Kinase Inhibitor Therapies for Fragile X Syndrome

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Absence of the Fragile X Messenger Ribonucleoprotein 1 (FMRP) causes autism spectrum disorders and intellectual disability, commonly referred to as the Fragile X syndrome. FMRP is a negative regulator of protein translation and is essential for neuronal development and synapse formation. FMRP is a target for several post-translational modifications (PTMs) such as phosphorylation and methylation, which tightly regulate its cellular functions. Studies have indicated the involvement of FMRP in a multitude of cellular pathways, and an absence of FMRP was shown to affect several neurotransmitter receptors, for example, the gamma aminobutyric acid (GABA) receptor and intracellular signaling molecules such as Serine-Threonine Protein kinase B (Akt), Extracellular signal-regulated kinases (ERKs), mechanistic target of rapamycin (mTOR), and glycogen synthase kinase (GSK3). Interestingly, many of these molecules function as protein kinases or phosphatases and thus are potentially amendable by pharmacological treatment.

fragile X syndrome autism intellectual disability phosphorylation protein kinases

1. Introduction

Fragile X Syndrome (FXS) is the most prevalent form of inherited intellectual disability (ID) and autism spectrum disorders (ASD), and globally affects approximately 1:4000 males and 1:8000 females [1][2]. Patients exhibit distinct physical features such as a long face, prominent jaws, and elongated ears, in combination with macroorchidisms [3]. Furthermore, FXS patients also display mild to severe cognitive impairments and behavioral problems, including attention deficit hyperactivity disorder (ADHD), anxiety, memory impairments, and autism spectrum disorder (ASD) [4]. Epileptic seizures are also observed in 20% of FXS patients [5]. At the molecular level, the FXS is caused by a trinucleotide CGG repeat expansion in the 5′ untranslated region of the *Fragile* X Messenger Ribonucleoprotein 1 gene (*FMR1*) [6]. This expansion is associated with loss of methylation boundaries, which are hypothesized to inhibit the spread of methylation of the *FMR1* promotor [7]. Loss of these insulator elements could contribute to total methylation of the promotor region and heterochromatin formation [8]. The spread of hypermethylation causes transcriptional silencing and an absence of the Fragile X Mental Retardation Protein (FMRP) [9].

FMRP is an RNA-binding protein that binds to polyribosomes supporting local protein synthesis [10]. Generally, FMRP represses the expression of specific mRNAs encoding pre- and postsynaptic proteins such as the N-methyl-D-aspartate receptor (NMDAR), metabotropic glutamate receptor 5 (mGluR5), postsynaptic density protein 95 (PSD-95), Homer1, PI3-Kinase Enhancer (PIKE), and voltage gated ion channels [11]. Loss of FMRP results in an increased protein translation of ~15–20%, heavily impacting various signal transduction pathways [1][12]. Indeed,

hyperstimulation of mGluR5 as a result of FMRP silencing has been linked to aberrant translation of several neuronal proteins, and has been associated with dendritic spine abnormalities, causing mGluR-dependent long-term depression (LTD), increased seizure susceptibility, and accelerated prepubescent growth [13]. Genetic and pharmacological reduction of mGluRs corrects the deregulation caused by the absence of FMRP [2]. FMRP-related defects were also discovered in several neurotransmitter receptors pathways, of which the gamma aminobutyric acid receptor (GABA-R) is widely discussed. GABA is the main inhibitory neurotransmitter in the adult mammalian brain [14]. It is strongly involved in the modulation of neuronal activity. FMRP shows affinity for mRNAs encoding different GABA-R subunits. Hence, deregulation of the GABAergic system is thought to play a pivotal role in behavioral abnormalities underlying the FXS [15]. Another example of an FRMP-dysregulated protein is matrix metalloproteinase 9 (MMP9). MMP9-mRNA was found to be part of the FMRP complex, which clusters in dendrites. Loss of FMRP results in elevated synaptic MMP9 levels in Fmr1 knock-out mice which, in turn, could contribute to impaired dendritic spine morphology and altered neuronal signaling [16]. MMP9 also cooperates with intracellular signaling molecules such as glycogen synthase kinase-3β (GSK3β), which is strongly activated in the Fmr1 knock-out mice hippocampus. Aberrant GSK3β signaling has also been linked to structural changes in dendritic spines [17]. Another relationship was found between FMRP and the amyloid precursor protein (APP), linking the pathophysiology of the FXS with Alzheimer's disease. Recently, it was shown that APP mRNA is repressed by FMRP [18]. Cleaving of APP by β-secretase results in the neurotoxic amyloid-β protein, of which the concentration is higher in FXS patients, possibly due to elevated APP synthesis [19]. Besides APP, another 4% of the total mRNAs in the brain show affinity for FMRP, including transcripts that regulate the synaptic cytoskeleton, such as activity-regulated cytoskeleton-associated protein (Arc), microtubule-associated protein 1B (Map1B), postsynaptic density protein 95 (PSD-95), and the ras-related C3 botulinum toxin substrate 1 (Rac1) [20]. These findings illustrate the involvement of FMRP in a multitude of intracellular pathways [1][21].

Most of the intracellular signaling pathways pivotal in the FXS are tightly regulated by protein kinases, which phosphorylate their protein targets [22]. Phosphorylation is a reversible post-translational modification (PTM) characterized by covalent addition of a phosphate moiety to an amino acid of a protein substrate [23]. This addition modifies the polarity of the protein, resulting in a more hydrophilic and polar character [24]. The change in polarity results in an altered conformation, inducing the formation of different protein–protein interactions or detachment from other complexes [25]. However, phosphorylation events are also strongly involved in the regulation of the biochemical activity of proteins, as well as the subcellular localization, degradation, and stabilization [26]. Any interference in the phosphorylation state will drastically affect their cellular function, and could potentially cause disease [27]. A much-debated example of a protein kinase in the FXS is mechanistic target of rapamycin (mTOR), which is involved in a translational pathway that is dysregulated by the loss of FMRP [28]. Phosphorylation of mTOR itself was reported to be increased in the hippocampus of juvenile Fmr1 knock-out mice, enhancing phosphorylation of multiple downstream targets such as ribosomal protein S6 kinase beta-1 (S6K), 4E-binding protein (4E-BP), and eukaryotic initiation factor complex 4F (eIF-4E) [29]. In addition to this cascade of phosphorylation events following the loss of FMRP, many other phosphorylation/kinase abnormalities have been reported in the FXS [30].

2. The Fragile X Syndrome: A Systemic Overview of the Molecular Pathophysiology

The FXS is an X-linked monogenetic disorder that is caused by a CGG trinucleotide repeat expansion in the 5′ untranslated region (UTR) of the *FMR1* gene [31]. The gene is located on Xq27.3 and is approximately 38 kb long [32]. During evolution, *FMR1* shows a high conservation in both rodents and primates [33]. Expression of the *FMR1* gene starts early during embryonic development, with the highest levels in brain, testes, ovaries, and thymus, concomitant with several symptoms underlying the FXS [34]. The *FMR1* gene has 17 exons which are alternatively spliced into 12 transcriptional variants [35]. Within the 5′ UTR, a CGG trinucleotide repeat is located. Expansion of this repeat lies at the molecular basis of the FXS and can be subdivided into four allelic classes based on the expansion size of the repeat: normal (6–40 repeats), intermediate (41–60 repeats), premutation carriers (61–200 repeats), and the full FXS mutation (>200 repeats) [36]. Normal and intermediate alleles are considered to be transmitted in a stable manner without affecting the genotype [37]. In contrast, premutations are unstable and expand when passed on to the offspring. The ultimate full mutation is hypothesized to occur in utero, even before the stage of the zygote [38]. Premutations are characterized by a poor methylation status [7]. These alleles are transcribed and represent high *FMR1* mRNA levels. However, FMRP expression is slightly reduced [39]. The full FXS mutation corresponds with epigenetic modifications such as CGG repeat methylation, associated with transcriptional silencing of *FMR1*. Here, methylation of CpG-specific cytosines covers the entire promotor region, leading to heterochromatin formation [40].

The full repeat expansion and subsequent hypermethylation of the *FMR1* gene result in transcriptional downregulation and an absence of FMRP [41]. Mammalian FMRP is a 71 kDa protein that comprises a variety of functional domains, including three RNA interacting domains of which two are K homology domains (KH1 and KH2), and an enriched cluster of arginine–glycine–glycine (RGG box). A nuclear localization signal (NLS) and nuclear export signal (NES) are also present within the amino acid sequence, enabling nucleocytoplasmatic shuttling [42]. A final feature is the presence of two tandem Agenet (Age) domains which have affinity for trimethylated lysine residues and could possibly interact with methylated histone H3K9 [43].

FMRP plays a crucial role as a negative regulator of translation of proteins that are involved in synaptic function, connectivity, plasticity, and dendritic morphology [44]. The way in which FMRP modulates translational repression is supported by several theories. First, monomeric FMRP is thought to dimerize in the cytoplasm, subsequently entering the nucleus through its NLS. Here, the dimer interacts with target-specific mRNAs through its KH-domains and RGG box. The FMRP–mRNA complex then leaves the nucleus by the NES. A first theory supports the concept of ribosome blocking. Subsequently, the FMRP–mRNA complex binds to the inter-ribosomal space, interfering with the binding of translation-specific initiation factors [41]. An alternative hypothesis suggests the involvement of the RNA-induced silencing complex (RISC). Here, FMRP represses the translation of synaptic mRNAs via miRNA interference. Only recently, FMRP was identified as a reader of N^6-methyladenosine (m^6A), a modification regulating mRNA function. Reading of the m^6A modification enables shuttling of methylated mRNA targets between the nucleus and the cytoplasm during neural differentiation. Moreover, FMRP also interacts with another m^6A reader YTH N^6-methyladenosine RNA Binding Protein 2 (YTHDF2), which ensures degradation of the mRNAs

previously stabilized by FMRP [45][46]. In the end, translationally silenced mRNAs are transported to the postsynaptic dendritic membrane, waiting for a translational activation signal [47]. The translation is, for example, initiated when the metabotropic glutamate receptor 5 (mGluR5) is activated upon ligand binding[48].

In response to ligand binding, several postsynaptic membrane receptors can be triggered, initiating an intracellular signaling cascade. Common receptors still under debate regarding their involvement in the FXS are the metabotropic glutamate receptors (mGluRs), AMPA receptors, GABA receptors type A and B, NMDA receptors, and TrkB receptors. Signal transmission of one of these receptors will cancel out the translation inhibitory effect of FMRP, establishing protein synthesis. Activation of these membrane receptors results in an intracellular signaling cascade. A first pathway showing higher activation is the phosphatidylinositol 3-kinase (PI3K) pathway, resulting in upregulation of Akt and mTOR signaling. Simultaneously, ERK and TrkB signaling have been shown to be highly active with converging effects towards protein synthesis. In the case of the FXS, the inhibitory effect of FMRP is lost, supporting local protein synthesis of proteins such as Arc, Map1B, CamKII, postsynaptic density-95 (PSD-95), matrix metalloproteinase 9 (MMP9), and glycogen synthase kinase 3 beta (GSK3β). Elevated Arc levels will cause increased AMPA receptor internalization, resulting in ion channel imbalance. This causes a receptor imbalance, resulting in enhanced mGluR long-term depression (mGLuR-LTD), which contributes to altered hippocampal synaptic plasticity. Furthermore, the upregulation of key regulatory proteins such as Arc, Map1B, and PSD-95 causes deregulation of the neuronal cytoskeleton [15][35].

In addition to the glutamatergic imbalance, the GABA receptor system is also impaired in FXS patients. GABA is the principal inhibitory neurotransmitter expressed in the adult mammalian brain and is strongly involved in the modulation of neuronal activity. Dysregulated GABAergic transmission is thought to play a role in behavioral abnormalities underlying the FXS. Epileptic seizures and sleeping disorders are common problems affecting FXS patients. At a cellular level, GABA can bind to two different types of receptors, being the ionotropic GABAA receptor and the metabotropic GABAB receptor. Here, FMRP shows affinity for the mRNAs coding for the GABAA receptor subunits α5, β2, and δ. Moreover, regulation of the mRNA subunit stability as well as inhibition of their degradation are FMRP-mediated effects. FMRP also shows affinity for the enzyme responsible for GABA synthesis, glutamic acid decarboxylase (GAD). Additionally, impairments of the GABAB receptor are also reported. Upon binding of GABA, presynaptic vesicles, carrying glutamate neurotransmitters, are inhibited from unloading their cargo, resulting in an inhibition of the postsynaptic mGluR signaling. In case of the FXS, there is an imbalance of neurotransmission, favoring excitatory mGluR5 signaling [2][12][15].

3. The Fragile X Messenger Ribonucleoprotein (FMRP) Is an RNA-Binding Molecule, Which Is Tightly Regulated by the Actions of Protein Kinases and Phosphatases

FMRP is a multifunctional protein that plays a role in translational repression through ribosomal stalling, where it is locally modulated by posttranslational modifications such as phosphorylation and methylation. Initially, mass spectrometry analyses of murine brains and cultured cells showed that FMRP is mainly phosphorylated between the amino acid residues 483 and 521. Within this sequence, primary phosphorylation takes place at the conserved

serine 499, which subsequently triggers phosphorylation of the nearby residues Ser489, Ser493, Ser496, Ser503, Ser510, and Ser513 [49][50]. Strict regulation of FMRP phosphorylation is crucial for controlling its ability to modulate the translation process. It is well established that signaling through neuronal GPCRs, including the metabotropic glutamate receptor (mGluR), impacts the phosphorylation status of neuronal proteins. Therefore, the translational activity of FMRP is regulated by the actions of the ribosomal protein S6 kinase 1 (S6K1) and protein phosphatase 2A (PP2A) system [6][51]. After mGluR stimulation, S6K1 is activated, and PP2A activity is inhibited, allowing phosphorylation of FMRP and its subsequent translational repression via ribosomal stalling [52]. In contrast to S6K1, PP2A initiates rapid FMRP dephosphorylation after group I mGluR stimulation, measured by enhanced PP2A activity. Furthermore, measurements up to five minutes after mGluR stimulation have shown mTOR-mediated PP2A inhibition, together with new phosphorylation events, indicating an activity-dependent induction of FMRP phosphorylation [53]. However, findings by Bartley et al. (2014) showed that FMRP phosphorylation at Ser 499 remained unchanged despite a higher mTORC1-S6K1 activity, thereby suggesting that other kinases may also play a role in FMRP phosphorylation [54][55]. Hence, casein kinase II (CKII) was discovered to phosphorylate FMRP at Ser499 within 2–4 h of synthesis, promoting dynamic phosphorylation of nearby residues by other kinase-phosphatase systems, also including S6K1/PP2A. Once FMRP is phosphorylated, it remains phosphorylated without affecting the half-life of the protein [50]. FMRP is also involved in activity-dependent protein translation which requires transport of mRNA into ribonucleoprotein organelles, called neuronal granules, which is facilitated by its C-terminal low-complexity disordered region. Here, post-translational modifications such as phosphorylation and methylation of FMRP show opposing activities after translation initiation. Both protein phosphorylation, as well as methylation, can modulate the ability of FMRP to bind mRNAs. For example, receptor stimulation promotes neuronal granule disassembly after FMRP dephosphorylation by PP2A. Here, methylation of FMRP causes a reduction in higher-order assembly formation with mRNA and polyribosomes, thereby activating protein translation. Reversely, PP2A activity is counteracted by the actions of S6K1, which induces FMRP phosphorylation, thereby promoting granule reassembly and translational silencing as well as FMRP demethylation [56]. These findings propose a model where FMRP is controlled by multiple phosphodynamic processes, all impacting the translational process [45][48][51].

4. A Predictive Network Analysis Suggests Functional Associations between the Abnormally Regulated Protein Kinases in the FXS

Mapping of kinase interaction networks is essential for understanding the cellular processes that protein kinases participate in, and subsequently could provide information about disease-associated signaling pathways [57]. For this reason, the UniProt IDs of the protein kinases whose activity was reported in FXS literature were submitted to the STRING database version 11.5 to identify network interactions. Several functional associations of FMRP and all the above-mentioned protein kinases were characterized by protein homology, co-expression, text mining, and database evidence. Generally, functional enrichment analysis revealed that the submitted kinases were strongly connected (protein–protein-interaction (PPI) enrichment $p < 0.0001$). Here, FMRP is involved in a tightly related

network of protein kinases with its main interactors being LIMK1, CaMKIIα, Akt1, S6K1, GSK3β, and TrkB. For DGKk, no functional associations with other protein kinases or with FMRP were predicted.

5. The Aberrant Kinase-Signature in the Fragile X Syndrome Translates to Abnormalities of the Phosphoproteome

Intracellular signaling is mostly mediated through phosphorylation of protein kinases, which are dysregulated in the FXS. However, despite great advances in studies of protein kinases in the FXS, an understanding of their dysfunction is far from clear and needs an integrative approach. For this reason, identification of phosphorylated proteins can be exerted by mass spectrometry-based phosphoproteomics. In this way, the activity of protein kinases can be indirectly quantified with phosphoproteomics as changes in protein phosphorylation result from dynamic alterations in protein kinase signaling [58]. Initially, quantitative proteome analyses have been performed in mouse and Drosophila models for the FXS, which were developed to identify differences in synaptic protein expression. Stable isotope labeling by amino acids in cell culture (SILAC)-based proteomics in Fmr1 knock-out and wild-type cortical neurons resulted in identification of 132 differentially expressed proteins in absence of FMRP, related to changes in synaptic structures, neurotransmission, and dendritic mRNA transport, together with autism- and epilepsy-related proteins [59]. Another study compared the proteomes of Fmr1 knock-out and wild-type hippocampal synapses, using isobaric tags for relative and absolute quantitation (iTRAQ). A series of 23 proteins were significantly different in expression with groups of proteins known to be involved in cellular differentiation, neurite outgrowth, and synaptic vesicle release [60]. Another SILAC study performed in a heterozygous dfmr1 Drosophila model resulted in profiling of 1617 proteins, identifying several proteins which were altered in expression such as actin-binding protein profilin and microtubule-associated protein futsch [61]. A final study investigated systemic protein expression in neocortical synaptic fractions from Fmr1 knock-out and wild-type mice at adolescent and adult stages of life. Over 100 proteins were upregulated in Fmr1-deficient mice at adolescence, but this was no longer the case in adult mice. The differentially expressed proteins were involved in processes affecting brain development, ASD, and intellectual disability, which were further integrated in an interactome showing a central role for PSD95 [62]. Besides these proteomic attempts to resolve global molecular processes in FXS, phosphoproteomic studies are still poorly applied in FXS, although phosphorylation abnormalities might be critical determinants of FXS pathologies, based on the above-mentioned kinase literature. Only one proteome-wide study on the phosphorylation abnormalities was reported, in which SILAC-based quantitative phosphoproteomics was used to analyze murine Fmr1 knock-out and wild-type fibroblastic cell lines derived from Fmr1-deficient embryos to identify proteins and phosphorylation sites dysregulated as a consequence of FMRP loss. FMRP-related changes in the levels of 5023 proteins and events were initially identified and mapped onto major signaling transduction pathways.

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