also be regulated by p38 mitogen-activated protein kinase (MAPK)-mediated inhibitory phosphorylation of serine-389 [53]. While GSK-3β and GSK-3β are expressed ubiquitously, GSK-3β2 is highly expressed in the central nervous system (CNS) and is found in highest concentrations during neurodevelopment [44].

GSK-3 inactivation has been associated with increased neuronal progenitor proliferation and suppressed neural differentiation. GSK-3 interacts with the canonical Wnt, sonic hedgehog (SHH), and Notch pathways to regulate proliferation [22, 24, 43]. The canonical Wnt/β-catenin signaling pathway involves Wnt binding to Frizzled receptors (Fzd) and Fzd binding to the protein Disheveled (Dvl). Dvl then binds and destabilizes the β-catenin destruction complex, which includes GSK-3. Therefore, GSK-3 regulates the canonical Wnt pathway by remaining bound to the Wnt complex, preventing β-catenin from translocating to the nucleus to induce gene transcription [54]. Inhibition of GSK-3 is necessary for β-catenin-mediated transcription. Wnt/β

-catenin signaling is vital for adult hippocampal neurogenesis and is critical for CNS developmental processes, such as synapse and dendrite formation.

GSK-3 and fragile X syndrome

GSK-3 activity has been found to be elevated in murine models of FXS [57]. A recent study found that lithium administration increased inhibitory phosphorylation of GSK-3 isoforms, reduced audiogenic seizure activity, and improved performance on open field, elevated plus maze, and passive avoidance tests in *Fmr1* knockout (KO) mice, and passive avoidance tests [52]. *Fmr1* KO mice also display impaired sociability. Mines *et al.* (2010) found that GSK-3 inhibition with lithium improved the previously impaired social interaction of Fmr1 KO mice with a novel mouse [58].

GSK-3 activity is also associated with mGluR5 in that mGluR5 normally activates the PI3K/Akt pathway, which induces inhibitory phosphorylation of GSK-3. However, it has been shown that mGluR5 signaling and GSK-3 activities are both elevated in Fmr1 KO mice [57]. A study utilizing both lithium and the mGluR inhibitor 2-methyl-6-phenylethynyl-pyridine (MPEP) found that the inhibition of mGluR also led to the inhibition of GSK-3. Both treatments led to decreased audiogenic seizure activity and improvement on open field tests. However, treatment with both lithium and MPEP did not have an additive effect, suggesting that the pharmacologic agents may target the same signaling pathway.

Likewise, long-term depression has been found to be increased in Fmr1 KO mice [60]. One study found that *Fmr1* knockout mice displayed less fear memory in contextual fear conditioning than wild-type mice and decreased long-term potentiation (LTP) in the anterior cingulated cortex and lateral amygdala (areas important for associative learning) [61].

Another way in which GSK-3 may be culpable in the cognitive deficits and altered brain pathology observed in FXS is through regulation of glycogenolysis. GSK-3 inhibits glycogen synthase, thus reducing glycogenolysis. Inhibition of glycogenolysis produces learning and memory deficits. Thus, the increased GSK-3 activity observed in FXS patients and *Fmr1* KO mice may cause intellectual disability through negative regulation of glycogenolysis in the CNS.

While it is evident that GSK-3 inhibition has a therapeutic effect in *Fmr1* KO mice and that GSK-3 plays a role in neuronal morphology and proliferation, it is not clear whether and to what extent GSK-3 is responsible for Fragile X neuronal and brain pathogenesis. Further analysis is required to glean the role of GSK-3 in neurodevelopment in murine models of FXS (**Figure 1**).

Potential therapy and future directions

FXS is generally believed to be a neuronal disorder due to the aforementioned behavioral and cognitive deficits and abnormalities in neuron morphology. Neuronal function is modulated by an array of immune cells and there is convincing evidence of neuronal dysfunction resulting from neuroinflammation [41, 42]. Though a role for immune activation and associated inflammation in autism is controversial [62-64], there is evidence of activated glia in autism [65-68] and disregulated plasma cytokines associated with FXS [21]. Additionally, reactive astrocytes have been found in many brain regions of *Fmr1* knockout mice. This pathology was attenuated with lithium treatment, providing further evidence of the involvement of GSK-3 in FXS [69]. Another study found that treatment of *Fmr1* KO mice with minocycline (an antibiotic that exerts anti-inflammatory effects), improved dendritic spine formation and performance on behavioral tests [70].

It has been speculated that maternal immune activation (MIA) may play a role in the development of autism through activation of inflammatory pathways *in utero* [71]. MIA can negatively impact fetal brain development and may impair social behavior [72]. A study of MIA in normal mice revealed an increase in interleukin-6 (IL-6) [74]. IL-6 is known to induce phosphorylation of Janus kinase-2 (Jak2) and signal transducer and activator of transcription-3 (STAT3), leading to the release of proinflammatory cytokines, such as TNF-α and IL-1β. Treatment with the bioflavonoid diosmin reduced inflammation in the brain tissue of MIA offspring [72]. Another study found that GSK-3 and STAT3 enhance production of IL-6 after immune activation, GSK-3 was also found to be critical in the interferon-γ (IFN-γ)-induced activation of STAT3 [51]. Therefore, it is possible that quelling the inflammatory

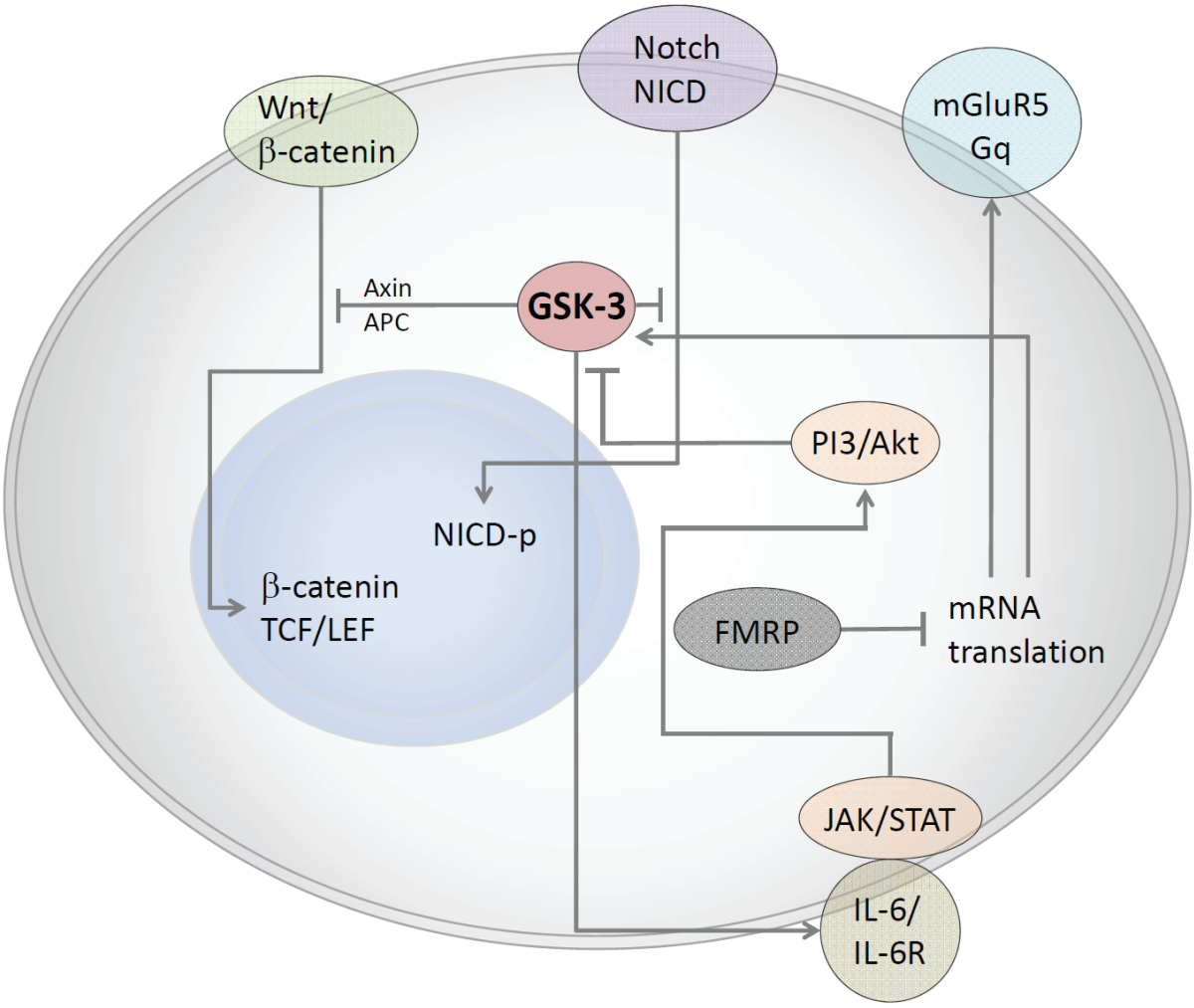

Figure 1. GSK3 regulates a variety of pathways involved in neurodevelopment. Active GSK3 regulates the canonical Wnt pathway by remaining bound to the Wnt/β-catenin destruction complex that includes APC and Axin. This complex targets β-catenin for proteasomal degradation. Inhibitory phosyphorylation of GSK3 releases β-catenin from the complex. It is then recruited to the nucleus by TCF/LEF where it induces gene transcription. GSK3β is known to phosphorylate and inhibit Notch, resulting in the inhibition of many Notch target genes. This inhibitory phosphorylation is reversed when Wnt1 is present. Inhibitory phosphorylation is induced by a number of kinases. The PI3/Akt pathway regulates GSK-3 activity by inducing inhibitory serine-phosphorylation. FMRP is deficient in Fragile X syndrome and is normally responsible for regulating mRNA transcription of metabotropic glutamate receptor-5 (mGluR5) and GSK3. It has been shown that inhibition of mGluR5 leads to the inhibition of GSK3. In addition to regulating neurodevelopmental pathways, it has been shown that GSK3 is also involved in pathways that promote inflammation. GSK3 induces IL-6 production, leading to the subsequent phosphorylation of Jak2/STAT3.

environment through modulation of GSK-3 is a mechanism by which a therapeutic effect may be achieved in *Fmr1* KO mice.

Another way to accomplish this may be to use bioflavonoids to inhibit GSK-3 activity. It has been shown that GSK-3β activity is decreased in pancreatic cancer cells when they are treated with various citrus flavonoids [73, 74]. The impetus for targeting GSK-3 in pancreatic cancer cells is that GSK-3β is over-expressed in the nucleus of these cells and causes nuclear factor -κB (NF-κB) to become active and induce an inflammatory cascade. Thus, attenuation of the inflammation leads to decreased cancer cell proliferation. Treatment with the bioflavonoid

luteolin has also been shown to reduce amyloid plaques in a transgenic (Tg2576) mouse model of Alzheimer's disease through modulation of GSK-3α [75]. To date, bioflavonoid-induced inhibition of GSK-3 has not been studied in other CNS-related disorders.

As noted earlier, lithium has been shown to be beneficial for *Fmr1* KO mice in reducing the occurrence and severity of audiogenic seizures and ameliorating behavior deficits. However, cessation of lithium treatment has led to the reemergence of the FXS phenotype in *Fmr1* KO mice [76]. Thus, lithium would have to be chronically administered to patients for the duration of the lifespan. Unfortunately, lithium can be highly toxic and may not be feasible as a prophylactic or therapeutic agent for pregnant mothers or young children [18, 77]. Bioflavonoids and other anti-inflammatory agents that serve as GSK-3 inhibitors may prove to be more viable and safe therapeutic options in the future.

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