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Review

Insights into $GABA_A$ergic system deficits in fragile X syndrome lead to clinical trials

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ABSTRACT

An increasing number of studies implicate the $GABA_A$ergic system in the pathophysiology of the fragile X syndrome, a frequent cause of intellectual disability and autism. Animal models have proven invaluable in unravelling the molecular mechanisms underlying the disorder. Multiple defects in this inhibitory system have been identified in *Fmr1* knockout mice, including altered expression of various components, aberrant $GABA_A$ receptor-mediated signalling, altered GABA concentrations and anatomical defects in GABAergic neurons. Aberrations compatible with those described in the mouse model were detected in *dfmr1* deficient *Drosophila melanogaster*, a validated fly model for the fragile X syndrome. Treatment with drugs that ameliorate the $GABA_A$ergic deficiency in both animal models have demonstrated that the $GABA_A$ receptor is a promising target for the treatment of fragile X patients. Based on these preclinical studies, clinical trials in patients have been initiated.

This article is part of the Special Issue entitled 'GABAergic Signaling in Health and Disease'.

1. Introduction

The fragile X syndrome is a frequent inherited cause of intellectual disability and syndromic autism (Santoro et al., 2012). The disease is caused by a loss of function mutation in the *FMR1* gene at Xq27.3 (Verkerk et al., 1991). Expansion of a CGG repeat in the 5′ untranslated region to more than 200 units, leads to hypermethylation of the *FMR1* promoter and the repeat. As a consequence, *FMR1* transcription is silenced and no fragile X mental retardation protein (FMRP) is synthesised. FMRP is a selective RNA-binding protein that regulates transport and translation of its target mRNAs (Heulens and Kooy, 2011; Santoro et al., 2012). Male patients present with mild to severe intellectual disability, physical features, including macroorchidism and facial dysmorphisms, and behavioural problems (Hagerman, 2002; Berry-Kravis, 2014). The latter include hyperactivity, impulsivity, attention problems, anxiety, mood lability and autistic features. Specific medical problems are childhood seizures and strabismus. As females still have one active copy of the *FMR1* gene, they are typically less severely affected and their clinical presentation is more variable.

GABA, the major inhibitory neurotransmitter in the adult mammalian brain, exerts its action through ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors (Bettler and Tiao, 2006). Both receptors have been implicated in the fragile X syndrome. Here, we focus on the $GABA_A$ergic system as the abnormalities in the $GABA_B$ system have been reviewed in detail elsewhere (Berry-Kravis, 2014; Braat and Kooy, 2014). $GABA_A$ receptors are heteropentameric ligand-gated chloride channels, as described in more depth elsewhere in this issue. The $GABA_A$ receptor is assembled as a non-random combination of receptor subunits; α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, π, ρ_{1-3} (Farrant and Nusser, 2005; D'Hulst et al., 2009a). This results in a wide variety of subtypes, with distinct expression patterns, different physiological properties and distinct sensitivity to pharmacological modulation. Moreover, the receptor subtypes have specific subcellular expression patterns. $GABA_A$ receptors that contain an α_{1-3}, $\beta_{2/3}$ and a γ_2 subunit are mainly synaptic, whereas α_{4-6}- and δ-containing receptors are mainly perisynaptically or

Abbreviations: ADAMS, Anxiety, Depression, and Mood Scale; APRA, Antibody-Positioned RNA Amplification; CGI-I, Clinician's Global Impression-Improvement; EMSA, Electrophoretic Mobility Shift Assays; FMRP, Fragile X Mental Retardation Protein; FS, Fast-Spiking; HITS-CLIP, High-Throughput Sequencing of RNAs Isolated by Crosslinking Immunoprecipitation; IPSC, Inhibitory Postsynaptic Current; KCC2, K^+–Cl^- Co-transporter 2; LTS, Low-Threshold-Spiking; MARCM, Mosaic Analysis with a Repressible Cell Marker; mGluR, Metabotropic Glutamate Receptor; NKCC1, Na^+–K^+–$2Cl^-$ co-transporter 1; PAR-CLIP, Photoactivable Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation; PARS, Paediatric Anxiety Rating Scale; PND, Postnatal Day; PPI, Prepulse Inhibition; RRE, RNA-Recognition Elements; RS, Regular-Spiking; SNAP-IV, Swanson, Nolan and Pelham-IV Questionnaire.

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extrasynaptically located (Farrant and Nusser, 2005). Functionally, two types of $GABA_A$ receptor-mediated signalling can be discerned, phasic and tonic inhibition. Phasic inhibition occurs when synaptic $GABA_A$ receptors are briefly activated by a high concentration of GABA, released from presynaptic vesicles. Binding of GABA to the postsynaptic receptors results in a chloride influx and hyperpolarisation of the postsynaptic membrane and so reduces the firing probability of action potentials. Tonic inhibition is mediated by extrasynaptic $GABA_A$ receptors. These receptor subtypes are relatively insensitive to desensitisation and have a high affinity for GABA as they are activated by low concentrations of GABA, a spill over from the GABA released in the synaptic cleft.

While $GABA_A$ receptors are inhibitory during adult life, they act as excitatory receptors during early development (Ben-Ari, 2002). The early depolarising effect is suggested to have a crucial trophic role and contribute to cell proliferation, migration, differentiation and synaptogenesis (Cellot and Cherubini, 2013). The developmental polarity switch from excitatory to inhibitory occurs following changes in intracellular chloride concentration, which are mediated by changes in expression of the ion transporters Na^+–K^+–$2Cl^-$ co-transporter 1 (NKCC1) and K^+–Cl^- co-transporter 2 (KCC2) (Ben-Ari, 2002; Cellot and Cherubini, 2013). The balance between the amount of the chloride importer NKCC1 and the chloride exporter KCC2 determines the intracellular chloride concentration and, therefore, the nature of $GABA_A$ receptor-mediated signalling. In immature neurons, the NKCC1 co-transporter maintains a high intracellular chloride concentration, leading to chloride efflux through $GABA_A$ receptors and GABA-mediated depolarisation. As neurons mature, the expression of the co-transporter KCC2 is up-regulated, while at the same time NKCC1 expression is down-regulated. Consequently, intracellular chloride concentrations decrease and GABA signalling becomes hyperpolarising.

An increasing amount of experimental evidence supports the key involvement of the $GABA_A$ receptor in the pathophysiology of the fragile X syndrome. The aim of this review is to summarise the defects in the $GABA_A$ergic pathway in the fragile X syndrome that have been reported at different levels, including at the expression, the functional, the neurotransmitter and the neuroanatomical level (Table 1).

2. Experimental evidence for $GABA_A$ receptor-associated defects in fragile X syndrome

2.1. *Mus musculus*

2.1.1. *mRNA and protein level*

Using whole-genome expression profiling, we initially identified a twofold reduction in δ subunit mRNA in the hippocampus and cortex of adult *Fmr1* knockout mice (Gantois et al., 2006). Subsequently, several studies used quantitative real-time PCR to assess $GABA_A$ receptor mRNA expression in adult *Fmr1* knockout mice (Table 1). Firstly, mRNA expression levels of 18 $GABA_A$ receptor subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, π, ρ_{1-2}) were measured in cortical and cerebellar samples (D'Hulst et al., 2006). This analysis revealed an underexpression (~35–50%) of 7 additional subunits (α_1, α_3, α_4, β_1, β_2, γ_1, γ_2) in the cortex, whereas no differential expression was detected in the cerebellum, consistent with the initial study. Additional studies have reported reduced α_5 (26%) and δ (35%) mRNA levels in the subiculum (Curia et al., 2009) and reduced β_1, β_2 and β_3 mRNA levels in cerebellum, hippocampus and cortex, respectively (Hong et al., 2011). Conversely, β_3 expression was reported increased in the hippocampus in the latter study. Protein levels were quantified using western blot or immunohistochemistry. Using an antibody against all β subunits, a (~35%) reduced expression of was found in adult cortex, hippocampus, diencephalon and brainstem, but not in the cerebellum (El Idrissi et al., 2005). Subicular protein amounts of the tonic $GABA_A$ receptor subunits α_5 and δ were diminished (with 13 and 28%, respectively) in *Fmr1* knockout mice (Curia et al., 2009). Protein levels have also been quantified during early postnatal development (Adusei et al., 2010; Kratovac and Corbin, 2013). The first study measured the expression of several $GABA_A$ receptor subunits (α_1, β_{1-3}, γ_2, δ) in forebrain homogenates during development, i.e. at postnatal day 5 (PND5), postnatal day 12 (PND12) and at two months of age (Adusei et al., 2010). Reductions in the amount of α_1, β_2 and δ subunit were found at some, but not all, developmental stages. The second study evaluated the expression of three α subunits (α_{1-3}) in the basolateral amygdala during the first three postnatal weeks (Kratovac and Corbin, 2013). Although the protein levels did not differ between both genotypes at any particular time point (PND10, PND14 and PND21), there were differences in developmental timing of α_1 and α_2 expression. For example, α_1 expression did increase significantly from PND10 to PND14 in *Fmr1* knockout samples, but not in the wild-type samples. These changes in the precise timing of expression of specific subunits during development might contribute to the observed differences in inhibitory neurotransmission.

Other components of the $GABA_A$ receptor system that have been studied include enzymes involved in GABA synthesis (*Gad1*/GAD67 and *Gad2*/GAD65), GABA transporters (*Slc6a1*/GAT1, *Slc6a12*/GAT2, *Slc6a13*/GAT3 and *Slc6a11*/GAT4), enzymes involved in GABA degradation (*Aldh5a1*/succinate semialdehyde dehydrogenase and *Abat*/GABA transaminase) and a protein important for the targeting and clustering of $GABA_A$ receptors at the postsynaptic membrane (*Gphn*/Gephyrin). mRNA expression of *Gad1*, *Slc6a1*, *Slc6a11*, *Aldh5a1* and *Gphn* was (~20–40%) reduced in the cortex of adult *Fmr1* knockout mice (Table 1) (D'Hulst et al. 2009b). Although $GABA_A$ receptor mRNA expression was not reduced in the cerebellum (D'Hulst et al., 2006), the mRNA levels of *Gad1*, *Slc6a1*, *Slc6a11* and *Aldh5a1* were (~20%) down-regulated in this brain region (D'Hulst et al., 2009b). Studies of the protein levels of the GABA-synthesising enzyme GAD65/GAD67 reported potentially conflicting results. Reduced GAD65/GAD67 levels were found in the amygdala of young *Fmr1* knockout mice (PND21) (Olmos-Serrano et al., 2010), while increased GAD65/GAD67 expression was observed in the adult cortex, hippocampus, diencephalon and brainstem (El Idrissi et al., 2005). Increased (50%) GAD65 expression was reported in the adult forebrain (Adusei et al., 2010). In addition, gephyrin protein level was (28%) reduced in adult forebrain (Adusei et al., 2010), but was not altered in the basolateral amygdala during early development (PND10, PND14 and PND21) (Kratovac and Corbin, 2013). Additionally, there were differences in the developmental timing of gephyrin expression. Finally, GABA transaminase and succinate semialdehyde dehydrogenase were reduced (45% and 28%, respectively) in the forebrain during development (PND12), but not in adult *Fmr1* knockout mice (Adusei et al., 2010).

2.1.2. *Functional level*

A first indication of functional $GABA_A$ receptor defects was that *Fmr1* knockout mice exhibit altered responses to the cholinergic compound carbachol (Table 1) (D'Antuono et al., 2003). In a subsequent study, $GABA_A$ receptor-mediated signalling was measured directly in the subiculum, showing a profound (~91%) impairment of tonic inhibition (Curia et al., 2009). In contrast, phasic inhibition, as measured by the characteristics of spontaneous inhibitory postsynaptic currents (IPSCs), was unaffected. The observed defects in tonic inhibition correlated well with reduced mRNA and protein levels of the extrasynaptic $GABA_A$ receptor subunits α_5 and δ.