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Fragile X syndrome: from gene discovery to therapy

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1. ABSTRACT

A dynamic mutation in the *fragile X mental retardation 1* gene, *FMR1*, was found to cause fragile X syndrome almost 20 years ago. Since, a wealth of information regarding the function of the gene has been gathered. It plays a role in RNA transport and stability and RNA-binding influences the function of a multitude of other genes. In this review, we focus on the recent knowledge of molecular and biochemical pathways shown to be relevant in the fragile X syndrome and how these insights have led to a first series of clinical trials in fragile X patients.

2. INTRODUCTION

Fragile X syndrome is the most common form of inherited mental retardation, with a prevalence of 1/2500 – 1/6000 (1, 2). Besides the cognitive delay, the syndrome is characterized by typical facial features, like a long face with prominent forehead, a protruding jaw and large ears. Other physical abnormalities are macroorchidism, connective tissue dysplasia, flat feet and sometimes hyperextensible joints, hand calluses and strabismus. Fragile X patients can exhibit different behavioural problems, including hyperactivity, sleep problems, autistic-like behaviour, anxiety and mood disorders, impulsivity and aggressive behaviour (reviewed by (3)). In 20 percent of the patients epileptic seizures can occur (4). Neuro-anatomically, no gross abnormalities have been reported. However, immature dendritic spines and an increased spine density are observed (5, 6).

In 1991, the disease-causing gene, *Fragile X Mental Retardation 1 (FMR1)*, was discovered (7). The disease is most often caused by expansion of a CGG triplet located within the 5' untranslated region of the *FMR1* gene to more than 200 repeats. Due to the dynamic mutation, the CGG repeat and the CpG island in the promoter region of the gene become hypermethylated, leading to transcriptional silencing of *FMR1*. At the cytogenetic level, the triplet expansion can be seen as a gap or break on the X chromosome when fragile X cells are grown under folate poor culture conditions. This so-called fragile site at Xq27.3 is called FRAXA (8).

Control individuals carry 5-50 CGG repeats. Repeats in this size range will be stably transmitted to the progeny. Individuals with 50-200 repeat units are carriers of a so-called premutation that is inherited unstably, but they do not suffer from the fragile X syndrome. Repeat expansion from a premutation to a full-sized syndrome-causing mutation (> 200 repeat units) occurs in all tissues except the male germline, explaining why the full mutation can only be inherited from the mother (9). Male carriers of a full mutation are always affected, whereas the phenotype of female carriers varies from affected to symptomless due to non-random X-inactivation. Male premutation carriers can develop a late-onset neurodegenerative syndrome called fragile X tremor/ataxia syndrome (FXTAS) (10). Female carriers of a premutation can develop fragile X-associated primary ovarian insufficiency (FXPOI) (11).

Whether the expansion from premutation to full mutation occurs post- or prezygotically is not yet elucidated. Evidence of a postzygotic model came from the findings that the full-mutation allele is absent in sperm of full-mutation males and the fact that 40 percent of fragile X males are mosaic (12, 13). However, these findings do not formally exclude the occurrence of prezygotic expansion. Evidence exists that somatic mosaicism is the result of variable contraction of somatic full-mutation alleles, rather than expansion of premutation alleles and full expansion may already exist in the maternal oocyte (14, 15). The latter two findings support a prezygotic model of repeat expansion.

Occasionally, fragile X syndrome results from deletions within or around the *FMR1* locus (reviewed by (16) and (17)). Both small deletions (less than 10 kb), which may be caused by CGG repeat instability and occur near the repeat, and large deletions (up to 13 Mb) caused by meiotic and mitotic recombination, can occur. In the latter case, genes located proximally and/or distally may also be lost resulting in additional phenotypes. In 1993, a fragile X patient with an intragenic point mutation, resulting in an Ile304Asn (I304N) substitution was reported (18). The patient has a severe fragile X phenotype with an IQ below 20. Thereafter, three unrelated patients were reported with a C to T substitution in intron 10, causing alternative splicing and leading to the introduction of a premature stop codon (19).

Molecular diagnosis of the fragile X syndrome is mainly based on detection of alterations in the *FMR1* gene (20). The length of the CGG repeat can be measured by polymerase chain reaction (PCR). However, this technique may not allow the accurate detection of repeat lengths in the full mutation range. Using Southern blot, the length and methylation status of *FMR1* can be determined. This is the preferred technique for detection of full mutations and repeat lengths in the upper end of the premutation range. Combination of these two techniques gives a detection sensitivity of 99 percent. DNA samples can be obtained from peripheral blood cells or, in case of prenatal research, from amniocytes or chorionic villi. A noninvasive test is by analysis of the presence of FMRP in the hair roots. The protein is absent from almost all hair roots of male fragile X patients and in more then 50 percent of the hair roots of female patients (21).

3. STRUCTURE AND FUNCTION OF FMRP

FMRP, the protein encoded by the *FMR1* gene, is an RNA-binding protein that is maximally 631 amino acids long. Twelve different protein isoforms exist, due to intensive alternative splicing especially in the 3' terminal half of the gene. The different FMRP isoforms have a molecular mass ranging from 70-80 kDa (22). FMRP contains 5 different functional motifs; two different RNA-binding domains: two hnRNP K-protein homology (KH) domains and an Arg-Gly-Gly (RGG) box, a nuclear localization signal (NLS), a nuclear export signal (NES) and two coiled coils (CC) involved in protein:protein interactions (23, 24). The human I304N mutation maps to the RNA-binding pocket present in the KH2 domain (25).

FMRP is widely expressed in various tissues with the highest expression in brain and testis (26). In neurons, FMRP expression is most concentrated in the perikaryon, proximal dendrites and the postsynaptic apparatus. In 1997, Feng and colleagues were able to detect presynaptic FMRP expression *in vivo* using immunoelectron microscopy (27). The hypothesis raised that FMRP might have a presynaptic function as well (28, 29). Very recently, Christie and colleagues were able to confirm the results showing expression of FMRP in axons and at presynaptic terminals (30). More specific, FMRP is a component of a novel presynaptic structure; the fragile X granule (FXG). The

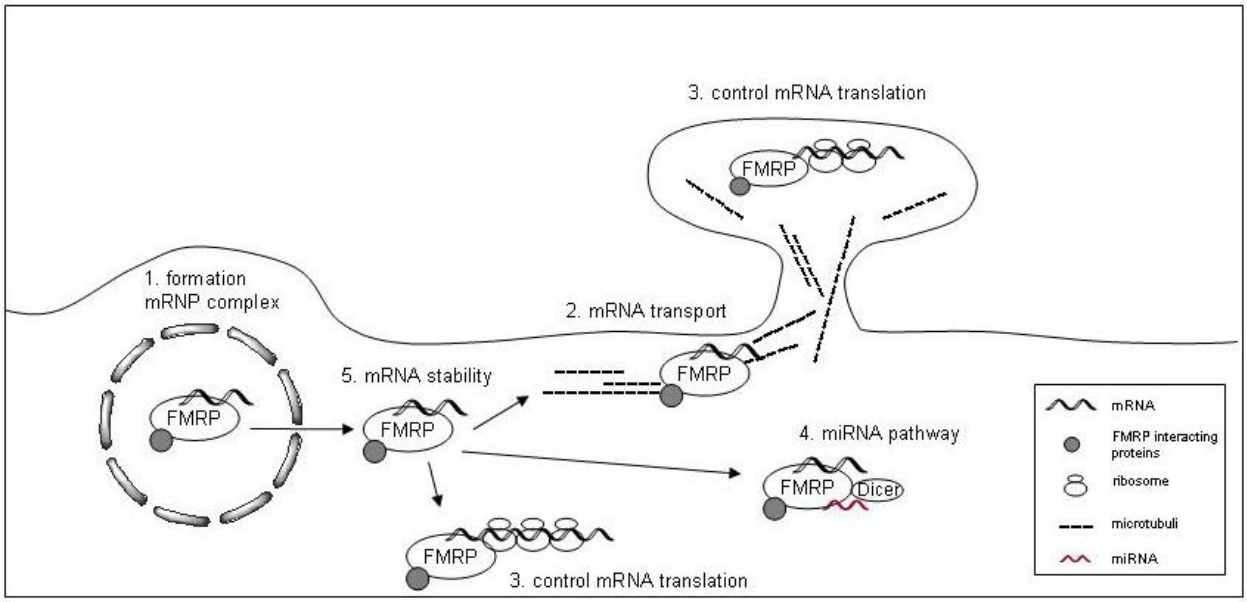

Figure 1. The functions of FMRP in the neuron. 1) After FMRP binds target mRNA and proteins in the nucleus, forming an mRNP particle, it is exported to the cytoplasm, where it can exert multiple functions. 2) The complex can stay in the cell body or move to dendritic spines, transporting the mRNA. 3) Subsequently, it can associate with translating ribosomes, regulating mRNA translation. 4) FMRP may also function as a translational regulator via its role in the miRNA pathway. 5) A last known function of FMRP is the involvement in mRNA stabilisation.

fragile X granule expression is restricted to axonal and presynaptic compartments in a subset of neurons and in specific periods of neuronal development and adult neurogenesis. Besides FMRP, all fragile X granules contain the fragile X related protein 1 (FXR1P), with a subset also containing the fragile X related protein 2 (FXR2P).

FMRP is associated with the 60S subunit of the ribosomes in an RNA-dependent manner (31, 32). When control lymphoblastoid cell lysates were treated with EDTA to dissociate the ribosomal subunits, FMRP was released as a large (more than 669 kDa) messenger ribonucleoprotein (mRNP) particle containing both poly(A)$^+$ mRNA and proteins. FMRP has been shown to bind its own mRNA and 4 percent of total brain mRNAs (33). FMRP has a preference for two classes of mRNAs that contain either a G-quartet structure or a U-rich sequence (34-37). Many mRNAs that bind FMRP are involved in neuronal functions like synapse formation, neurite outgrowth and neuronal development (33, 34). Proteins present in the FMRP-mRNP complex, found by yeast-two hybrid or co-immunoprecipitation experiments, are the fragile X related proteins (FXR1P, FXR2P), the nuclear FMRP interacting protein 1 (NUFIP1), 82 kDa FMRP interacting protein (82-FIP) and microspherule protein 58 (MSP58) (38-42). These proteins might modulate the affinity of FMRP for different classes of mRNAs. Binding of these proteins to FMRP can induce conformational changes, thereby exposing the RNA-binding domains differentially. Additional RNA-binding proteins such as nucleolin, YB-1/p50, Pur-alpha and Staufen have been detected in complex structures containing FMRP, but it is not known whether these bind FMRP directly (39, 43, 44). A few non-RNA-binding proteins have been shown to interact directly with FMRP too, including the actin-based motor protein myosin Va, Ran-BPM and Lgl, which are cytoskeleton associated proteins and CYFIP1 and CYFIP2, which link FMRP to the Rho GTPase pathway (44-47).

Based on the expression profile and the functional domains of FMRP, until now, three different functions are ascribed to FMRP (Figure 1). The presence of both an NLS and an NES signal motif within FMRP suggests that FMRP is a shuttle protein and that it travels between the nucleus and the cytoplasm. In the nucleus, FMRP binds to RNAs and proteins to form the mRNP particle and is then exported to the cytoplasm where it could associate with translating ribosomes (24). The mRNP complex can stay in the neuronal cell body or it can move to the dendritic spines via the microtubule structures present in the dendrites. In this way, FMRP can control the local protein synthesis at the synapses, influencing synaptic function, structure and plasticity. In addition, FMRP can act as a negative regulator of translation by inhibiting the assembly of 80S ribosomal initiation complexes (48) or it can either favour or prevent translation by acting as a nucleic acid chaperone (49, 50). Regulation of translation is influenced by phosphorylation of FMRP (51). Phosphorylated FMRP might be associated with stalled ribosomes, whereas non-phosphorylated FMRP associates with actively translating ribosomes, triggering translation of the associated messages. The recent observation of axonal fragile X granules, suggests that FMRP has also a function in presynaptic translation. The presence of mRNAs known to bind to FMRP in axons supports this

idea. However, polyribosomes have not yet been detected in presynaptic compartments (52).

Another manner in which FMRP could exert its translational regulation is by microRNA mediated translational suppression. MicroRNAs (miRNAs) are small (18-25 nucleotides in length) non-coding RNAs that are genomically encoded (53). These small RNAs bind to the 3' UTR of target mRNA, leading to mRNA degradation or translational silencing. Known biological roles include neuronal development and regulation of synaptic plasticity (54, 55). Mammalian FMRP as well as the drosophila ortholog, dFmrp, associates with several components of the miRNA pathway including Dicer, Ago1/2 (Argonaute) and miRNAs (56-58). FMRP facilitates the interaction between miRNA and target mRNAs and ensures proper translational suppression. The association of FMRP with the miRNA pathway is regulated by the phosphorylation of FMRP (59). Phosphorylated FMRP could not capture Dicer, resulting in the inhibition of the conversion of pre-miRNA into mature miRNA by this protein.

Recently, a third cytoplasmic regulatory function for FMRP was found, namely control of mRNA stability. In mice, Zalfa *et al.* found that FMRP binds and so prevents the decay of the mRNA encoding PSD-95 (60). This interaction occurs through the 3' untranslated region of the *PSD-95* mRNA.

4. ANIMAL MODELS TO STUDY THE FRAGILE X SYNDROME

4.1. Mouse models (*Mus Musculus*)

The human *FMR1* gene shows a high conservation in its nucleotide and amino acid sequence with the murine *Fmr1* gene (7, 61). In addition, the RNA and protein expression pattern is very similar, making the mice a good model for the fragile X syndrome (26).

4.1.1. Fragile X mouse

The first fragile X mouse was created by the Dutch-Belgian Fragile X Consortium in 1994 (62). *Fmr1* knockout mice were created by homologous recombination of a targeting vector into the mouse germline. In this way, exon 5 was interrupted by a neomycin cassette, leading to lack of the normal *Fmr1* RNA and absence of the Fmrp protein. Like in male human patients, *Fmr1* knockout mice develop progressive macroorchidism and show behavioural and cognitive abnormalities, including mild spatial learning and memory deficits (62-66), slightly increased locomotor activity (62, 67, 68) and altered sensorimotor integration (67, 69, 70). In contrast to humans, fragile X mice show increased exploratory behaviour (62, 67, 71) and some tests show a reduced anxiety-related response (65, 67, 68, 70-72). *Fmr1* knockout mice have no spontaneous epileptic seizures but are more sensitive to audiogenic induced seizures (69, 73). No gross pathological abnormalities in brain were observed, but increased spine density and excess of long and thin immature spines, has also been described (74-76). A decreased expression of the mGluR1 receptor was found in the cerebral cortex and a reduced long-term potentiation (LTP) was found in the cortex of the fragile X mouse (77-79). Moreover, electrophysiological measurements showed an increase in mGluR-dependent long-term depression (LTD) in the hippocampus of *Fmr1* KO mice (80).

Because the first knockout mouse has still an intact *Fmr1* promoter, aberrant *Fmr1* transcription producing abnormal RNA species, can be found. Therefore, a conditional *Fmr1* knockout mouse was generated by flanking the promoter and the first exon of *Fmr1* with *loxP* sites (81). This new *Fmr1* knockout mouse does not express any Fmrp and also lacks detectable *Fmr1* transcripts. Another advantage is that with this conditional knockout mouse, a null allele in specific cell types and at specific time points in development can be created. These mice show macroorchidism and altered hippocampal synaptic plasticity. A complete cognitive and behavioural assessment has not been performed as yet (82, 83).

4.1.2. Rescue mouse

Several attempts were made to rescue the fragile X phenotype by introduction of the *Fmr1* gene in the knockout mouse (84). Human FMR1 cDNA constructs were used to create the first rescue model (85). A slight restoration in Fmrp expression was detected, however no phenotypic, cognitive or behavioural rescue was observed. Therefore, *Fmr1* knockout mice carrying a yeast artificial chromosome (YAC) transgene containing the whole human *Fmr1* gene were generated (86). Macroorchidism and some behavioural symptoms like increased activity and reduced anxiety-like responses were rescued. Recently, partial rescue of the audiogenic seizure susceptibility was reported (87). Despite this encouraging finding, abnormal behaviour was also observed. It is evident that the cell specificity as well as the quantity of the FMRP should be strictly regulated in order to rescue all characteristics of the fragile X syndrome.

4.1.3. CGG repeat mouse

A CGG repeat mouse was made to better understand the timing and mechanism involved in the *FMR1* CGG repeat instability and methylation (88). The endogenous mouse CGG repeat was replaced by a human CGG repeat carrying 98 CGG units. This repeat shows mild instability upon both maternal and paternal transmission and until now it reached a length of 230 repeats. This repeat is in the human full mutation range. However, methylation of the repeat in this mouse model is absent; suggesting that modelling the fragile X full mutation requires additional repeats or other genetic manipulation. Furthermore, this knock in mouse displays biochemical, phenotypic and neuropathological characteristics of FXTAS (88-91). *Fmr1* mRNA levels are elevated and Fmrp levels are decreased. Ubiquitin-positive intranuclear inclusions were detected in brains of expanded CGG repeat mice.

A second knock in premutation mouse model was generated by Entezam and colleagues (92). Here, serial ligation of short, stable CGG-CCG repeat tracks was used to expand the endogenous CGG repeat. This model shows key features seen in humans including a direct relationship between repeat number and *Fmr1* mRNA levels, an inverse

relationship with FMRP levels, the presence of ubiquitin and lamin-positive neuronal intranuclear inclusions and Purkinje cell pathology. The repeat instability is high and transmission occurs both maternally and paternally. Large mutations into the full mutation range are seen that occur within a single generation. However, no DNA methylation of these alleles was observed.

4.1.4. I304N mouse

To get more insight into the RNA-binding properties of the RNA-binding KH2 domain of FMRP, a mouse model of the human fragile X syndrome I304N mutation was very recently made (93). The I304N knock-in mice show a comparable phenotype with the *Fmr1* knockout mice, including macroorchidism, behaviour problems, audiogenic seizures and altered synaptic plasticity. This supports the conclusion that the I304N mutation is sufficient to phenocopy the fragile X syndrome. The mutant protein shows a reduced expression compared to wild type FMRP and has lost polyribosome association and KH2 domain RNA-binding. Identifying FMRP KH2 RNA ligands is essential to understand the pathogenesis of the syndrome.

4.2. Fly models (*Drosophila melanogaster*)

The drosophila homologue of *FMR1*, *dFmr1* or *dFxr* (*drosophila fragile X related* gene), is a single gene, homologous to the three members of the mammalian *Fmr1* gene family, consisting of *FMR1*, *FXR1* and *FXR2*. It displays extensive amino acid sequence identity with the vertebrate genes, especially in the functional domains and it possesses similar RNA-binding activity (94).

Zhang and colleagues (95) developed a fragile X fly model. *dFmr1* null mutants are viable and anatomically normal. However, locomotory and central (optic lobe) and peripheral (neuromuscular junction, NMJ) synaptic transmission defects were observed. They also reported a modest increase in the number of the arboreal branches at the NMJ and in the number of peripheral synaptic boutons. In addition, *dFmr1* null mutants show an abnormal eclosion and circadian rhythm and an aborted courtship ritual (96-98). The relative severe phenotype of the *dFmr1* mutant might be due to the absence of the entire *Fmr1* gene family in the fly. One limitation of the fragile X fly model is the lack of good learning and memory assays (99). In one study, using the conditioned courtship paradigm assay, cognitive impairment (lack of memory) was found to be a phenotype of the fragile X fly too (100).

In accordance to the I304N mouse, an I244N fly and an I307N fly were created in which a conserved isoleucine was replaced by an asparagine in the KH1 and KH2 domain respectively (101). Only a partial loss-of-function phenotype was observed.

4.3. Zebrafish models (*Danio rerio*)

The amino acid sequence alignment between human FMRP and zebrafish Fmrp shows an overall identity of 74 percent (102). The zebrafish has orthologues of all three *FMR1*-related genes. Studies in zebrafish may thus be relevant to understand the human syndrome (103). In addition, the embryonic development is well known and the embryo is transparent and develops outside the mother. Thus, the zebrafish model has a benefit in the study of the involvement of FMRP in the processes during early embryonic development. This model also provides a highly efficient drug screening tool because drugs can be applied directly to the fish water.

Using morpholino knockdown of the *fmr1* gene, Tucker *et al.* (103) observed changes in neurons and neurite branching in the central and peripheral nervous systems. However, this method is not 100 percent efficient and can have a residual expression of 10-20 percent. Using TILLING, two independent *fmr1* knockout alleles were generated (104). The first allele, hu2787, defines a stop mutation in exon 5 of *fmr1*. The second allele, hu2898, has a mutated splice acceptor site at the end of the 7th intron, leading to the use of an alternative splice acceptor site. This induces a frameshift and a stop codon. Both mutant alleles result in an *fmr1* knockout zebrafish with complete loss of Fmr expression. In contrast to the results found in *fmr1* morphant embryos, no craniofacial or neurite branching defects were found in *fmr1* knockout zebrafish. In fact, no phenotype at all was observed. It was suggested that the morpholino-induced phenotype may not be related to loss of Fmr, but is a result of off-targeting effects and it remains at present unsolved why both zebrafish models show such a different phenotype.

5. MAJOR MOLECULAR PATHWAYS INVOLVED IN THE FRAGILE X SYNDROME

5.1. The GABAergic pathway

There is ample evidence that the $GABA_A$ receptor pathway is involved in the fragile X syndrome. We found an altered expression of several components of the GABAergic system, including 8 out of 20 mRNAs of known subunits of the $GABA_A$ receptor (*alpha 1*, *3* and *4*, *beta 1* and *2*, *gamma 1* and *2* and *delta*) and proteins and enzymes involved in synthesis (*Gad1*), transport (*Gat1* and *Gat4*) and degradation of GABA (*Ssadh*) and in the clustering and targeting of the $GABA_A$ receptors at the post-synaptic membrane (*Gephyrin*) in the *Fmr1* knockout mouse (105, 106).

Other groups demonstrated decreased protein levels of the $GABA_A$ receptor subunits alpha 5, beta and delta (the only subunits analyzed) in cortex, hippocampus, diencephalon and brainstem (107, 108). Electrophysiological studies demonstrated that absence of FMRP is associated with apparently normal striatal glutamate-mediated transmission, but abnormal GABA transmission (109). In addition, there is an enhanced GABA-mediated synaptic inhibition, secondary to loss of presynaptic FMRP-mediated control of transmitter release. Furthermore, electrophysiological recordings demonstrated that tonic $GABA_A$ currents were down regulated in *Fmr1* knockout mice whereas no significant differences were observed in phasic currents (108). Defects in neocortical GABAergic inhibitory circuits were also described in anatomic and behavioural studies of fragile X knockout mice (110, 111).

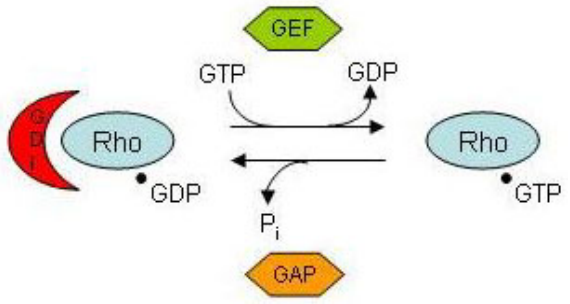

Figure 2. Regulatory mechanisms for the activation and inactivation of Rho GTPases. Rho GTPases act as molecular switches and cycle between an active GTP-bound state and an inactive GDP-bound state. Rho guanine nucleotide exchange factors (GEFs) facilitate the exchange of Rho-GDP into Rho-GTP. GTPase-activating proteins (GAPs) inactivate Rho GTPases by increasing the intrinsic GTPase activity and guanosine nucleotide dissociation inhibitors (GDIs) sequester GDP-bound Rho GTPases, maintaining the Rho GTPases in the inactive state.

$GABA_A$ receptors are the main inhibitory receptors in brain and are implicated in anxiety, depression, epilepsy, sleep problems and learning and memory (112). So, underexpression of the $GABA_A$ receptor system can explain many of the behavioural symptoms of the fragile X syndrome (113).

5.2. mGluR related pathways

5.2.1. The mGluR theory

In fragile X mice, an increase in postsynaptic group 1 metabotropic glutamate receptor (Gp1 mGluR1/5)-dependent LTD was found (114). This type of LTD requires the rapid translation of pre-existing mRNA in the dendritic spines and stimulates the loss of surface expressed synaptic AMPA and NMDA receptors. FMRP is present in the dendrites, binds mRNA and actively translating ribosomes and plays a role in the translation of these mRNAs. Moreover, *Fmr1* mRNA itself is rapidly translated in response to activation of mGluRs. This observation led to the assumption that FMRP plays an important role in the protein-synthesis dependent mGluR LTD. The theory suggests that mGluR activation normally stimulates synthesis of proteins involved in stabilization of LTD. FMRP functions as negative regulator of translation and puts a brake on LTD. In absence of FMRP, the brake on LTD diminishes, resulting in an increased LTD.

LTD, together with LTP, are mechanisms responsible for the long-lasting changes in synaptic strength in response to synaptic activation. Consequences of an increased LTD are weakening and sometimes totally disappearing of the synaptic connections. Inappropriate forming of the synapses may lead to immature dendritic spines, developmental delay, cognitive impairment, anxiety and epilepsy, all features of the fragile X syndrome.

5.2.2. The ERK pathway

Synaptic protein synthesis is regulated by a number of signalling pathways. One of the key players in most of these signalling cascades is the extracellular-signal-regulated kinase (ERK). After neurotransmitter binding with mGlu receptors, ERK is phosphorylated and activated through the MAPK pathway, leading to translation initiation.

Kim and colleagues showed that ERK and the upstream effector molecule MAPK/ERK kinase (MEK) in *Fmr1* knockout synaptoneurosomes are rapidly dephosphorylated upon mGluR stimulation, whereas they are phosphorylated in WT mice (115). The rapid deactivation is caused by the overactivity of two phosphatases; protein phosphatase 2A (PP2A) and tyrosine phosphatase. Interestingly, *PP2A* mRNA and *tyrosine phosphatase* mRNA are ligands of FMRP. Moreover, it was found that all these molecules play an important role in the phosphorylation status of FMRP. The phosphatase responsible for the dephosphorylation of FMRP is PP2A (116), whereas the phosphorylation of FMRP occurs through protein S6 kinase 1 (S6K1), which is ERK and mammalian target of rapamycin (mTOR) signalling mediated (117, 118). In this way, those kinases as well as phosphatases have an additional effect on protein translation via their interactions with FMRP.

5.2.3. Glycogen synthase kinase

Glycogen synthase kinase 3 (GSK3) is a central metabolic regulatory enzyme. After mGluR stimulation, GSK3 is inhibited by serine-phosphorylation through the PI3K/Akt or MEK/ERK pathways. Increased activity of GSK3 in several regions of the fragile X mouse brain, caused by an impaired phosphorylation of GSK3, were detected (119). This is an unexpected finding as elevated mGluR signalling in the fragile X syndrome predicts an inactivation of GSK3.

5.3. The Rho GTPase pathway

Rho GTPases are Ras Homology proteins that regulate actin dynamics, gene transcription, cell cycle progression and cell adhesion (120). There are 3 main subfamilies: Rho, Rac and Cdc42. In neurons, Rho GTPases are implicated in axon and dendrite outgrowth as well as in the formation and maintenance of dendritic spines and consequently synapse formation via the regulation of the actin cytoskeleton. They act as a molecular switch and cycle between an active GTP-bound and an inactive GDP-bound state (Figure 2). The activation is facilitated by Rho guanine nucleotide exchange factors (RhoGEFs) by the exchange of GDP into GTP. GTPase-activating proteins (GAPs) inactivate Rho GTPases by increasing the intrinsic GTPase activity and guanosine nucleotide dissociation inhibitors (GDIs) sequester GDP-bound Rho GTPases, maintaining the Rho GTPases in the inactive state.

Mental retardation is often associated with abnormalities in dendritic spines (121). In addition, disruption of several genes in the Rho GTPase pathway were found in patients with specific forms of mental retardation, including *oligophrenin 1*, *PAK3*, *ARHGEF9*, *LIMK-1*, *ARHGEF6* and many others (reviewed by (122)). For some of these genes, the effect on dendritic spine

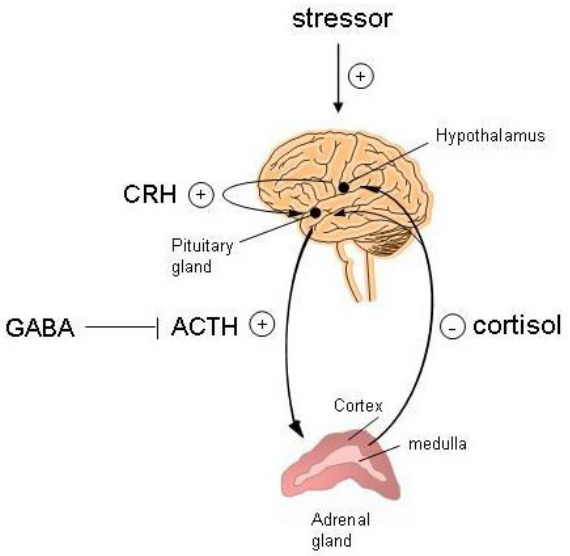

Figure 3. Stress responses and control of the hypothalamic-pituitary-adrenal axis. The hypothalamic-pituitary-adrenal (HPA) axis controls stress reactions by secreting corticotrophin-releasing hormone (CRH) by the hypothalamus. On his turn, CRH stimulates the pituitary gland to secrete adrenocorticotropic hormone (ACTH) which stimulates the production of glucocorticoids, such as cortisol, in the adrenal cortex. Cortisol inhibits HPA activity through negative feedback to both the hypothalamus and the pituitary gland. In addition, a non-glucocorticoid mechanism of inhibition is regulated by the neurotransmitter GABA, which inhibits ACTH release. Both inhibitory mechanisms are disturbed in the fragile X syndrome.

morphology *in vivo* was demonstrated in knockout mice. *LIMK-1* knockout mice show an altered head/neck ratio of spines (123) and *oligophrenin 1* knockout mice exhibit a reduction in mature dendritic spines (124). Both knockout mice models show impaired cognition. These observations lead to the hypothesis that an aberrant Rho signalling might be responsible for the abnormal maturation of dendritic spines and could contribute to the mental retardation in the fragile X syndrome.

The identification of CYFIP as a cytoplasmic FMRP interacting protein identified a first functional link between FMRP and the control of actin (46). CYFIP1/2 interacts with Rac1 which plays a role in the dynamic reorganisation of the actin cytoskeleton (125). In *Drosophila melanogaster*, *dRac* mRNA was found in the dFmrp-RNP complex (126). Schenck and colleagues proposed a model in which dRac1 activation upon unknown extracellular signals positively regulates dFmrp action via CYFIP on neuronal morphogenesis (127). Moreover, Rac1 activation leads to relocalization of four FMRP main interactors (CYFIP1, FXR1P, NUFIP and 82-FIP) to actin-containing domains called actin rings (128). In Fmrp deficient fibroblast cells, there is an enhanced Rac1-induced actin remodelling. This correlates with reduced expression levels of phospho-ADF/Cofilin (P-Cofilin), an effector protein of Rac1, and an increased level of the catalytic subunit of protein phophatase 2A (PP2Ac), which controls P-Cofilin dephosphorylation, in these cells. This altered expression level might be the consequence of translational repression of *Pp2Ac-beta* mRNA due to Fmrp binding of this message.

A further link between FMRP and Rho GTPases was provided by Gantois and colleagues (129). They found a 2-fold underexpression of a RhoGEF, *Leukemia-associated RhoGEF* (*Larg*/*ARHGEF12*) in the hippocampus of fragile X mice. LARG specifically activates RhoA (130). In general, RhoA has an inhibitory effect on neuronal growth. Constitutively active RhoA leads to a decrease in spine density and spine length, while an increased spine density and length was found with dominant negative RhoA (131). A decreased density and an immature form of spines are observed in many forms of mental retardation. However, an increased density is specific for the fragile X syndrome. This gives an indication that LARG, via RhoA, might be involved in the aberrant neuro-anatomic morphology. Underexpression of LARG could lead to less activation of RhoA and, as a consequence, cause changes in neuron outgrowth and formation of immature spines.

p21-activated kinase (PAK) is the final known link between FMRP and Rho GTPases (132). PAK is a downstream effector of Rac and consequently is a regulator of the actin cytoskeleton. In transgenic mice expressing a dominant negative form of PAK, the dendritic spine morphology is opposite to that seen in fragile X patients and mice. In an intercross of this transgenic mouse with the fragile X mouse, several features of the fragile X syndrome were fully or partially rescued including the dendritic spine morphology, altered cortical LTP and some behavioural abnormalities. Moreover, an interaction between PAK1 protein and FMRP was demonstrated, but how this interaction influences the FMRP function is still unknown.

5.4. The neuroendocrine system

5.4.1. The hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis is a major part of the neuroendocrine system. It controls stress responses and influences anxiety-related behaviour and emotions. There is evidence of dysregulation of the HPA axis in the fragile X syndrome (reviewed by (133)).

In response to stress, the hypothalamus secretes corticotrophin-releasing hormone (CRH), which stimulates the pituitary to secrete adrenocorticotropic hormone (ACTH), which then stimulates the adrenal gland to secrete glucocorticoids such as cortisol (Figure 3). Elevated levels of salivary cortisol and increased cortisol reactivity when performing social tasks were found in male fragile X patients (134). The level of cortisol was positively associated with the severity of the behavioural, social and attention problems in the patients. An exaggerated stress response was also described in the fragile X mouse (135, 136). It is possible that the HPA axis abnormalities do not only influence the behaviour but also the dendritic spine morphology observed in the fragile X syndrome as it was

recently shown that increased corticosteroids levels and stress can also increase the spine density in rats (137, 138).

When the stressor disappears, the stress response must be terminated (Figure 3). Glucocorticoids inhibit the stress-induced HPA activity by a negative feedback mechanism. In fragile X patients and mice, this feedback system is disturbed. Several explanations have been suggested. First, glucocorticoid receptor alpha is a predominantly cytoplasmic, low-affinity receptor for corticosteroid hormones and its mRNA is a ligand of FMRP (139). In hippocampal dendrites of *Fmr1* knockout mice, a decreased concentration of glucocorticoid receptors was found. This suggests diminished responsiveness to corticosteroids which may disrupt corticosteroid feedback regulation. Second, annexin-1 (Anx-1) was found to be abnormally expressed in leukocytes from male patients (140). Anx-1 is a glucocorticoid-modulating protein that mediates the negative feedback mechanism exerted by glucocorticoids on the HPA axis (141). The aberrant posttranslational modification of Anx-1 may lead to abnormal function of the protein and an altered feedback mechanism. Third, GABA is a known inhibitor of ACTH release and there is evidence that $GABA_A$ receptor agonists can reduce the stress response of the HPA axis (142). A decreased GABA expression should lead to more stress and anxiety in concordance to our observations of the underexpressed GABAergic system in fragile X animal models and the related fragile X phenotype (106).

5.4.2. Melatonin homeostasis

Sleep disturbances are reported in up to 77 percent of children with fragile X syndrome (143). They have problems with falling asleep and during night-time they often come awake. It was suggested that this could be caused by abnormal melatonin levels. Melatonin is a sleep hormone secreted from the pineal gland and regulated by the hypothalamus via the sympathetic nervous system. It is secreted in response on darkness and is important in the regulation and maintenance of sleep. One study with fragile X boys reported a melatonin deficiency (144). In contrast, a second study reported elevated levels of melatonin both during the day and at night (145). It was suggested that this could be due to malfunctioning of melatonin receptors, leading to increased melatonin production to compensate for the reduced receptor reactivity. However, no molecular evidence is found yet. Increased melatonin production might also be related to an overactive sympathetic nervous system in fragile X patients (146). The sympathetic nervous system innervates the pineal gland and stimulates the production of melatonin. As a consequence, overactive neurons may lead to elevated melatonin levels.

5.4.3. Other hormones in the neuroendocrine system

The levels of several other hormones secreted by the hypothalamic-pituitary system, like gonadotropins, testosterone and thyroid hormone, were investigated (reviewed by (133)). Elevated levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and a blunted thyroid-stimulating hormone (TSH) response to thyrotropin-releasing hormone (TRH) were found. These findings may contribute to the physical features of the fragile X syndrome such as macroorchidism and pre-pubertal growth.

5.5. The cholinergic system

Another pathway believed involved in the cognitive-behavioural deficits of the fragile X syndrome is the cholinergic system. *FMR1* is highly expressed in cholinergic neurons of the nucleus basalis during early neurodevelopment (147) and cholinergic pathways are involved in specific cognitive functions, including executive attention, learning and memory (148). An aberrant cholinergic function was found in the fragile X mouse and fly model (149, 150). Functional magnetic resonance imaging (fMRI) in fragile X girls showed reduced neuronal activity in hippocampus and basal forebrain, two cholinergic brain regions (151). Using magnetic resonance spectroscopy (MRS) decreased choline levels were found in the dorsolateral prefrontal cortex in male fragile X patients (152).

6. TREATMENTS IN THE FRAGILE X SYNDROME

6.1. Non-pharmacological interventions

The cognitive and behavioural problems that fragile X patients encounter have a major impact on their daily tasks. Many behavioural characteristics are influenced by environmental factors. By manipulating these factors quality of life can be dramatically improved (153). For fragile X patients, it is important to have routine and an appropriate educational training. In several studies with fragile X children, it was already shown that a higher-quality in home and school environment is associated with less autistic behaviour, better adaptive behaviour and higher IQ scores (154-156). Non-pharmacological interventions, such as speech therapy, social skills training, occupational and sensory integration therapies, may be an effective treatment for the behavioural problems associated with the fragile X syndrome.

6.2. Symptom-based pharmacological treatment

Environmental interventions are often combined with a symptom-based pharmacological treatment to improve behaviour (Table 1). However, these medications are rather supportive and do not specifically target the underlying neuronal mechanisms dysregulated in the fragile X syndrome (157).

Stimulants are the most frequently used class of medication in boys with the fragile X syndrome (157). Central nervous system (CNS) stimulants are drugs that increase the activity of the CNS. They are targeted to symptoms of distractibility, hyperactivity and impulsivity. Two used stimulants are methylphenidate (Ritalin) and dextroamphetamine (Adderall) (158). Besides the improvement in attention deficit hyperactivity disorder (ADHD)-like symptoms, some individuals show side effects like anxiety, mood lability or aggressive tendencies after intake of stimulants (157). In Europe, the use of stimulants is not allowed or is discouraged. The use of the nonstimulant L-acetyl-carnitine can be a good alternative. Two controlled trials have shown its efficacy in treatment

Table 1. Current symptom-based therapy

Drug Class / Drug	Symptom	Side effects	Remarks
CNS stimulants - Methylphenidate (Ritalin) - Dextroamphetamine (Adderall)	Distractibility, hyperactivity, impulsivity	Anxiety, mood lability, aggression, appetite suppression, insomnia, tics	Not allowed/discouraged in Europe Response rate lower in adults and children younger than 6 years old.
L-acetyl-carnitine	Distractibility, hyperactivity, impulsivity	No	Phase II clinical trial
Alpha 2-adrenergic receptor agonists - Clonidine (Catapres) - Guanfacine (Tenex)	Overarousel, hyperactivity, impulsivity,attention deficit, aggression, sleep problems (clonidine)	Sedation (clonidine)	It replaces stimulants in younger or neurological more affected children.
Selected serotonin reuptake inhibitors (SSRI) - Fluoxetine (Prozac) - Sertraline (Zoloft)	Anxiety, compulsive and perseverative behaviours, aggression, mood changes	Hyperactivity, restlessness, aggression, appetite, insomnia, nausea, impotence	Use less activating SSRIs in hyperactive patients.
Tricyclic antidepressants - Venlafaxine (Effexor)	Anxiety, attention problems, sleep problems	Cardiac dysrythmias (rarely)	Do not use in patients with active seizures.
Atypical antidepressants - Bupropion (Wellbutrin)	Anxiety, mood changes (Bupropion)		
Anticonvulsants - Carbamazepine (Tegretol, Carbatrol)	Seizures, mood changes, sleep problems	Sedation, hypotonia, clumsiness, weight alterations, depression, cognitive suppression, disinhibition	Avoid phenytoin in children: gum hypertrophy, tissue overgrowth and dental problems. Avoid phenobarbital and gabapentin: exacerbate behavioural problems.
Antipsychotics - Haloperidol (Haldol) - Risperidone (Risperdal) - Aripiprazole (Abilify)	Aggression, mood instability, anxiety, sleep problems	Weight gain, sedation, nausea, constipation, parkinsonism, tardive dyskinesia	For patients with extreme behaviours. Preference for atypical antipsychotics: less sedation, more favourable motor side effect profile.
Benzodiazepines - Diazepam (Valium)	Seizures, anxiety	Sedation, ataxia, tolerance, dependence	

of ADHD-like symptoms in boys with the fragile X syndrome (159).

To diminish overarousal symptoms, patients can be treated with the alpha 2-adrenergic receptor agonists clonidine and guanfacine (157). They are thought to dampen sensory input perceived by the brain and show about 70 percent efficacy in boys with the fragile X syndrome. Clonidine can be quite helpful for sleep problems too, although sedation can also be a problematic side effect. The agonists can also be used replacing stimulants in younger or neurological more affected children (159).

Antidepressants, particularly selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine (Prozac), are used to manage anxiety, compulsive and perseverative behaviours, and mood disorders (157). However, fluoxetine is often not the first choice, especially for hyperactive patients, because it is able to provoke restlessness and aggression (159).

Seizures occur in about 20 percent of the fragile X patients and can be treated by anticonvulsants. Side effects include sedation, hypotonia, clumsiness, weight alterations and depression (157). Sometimes, the side effects are more pronounced then the seizure improvement. A controlled follow-up and an individually optimized medication profile are necessary.

For patients who exhibit more extreme behaviours, like very aggressive behaviour and mood instability, antipsychotic drugs, such as Risperidone, can be desirable (157). However, their use is limited in comparison with stimulants and antidepressants, due to the many side effects. The newer antipsychotic drugs have a much safer profile than older commonly used medications and are even used for sleep problems.

6.3. Targeted pharmacological treatment

So far, no specific medical treatment exists for the fragile X syndrome. The more we learn about the neuronal functions of FMRP and the molecular pathways that are involved in the fragile X syndrome, the better we can address the symptoms by using drugs that interact specifically with these pathways. An overview of all ongoing and planned drug trials is presented below (Table 2).

6.3.1. Drugs interacting with the $GABA_A$ receptor

$GABA_A$ receptors are inhibitory receptors involved in anxiety, epilepsy, depression, sleep problems and learning and memory; all symptoms of the fragile X syndrome. Therefore, the $GABA_A$ receptor might be a good target for the treatment of this disorder. The pharmacology of the $GABA_A$ receptor is well documented and several types of drugs, acting through the $GABA_A$ receptor, are discussed below.

Like other types of ionotropic receptors, $GABA_A$ receptors are pentamers assembled from a combination of 19 possible known subunits; alpha 1-6, beta 1-3, gamma 1-3 delta, epsilon, pi, theta and rho 1-3 (160). Many drugs interacting on the $GABA_A$ receptor exist and it is the subunit composition of the receptor that makes the pharmacological characteristics of the receptor subtype. This composition depends on the type of neuron and the position in the brain (161). Besides a direct activation of

Table 2. Future targeted therapy

Drug Class / Drug	Symptom	Side effects	Specific FXS target	Used by	Remarks
Neuroactive steroids - Ganaxolone	Epilepsy, anxiety, cognition	Somnolence	$GABA_A$ R delta subunit	Catamenial epilepsy, partial-onset seizures, infantile spasms (phase II clinical trial)	Clinical trial is planned.
mGluR5 antagonists - Fenobam - STX107	Anxiety, hyperactivity, mood changes	Mild sedation (fenobam)	mGluR5		Phase I clinical trial
Ampakines - CX516 (Ampalex)	Cognition, memory	Rash	AMPA receptor	Alzheimer's disease, schizophrenia (phase II clinical trial: improvements observed)	Phase II clinical trial: no significant difference between placebo- and drug group found. More potent ampakines needed.
Lithium	Behaviour and cognition, mood stabilisation	Well-tolerated	mGluR pathway	Various psychiatric disorders	Add-on pilot trial.
$GABA_B$ receptor agonists - Arbaclofen (STX209)	Seizures, irritability, aggression	?	$GABA_B$ receptor		Phase II clinical trial
Antiglucocorticoids - Mifepristone (RU-486, Mifeprex)	Stress, overarousel, hyperactivity	Rash	HPA axis		Open-label pilot study: behavioural problems worsened in 1 patient: need to test a larger group
Melatonin	Sleep problems	No	Melatonin homeostasis	ASD, developmental disabilities, insomnia	Randomized, double-blind, placebo-controlled, crossover trial.
Acetylcholinesterase inhibitor - Donepezil (Aricept)	Executive function, hyperactivity, irritability	Very mildly	Cholinergic system	Alzheimer's disease	Phase II clinical trial
Tetracycline antibiotic - Minocycline	Cognition, language and social skills, anxiety	Gastrointestinal problems, hyperactivity, mood changes, sleep problems	MMP-9	Alzheimer's disease, ALS, MS	Preliminary survey. More effective in younger children.

the $GABA_A$ receptor by binding of GABA to the GABA binding site, the $GABA_A$ receptor can also be allosterically modulated by many drugs like benzodiazepines, barbiturates, steroids, anaesthetics and convulsants (162, 163).

Pharmacological evidence that treatment via the GABAergic system might be effective in the fragile X syndrome came from experiments performed by Chang and colleagues (150). They discovered that *dFmr1*$^{-/-}$ flies die during development when reared on food containing increased levels of glutamate. By performing a chemical genetic screen, three compounds implicated in the GABAergic pathway were able to rescue lethality. These compounds were GABA itself, nipecotic acid, a GABA reuptake inhibitor, and creatinine, a potential activator for the $GABA_A$ receptor. Moreover, GABA treatment could restore multiple phenotypes in *dFmr1* mutant flies.

The most used GABAergic drugs for fragile X therapy are the benzodiazepines, such as diazepam (Valium). Benzodiazepines have anticonvulsant and anxiolytic effects, but display side effects like sedation, ataxia, tolerance and dependence (164). Only $GABA_A$ receptors with a beta, gamma 2 and either an alpha 1, alpha 2, alpha 3 or alpha 5 subunit contain a benzodiazepine binding site (165). Knowledge of underexpression of specific $GABA_A$ receptor subunits in the fragile X syndrome enables treatment of fragile X patients in a more specific way using subunit-selective agonists. It is known that the sedative effect of benzodiazepines is mediated by alpha 1 containing $GABA_A$ receptors (166), while alpha 2 and alpha 3 containing subtypes are responsible for the anxiolytic effects (167, 168). More selective $GABA_A$ receptor agonists are currently under investigation (169, 170). One example is TPA-023 (MK-0777), which has antagonistic efficacy for the alpha 1 and alpha 5 subtypes, but is a partial agonist at the alpha 2 and alpha 3 subtypes (171). Its anxiolytic activity and lack of sedation were proved in rodent and primate animal models. TPA-023 showed also anticonvulsant activity in a mouse pentylenetetrazole seizure model. No typical benzodiazepine side effects were observed after prolonged treatment.

Another type of drugs are the neuroactive steroids, which are positive allosteric modulators of $GABA_A$ receptors (172). Neuroactive steroids are naturally occurring metabolites, or synthetic analogs, of steroid hormones like progesterone, that lack hormonal activity, but instead modulate neuronal function through interaction with a unique recognition site on the $GABA_A$ receptor. Use of a delta subunit knockout mouse revealed that especially the delta subunit containing $GABA_A$ receptor subtypes are sensitive to neuroactive steroids (112). Ganaxolone (Marinus Pharmaceuticals, Inc., Connecticut) is the 3-beta-methyl-substituted analog of the endogenous neuroactive steroid allopregnanolone. The 3-beta substitution prevents metabolism and enhances the bioavailability without altering the primary pharmacological properties. The

anticonvulsant activity of ganaxolone is already proofed in *in vitro* and in *in vivo* tests. The safety and tolerability profile in humans seems to be favourable and ganaxolone is now tested in several phase II clinical trials for the treatment of different types of epilepsy, such as catamenial epilepsy, partial-onset seizures, infantile spasms (reviewed by (173, 174)). All clinical trials show that patients treated with ganaxolone have a trend in decreased seizure frequency. The most frequently reported side effect is somnolence, which is dose-limiting. At this moment, our group is validating the efficacy of ganaxolone as an antiepileptic drug in the *Fmr1* knockout mouse. Clinical trials with ganaxolone in children and adults with fragile X syndrome are planned (175). The focus will be on anxiety and improvement of seizures.

6.3.2. Drugs interacting with the mGluR pathway

The mGluR theory predicts that fragile X symptoms should be treatable with mGluR5 antagonists in order to dampen mGluR5-mediated dendritic translation and/or by treatment with AMPA-receptor activators to enhance their activity at the synapse (Figure 4).

6.3.2.1. MPEP

MPEP (2-methyl-6-(phenylethynyl)-pyridine) is an mGluR5 antagonist and its efficacy was tested in fragile X animal models. When *dFmr1* mutant flies were reared on food containing MPEP, fragile X phenotypes such as courtship, memory and mushroom defects could be partially reversed (100). In mice, MPEP shows anticonvulsant activity, reducing the percentage of seizures from 60 percent to 10 percent in *Fmr1* knockout mice. MPEP was also able to rescue the open field phenotype and the prepulse inhibition of acoustic startle (PPI) phenotype observed in fragile X mice. In addition, after MPEP treatment of primary hippocampal neurons *in vitro*, less immature spines were measured (82, 176). In the morpholino-induced knockdown zebrafish, treatment with MPEP appeared to rescue the neurite branching and craniofacial defects (103). Unfortunately, the drug is not approved for use in patients.

6.3.2.2. Fenobam

Fenobam is a selective and potent mGluR5 antagonist and shows anxiolytic activity (177). It binds to the same allosteric modularity site at the mGluR5 receptor as MPEP. Consequently similar effects were observed on the spine morphology of *Fmr1* knockout hippocampal cultures after treatment with fenobam as after treatment with MPEP (82). In 2009, a first clinical study using a single oral dose of fenobam was conducted to provide an initial evaluation of safety and pharmacokinetics in adults with fragile X syndrome (178). No significant adverse reactions were reported. Improvements in mood, calmed behaviour and a 20 percent improvement in PPI were observed in a subset of fragile X patients.

6.3.2.3. STX107

STX107 (Seaside therapeutics, Masssachusetts) is a highly potent, selective mGluR5 antagonist. A phase I clinical trial of STX107 was initiated in 2009 to determine the basic pharmacokinetic parameters and to evaluate safety, tolerability and optimal dosage in healthy volunteers.

6.3.2.4. CX516

CX516 (Cortex Pharmaceuticals, Inc., California) is a positive AMPA receptor modulator or ampakine (179). By binding the AMPA receptor, it increases the response amplitude and enhances glutamate-induced long-term potentiation and synaptic strength (180). Previous studies have shown that the use of CX516 leads to enhancement of learning and memory in animal models and in humans. In 2006, Berry-Kravis and colleagues have set up the first phase II clinical trial in fragile X patients (181). It was a double-blind, placebo-controlled study in which CX516 was given for 4 weeks to the fragile X patients. Despite the promising results in patients with Alzheimer's disease and schizophrenia on behavioural and cognitive symptoms, no significant improvement in behaviour or cognition in fragile X patients treated with CX516 was found. It is possible that longer treatment trials must be performed in order to know the full effects or that the dosing was inadequate. Further clinical testing was terminated due to the low potency and the very short half-life of CX516 in humans. Clinical trials with more potent ampakine compounds are considered.

6.3.2.5. Lithium

The mGluR is a G-protein coupled receptor (GPCR) coupled to the Gq-protein. After activation with glutamate, the Gq signalling stimulates phospholipase C (PLC) to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (182). IP_3 causes calcium release from intracellular stores and DAG activates the second messenger signal cascade by activating the Ca^{2+}-dependent protein kinase C (PKC). The PKC pathway is implicated in dendritic mRNA translation by regulating transcription factors and translation initiation (183). Lithium might restore the translational activity in the fragile X syndrome by inhibiting the inositol phosphate (IP) turnover and thus depleting the PLC substrate and downregulating the PLC signalling pathway (159). Lithium also directly inhibits and increases the inhibitory phosphorylation of GSK3, the enzyme that is hyperactive in the fragile X mouse brain (119).

Lithium treatment in fragile X mice was able to increase the phosphorylation of GSK3 and resulted in a reduction of the incidence and severity of audiogenic seizures and modified the open field behaviour (119). In the fragile X fly, treatment was shown to improve defects in courtship behaviour and memory (100). An open-label trial in male fragile X patients resulted in a significant improvement in behavioural functioning, adaptive behaviour and verbal memory (184).

6.3.3. Arbaclofen (STX209)

$GABA_B$ receptors are metabotropic GABA receptors and act both on pre- and postsynaptic sites (185). Presynaptically, $GABA_B$ receptor activation leads to reduced neurotransmitter release through inhibition of presynaptic Ca^{2+} channels. The presynaptic $GABA_B$

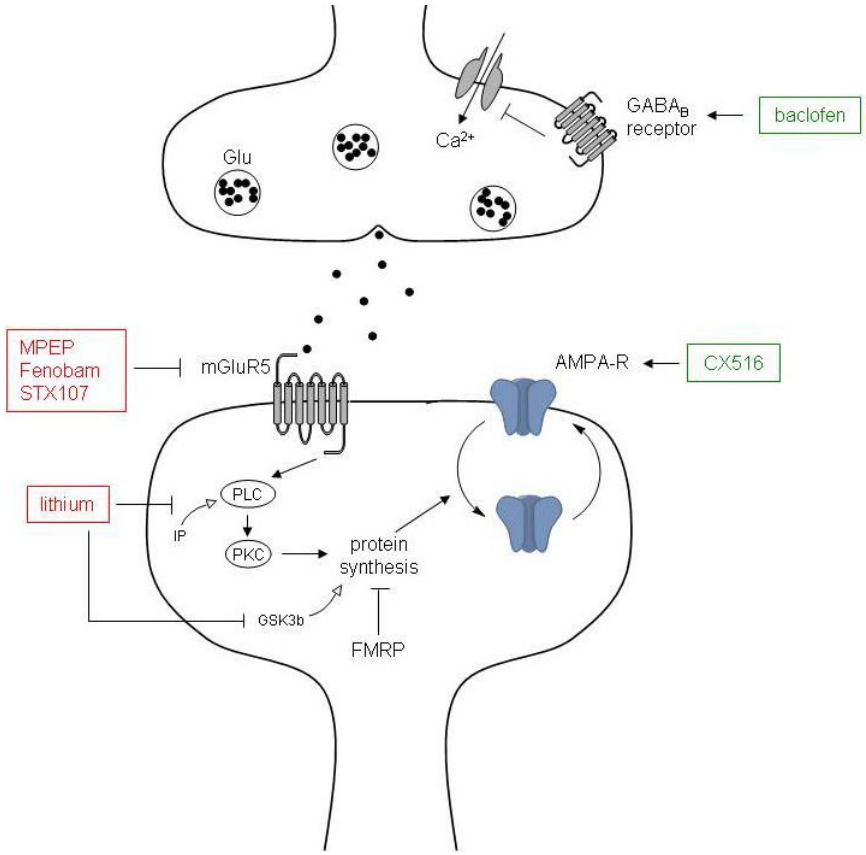

Figure 4. Drugs interacting with the mGluR pathway. The mGluR theory suggests that mGluR activation normally stimulates synthesis of proteins involved in long-term depression (LTD) via internalisation of surface expressed synaptic AMPA and NMDA receptors. FMRP functions as negative regulator of translation and puts a brake on LTD. In absence of FMRP this mechanism is disturbed, leading to an exaggerated mGluR response. Several drugs interacting at this pathway exist and are used in clinical trials. mGluR signalling can be reduced by MPEP, fenobam or STX107, all of which are mGluR5 antagonists and by lithium which interferes with the second messengers and the effector molecules of the mGluR pathway. CX516 is a positive AMPA receptor modulator, enhancing long-term potentiation. Baclofen interacts indirectly with the mGluR signalling by suppressing the glutamate release through presynaptically $GABA_B$ receptor stimulation.

expression is most abundant on excitatory glutamatergic synapses. Thus, $GABA_B$ receptor activation may suppress glutamate release. Therefore, stimulating the $GABA_B$ receptor should diminish the excessive metabotropic glutamate signalling in the fragile X syndrome (Figure 4). Recently, it was shown that treatment with a $GABA_B$ receptor agonist, baclofen, is able to inhibit the audiogenic seizures in *Fmr1* knockout mice (186). This is also in line with the observation that audiogenic seizures are influenced by different kinds of G-protein coupled receptors including mGluR5 and $GABA_B$ receptors (187). While the mGluR signalling seems to induce seizures, the $GABA_B$ receptors seem to play an inhibiting role. A phase II clinical trial for arbaclofen was already initiated in adolescents and adults with fragile X syndrome.

6.3.4. Drugs interacting with the neuroendocrine system

6.3.4.1. Mifepristone

It was suggested that the aberrant stress response observed in fragile X patients could be caused by elevated levels of the stress hormone cortisol. Mifepristone (RU-486) is an antiglucocorticoid medication that blocks the glucocorticoid type II receptor. This can initially prevent the negative effects of the overabundance of cortisol. Prolonged treatment leads to an up-regulation of the glucocorticoid receptor in hypothalamic and limbic structures, thereby enhancing the glucocorticoid receptor negative feedback control of the HPA axis (188). An open-label pilot study with mifepristone improved behaviour problems in 2 out of 10 participants and worsened it in one patient (153). Titration of the medication in a double-blind trial of mifepristone in a larger group of participants is planned.

6.3.4.2. Melatonin

Sleep problems are a major problem in children with fragile X syndrome (145). Despite the use of melatonin in children with autism spectrum disorders (ASD) and in children with developmental disabilities (189-191), the first clinical trial of melatonin in fragile X subjects was only reported in 2009 by Wirojanan and

colleagues (143). A significant improvement in total night sleep duration, sleep latency time and sleep-onset time was observed in patients treated with melatonin compared to placebo treated patients.

6.3.5. Donepezil

Evidence was provided that cholinergic functions are diminished in the fragile X syndrome. Enhancing the acetylcholine function is another approach to treat the disorder and can be obtained by inhibiting the degradation of synaptic acetylcholine by acetylcholinesterase. Fragile X patients enrolled in an open-label trial of donepezil, an acetylcholinesterase inhibitor, demonstrated significantly improved executive function and significantly decreased problem behaviours (152). More specifically, they reported an improvement in working memory and mental flexibility, hyperactivity and irritability, and general cognitive-behavioural status. Side effects were very mild. A randomized controlled study will be set up this year.

6.3.6. Minocycline

Minocycline is a broad spectrum tetracycline antibiotic, but also has potential as a neuroprotective agent which is shown in animal model for neurodegenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis (ALS) (192, 193). Also clinical trials have shown its therapeutic potential in treating neurodegenerative disorders (194). One mechanism of action of minocycline in neuroprotection is by inhibiting matrix metalloproteinases (MMPs). Some MMPs, such as MMP-9, can influence the spine morphology by cleaving extracellular matrix (ECM) or membrane proteins that are implicated in synapse formation and dendritic spine maturation (195). MMP-9 expression levels are elevated in the hippocampus of the fragile X mouse and minocycline treatment was able to normalize the MMP-9 expression levels (196). Moreover, minocycline treatment rescued the immature dendritic spine profile of fragile X hippocampal neurons *in vitro* and *in vivo* and has benefits on the behavioural problems in the fragile X mouse. A first clinical trial for the fragile X syndrome showed especially improvements in general cognition, language, attention, social communication and anxiety (197). Treatment was more effective in younger children and the most common side effects were gastrointestinal problems.

6.4. Additional clinical trials

Some more clinical trials were announced on the website www.clinicaltrials.gov. Aripiprazole (Abilify, Bristol Meyers Squibb, New York) is an atypical antipsychotic drug (198). It is a dopamine D2 receptor partial agonist and has partial agonist activity at serotonin $5HT_{1A}$ receptors and antagonist activity at $5HT_{2A}$ receptors. It is extensively used in treatment of depression and schizophrenia (199). The purpose of this clinical trial is to determine the effectiveness and tolerability of aripiprazole in children and adolescents with fragile X syndrome. They suppose aripiprazole will be effective in decreasing aggression, SIB, agitation and interfering repetitive behaviour. Riluzole (Rilutek, Aventis Pharma S.A., Santo Domingo, Dominican Republic) is a glutamate release inhibitor. Open-label trials with riluzole demonstrated the effectiveness of the drug in patients with obsessive-compulsive disorder (OCD) (200). Given the overlap between repetitive behaviour in the fragile X syndrome and OCD symptoms, a phase IV clinical trial is planned. A phase II randomized, double-blind multiple ascending dose study with the drug RO4917523 (Hoffmann-La Roche Ltd) is just opened for patient recruitment. This drug is an NMDA receptor antagonist and it will be tested in clinical trials for patients with treatment resistant depression too (201). The safety, tolerability, pharmacokinetics and efficacy of RO4917523 will be evaluated. A phase II trial with the drug NPL2009 (Neuropharm Group plc, United Kingdom), an mGluR5 antagonist, was completed in 2008. The safety and effects of the drug were evaluated in prepulse inhibition tests and continuous performance tasks in adult patients. The drug was well tolerated and in 50 percent of the participants, improvement was observed (202).

7. PERSPECTIVES

Until recently, treatment of fragile X patients was aimed at managing the various symptoms of the disorder. Insights in the underlying mechanisms of the fragile X syndrome resulted in the identification of affected molecular and biochemical pathways and in the discovery of new rational therapeutic targets. Clinical trials with drugs interacting with the mGluR pathway, neuroendocrine system and some other pathways have begun and showed very promising results. More clinical trials with drugs working on these and other pathways such as the GABAergic system are planned in the near future.

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