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The fragile X syndrome is perhaps the most intensively studied autism and intellectual disability (ID) syndrome (reviewed in Hagerman et al., 2017; Willemsen and Kooy, 2017). This is due to combination of unique characteristics of the disorder. First, its X-linked inheritance pattern allowed the identification of exceptionally large pedigrees with multiple patients (Martin and Bell, 1943), second its fragile site provided a cytogenetic recognition mark on the X-chromosome (Lubs, 1969) and third, the peculiar inheritance pattern showing anticipation, resulting in more affected patients in the latter generations of any pedigree. The molecular mechanism behind this so called Sherman paradox was unravelled in 1991, when several groups reported a CGG-repeat at the fragile site that expanded in the disease pedigrees through multiple generations (Kremer et al., 1991; Oberlé et al., 1991; Verkerk et al., 1991). Subsequent analysis learned that the repeat is polymorphic in the population with repeat sizes of up to 45 repeats, while patients carry a full mutation that expended to a length of over 200 repeats. As a consequence of the repeat expansion, the repeat becomes methylated, inhibiting the expression of the associated *FMR1* gene and thus preventing expression of the fragile X mental retardation protein FMRP. The disorder was also one of the first that were discovered as caused by repeat expansion (reviewed in Nelson et al., 2013).

The fragile X syndrome is also one of the most frequent “rare” genetic disorders, with a frequency currently estimated at approximately 1/5000 patients (Hunter et al., 2014). It occurs in all populations. The ubiquitously expressed FMRP seem to play a role in a multitude of processes in the cell. The protein has two K homology domains (KH1 and KH2) and an arginine-glycine-glycine (RGG) box. Though both types of RNA binding domains, it binds a subset of neuronal mRNAs. Various types of high-throughput sequencing of bound RNAs isolated by different methodologies revealed that the mRNA targets of FMRP encode pre- and post-synaptic proteins and that several targets are implicated in autism spectrum disorders suggesting a molecular overlap between fragile X syndrome and other neurodevelopmental disorders (Darnell et al., 2011; Suhl et al., 2014). The protein also has both a nuclear localisation and export signal that allows it to shuttle its mRNA targets between the nucleus and cytoplasm [11] . Functional studies have shown involvement in a number of processes in the cell, including RNA transport, stability, in local protein translation etc.

The numerous functions of FMRP inspired many to look for interfering agents to correct the defective pathways in order to try and improve the lives of the patients. Pathways that have been interfered with include several neurotransmitter receptors, including for glutamate, gamma aminobutyric acid (GABA), endocannabinoid (eCB) and muscarinic acetylcholine (mACh), in intracellular signalling pathways such as glycogen synthase kinase-3 (GSK3), extracellular signal related kinase (ERK), mammalian target of rapamycin (mTOR) and p21-activated kinase (PAK) and in the matrix metalloproteinase 9 (MMP-9), an extracellular proteases (reviewed by Braat and Kooy, 2014; Ligsay and Hagerman, 2016; Willemsen et al., 2004). While interference with all of these pathways has shown be effective at least in animal models, two pathways are generally accepted as most promising for future trials: the glutamatergic and the gabaergic (Berry-Kravis et al., 2018). The discovery that Long-term depression is enhanced in the fragile X mouse model led to the development of the so-called mGLuR theory, which states that as the clinical symptoms of the fragile X syndrome are due to the absence of translational inhibition by FMRP in the synapse (Bear et al., 2004; Huber et al., 2002). In the absence of the break, the postsynaptic translation in enhanced in patients, leading to an increased internalisation of AMPA receptors, a molecular explanation for the enhanced LTD. The theory predicts that by dampening mGluR group 1 receptor signalling, the excess translation and thus the clinical consequences of the disorder can be restored. Pharmalogical and generic rescue experiments have

provided substantial experimental evidence for this hypothesis (Dölen et al., 2007; Michalon et al., 2012), but trials in humans failed to meet the expectations raised by the animal work as yet (Berry-Kravis et al., 2012; Berry-Kravis et al., 2017). The initial observation of a reduced expression of approximately half of the subunits of the ionotropic GABA(A) receptor in the fragile X mouse model led to the hypothesis that a decreased GABA signalling could be underlie a range of the clinical symptoms of the fragile X syndrome (D'Hulst and Kooy, 2007; D'Hulst et al., 2006; Gantois et al., 2006). Stimulated by the availability of various subtype specific agonists of the receptor treatment and more fundamental insights that led to the initiation of clinical trials. This review aims to highlight the GABAergic system abnormalities in the fragile X syndrome.

Fragile X patients and epilepsy

Fragile X syndrome patients suffer from poor language development, intellectual disability, autism and behavioural alterations such as hyperactivity and anxiety. Physical features include prominent ears and a long face, hyperlaxity of the small joints and macroorchidism that develops from puberty. This clinical picture can vary depending on sex, age and molecular variation (level of methylation or the presence of mosaicism of repeat size or methylation), which leads to differences in production of the FMR protein (Hagerman et al., 2017). The clinic has been extensively reviewed recently, and we here concentrate on the seizures co-morbid with the fragile X syndrome as these have been relatively ill-documented. Epilepsy is one of the most substantial medical comorbid problems in Fragile X syndrome. Seizures have in the past been reported in 10-40% of Fragile X patients (Musumeci et al., 1991; Partington, 1984; Wisniewski et al., 1991). A more recent survey on approximately 1400 full mutation Fragile X individuals narrowed this prevalence down to 12% in total, 14% in males and 6% in females, with an onset mainly between 4 and 10 years of age. (Berry-Kravis et al., 2010)

In general, the epilepsy in Fragile X syndrome is limited to childhood and adolescence with seizures disappearing before the age of 20 years and the average age of seizure remission is 9,5 years for boys and 5,5 years for girls. Epilepsy in adult patients has been described however in several papers (Kenmuir et al., 2015; Sabaratnam et al., 2001). The spectrum of seizures in fragile X syndrome is quite large but complex partial seizures are most commonly seen. Simple partial and generalized tonic-clonic seizures also occur (Berry-Kravis 2002). Abnormal epileptiform EEG findings can be observed both in FXS patients with seizures and without seizures. The most common EEG morphology shows centrotemporal spikes as seen in benign focal epilepsy of childhood (BFEC or benign rolandic epilepsy). As in normally developing individuals the centrotemporal spike pattern is not necessarily associated with clinical seizures. Other and less common EEG patterns in FXS are spikes in temporo-occipital or frontal areas, focal rhythmic frontal slow waves, generalized discharges, unspecified epileptic discharges and generalized slowing of the EEG (Berry-Kravis, 2002). Epilepsy in Fragile X is considered mild to moderate in severity and is generally easily controlled with anticonvulsants. Studies show that almost no patients are on more than one medication. Most commonly used are valproic acid, lamotrigine, levetiracetam and oxcarbazepine. However, severe epilepsy resistant to anticonvulsant therapy has also been described (Incorpora et al., 2002; Kenmuir et al., 2015). To what extend seizures in early live affect developmental outcome is not known, but in fragile X knockout mice it has been reported that a single seizure in early life can negatively influence the outcome of behavioural and cognitive testing (Hodges et al., 2019).

The GABA receptor.

GABA receptors are abundant in the brain and are held responsible for the majority of inhibitory transmission. The GABA(A) receptor is a pentameric ion channel that is permeable to chloride ions depending on its confirmation, that on its turn is dependent on binding of GABA and a several phamalogically relevant drugs (D'Hulst et al., 2009a; Farrant and Nusser, 2005). The receptor is composed of five out of 19 potential subunits, including α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, π and ρ_{1-3}, eleven of which make up 95% of all receptors (Sequeira et al., 2019). The stoichiometry is non-random and favours receptors containing 2α, 2β and one of either γ, δ, ε or π subunits but exceptions to this rule are observed. The subunit composition is highly brain-region dependent and strongly influences drug sensitivity. The GABA(B) receptor is consist of a heterodimer of R1 and R2 subunits. The inhibitory effect of this metabotropic receptor is mediated though activation of K^+ and blocking Ca^+ channels.

The GABAergic system is compromised in the fragile X syndrome

GABAergic abnormalities have been discovered in the knockout mouse model of the disorder in an unbiased genome-wide expression profiling screen, where the delta subunit was found underexpressed at the RNA level (Gantois et al., 2006). Follow-up studies found that approximately half of the subunits and a subset of GABA-synthesising and metabolizing enzymes were also reduced in expression in fragile X mouse brain (D'Hulst et al., 2006; D'Hulst et al., 2009b). A subset of these subunits has been analysed on Western blot and without exception, reduced expression at the protein level was also observed (Adusei et al., 2010; Braat et al., 2015; El Idrissi et al., 2005; Olmos-Serrano et al., 2010). These studies firmly confirmed a reduction in expression of several GABA subunits in the fragile X mouse model. A transgenic reintroduction of the FMR1 gene in the fragile X knockout mouse restored the expression of the alpha1 and delta subunits to a level observed in control mice, further substantiating the validity of the observations (Braat et al., 2015). The amount of the neurotransmitter GABA itself is also reduced in several brain regions of the mouse model (Braat et al., 2015; Davidovic et al., 2011). Apart from in mice, GABAergic deficits were also found in a *Drosophila melanogaster* fragile X syndrome model (Chang et al., 2008; D'Hulst et al., 2006; Franco et al., 2017; Gatto et al., 2014). A single PET scan study in patients demonstrated underexpression in multiple brain regions of GABA receptors targeted by [^{11}C]flumazenil, a strong ligand of the benzodiazepine site of the receptor (D'Hulst et al., 2015).

Functional consequences of the GABA reduction

The abnormalities in GABA compositions appear not directly linked to major anatomical abnormalities, though in neocortical inhibitory circuits in the fragile X knockout mouse, a mild but significant reduction in the densities of parvalbumin-positive neurons has been reported in two independent studies (Lee et al., 2019; Selby et al., 2007). Whole-cell voltage clamp recordings of CA1 pyramidal neurons in the hippocampus of Fmr1 knockout mice using showed significantly reduced eIPSC amplitudes in response to stimuli of various intensities, indicating an average reduction of about 30% in GABAergic transmission. In further electrophysiological studies, a significant reduction in the amplitude of evoked inhibitory postsynaptic currents (eIPSCs), but also in the amplitude and frequency of both, sIPSCs and mIPSCs.was recorded. Such findings are compatible with a combination of pre- and postsynaptic

changes underlying the deficit in GABA receptor-mediated inhibition in the hippocampus (Sabanov et al., 2017). Although binding of several GABA(A) receptor subunits by FMRP has been demonstrated the mechanism of underexpression of the various components of the GABAergic system remains elusive (Braat et al., 2015; Miyashiro et al., 2003).

Pharmalogical rescue of GABAergic abnormalities suggest the receptor is a valid target for treatment

Several lines of evidence suggest that agonist of the GABA(A) receptor can rescue some of the abnormalities of the fragile X syndrome at least in animal models. In a large-scale drug screen in *D. melanogaster*, GABA agonists, including GABA itself, were more effective in rescuing the symptom of the fragile X fly model than any other drug (Chang et al., 2008). In mice, seizures could be prevented following treatment with a single dose of ganaxolone, a neurosteroid agonist of the receptor (Heulens et al., 2012). The drug also corrected marble burying behaviour in a dose dependent way and in part prepulse inhibition deficits (Braat et al., 2015). Gaboxadol (also called OV101 and THIP), an agonist with a preference for delta-subunit-containing extrasynaptic GABA(A) receptors was reported to completely restore hyperactivity, anxiety, aggression, and repetitive behaviors in a second fragile X knockout mouse model (Cogram et al., 2019). In the latter model, the Fmr1 gene was inactivated by removing exon 1 and concomitant promotor sequences, as opposed to the more commonly used fragile X mouse model, where exon 5 has been interrupted by a neomycin cassette (Bakker et al., 1994; Mientjes et al., 2006). Both models though are reported to generate no functional FMRP.

A first clinical trials shows encouraging post-hoc results in the more severely affected patients

Ganaxolone was also used as a drug to treat a series of fifty-nine eligible children and adults in a randomized double-blind, placebo controlled trial (Ligsay et al., 2017). Fifty-five participants completed at least the first arm and 51 participants completed both treatment arms. Post-hoc analyses revealed positive trends in areas of anxiety, attention, and hyperactivity in participants with higher baseline anxiety and lower full-scale IQ. However, there were no statistically significant improvements observed on the primary outcome measure (Clinical Global Impression-Improvement), the key secondary outcome measure (Pediatric Anxiety Rating Scale-R), or any other secondary outcome measures in the overall study population. scores. No serious adverse events occurred, although the frequency of such adverse events was slightly higher in the ganaxolone treatment arms.

The future of GABAergic treatment

At the moment, we are faced with the controversy that there can be little doubt that the GABAergic system is compromised in the fragile X syndrome and in fact in many other neurodevelopmental disorders (Braat and Kooy, 2015) but that the treatment with a GABA(A) agonist failed to meet the expectations. An explanation for this discrepancy is not at hand. Perhaps the drug used has not the optimal subtype-specific efficiency and different agonists may have a stronger effect. The other explanation may be that the GABAergic pathway is not the only pathway disturbed. In fact, single-cell and neuronal network alterations in an in vitro model of Fragile X Syndrome could only be explained by a combination of increased excitation and reduced inhibition (Moskalyuk et al., 2019). Such observations suggest that perhaps combination therapies are to be encouraged.

Whatever trials are planned, the outcome measures a a serious point of concern. The currently used outcome measures are dependent on patient or parental questionnaires and, apart from the problems

inherent to questionnaires, suffer from a large (up to 30%) placebo effect, potentially hiding mild improvements in the patient population. More objective measures required, but not easy to implement given the severity of the condition. Perhaps the EEG abnormalities or even the seizures in general could be an outcome parameter of future trials, as these are much more objectively to measure than behavioural abnormalities. Although seizure treatment in patients is not generally considered a priority in fragile X syndrome patients, it has recently been show that in mice, a single seizure in early life, leads to long-term behavioural changes in later life.

In summary, there is overwhelming evidence for GABAergic abnormalities in the fragile X syndrome. Future work will potentially shed more light on the subtype-specificity of the abnormalities and of the additional pathways that are affected in this disorder, in order to facilitate future clinical trials.

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