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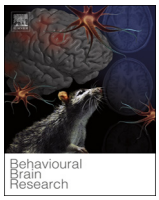

Review

Modelling fragile X syndrome in the laboratory setting: A behavioral perspective

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ABSTRACT

Fragile X syndrome is the most common form of inherited mental retardation and the most frequent monogenic cause of syndromic autism spectrum disorders. The syndrome is caused by the loss of the Fragile X Mental Retardation Protein (FMRP), a key RNA-binding protein involved in synaptic plasticity and neuronal morphology. Patients show intellectual disability, social deficits, repetitive behaviors and impairments in social communication. The aim of this review is to outline the importance of behavioral phenotyping of animal models of FXS from a developmental perspective, by showing how the behavioral characteristics of FXS at the clinical level can be translated into effective, developmentally-specific and clinically meaningful behavioral readouts in the laboratory setting. After introducing the behavioral features, diagnostic criteria and off-label pharmacotherapy of FXS, we outline how FXS-relevant behavioral features can be modelled in laboratory animals in the course of development: we review the progress to date, discuss how behavioral phenotyping in animal models of FXS is essential to identify potential treatments, and discuss caveats and future directions in this research field.

1. Introduction

In 1943, Martin and Bell described for the first time a sex-linked form of mental retardation affecting eleven sons of a family, all born from mothers with normotypic levels of intelligence [1]. More than 25 years later, the existence of a constriction near the end of the long arm of the X chromosome of males affected by intellectual disabilities was reported by Lubs [2]. Later, this variant was localized to Xq27.3 [3] and become known as "fragile site" on the X chromosome. Seven members of the original family of sex-linked mental retardation reported by Martin and Bell in 1943 were re-examined: five of them were found to carry this fragile site, together with typical facial features (i.e., long face, large ears and prominent jaw) and macroorchidism [4]. This condition was named as "Martin-Bell syndrome", now known as Fragile X Syndrome (FXS) [5]. In 1991, the gene responsible of FXS was cloned and named *Fragile X Mental Retardation 1* gene (*FMR1*) [6].

FXS is the most commonly inherited form of developmental and intellectual disability [7,8]. Several population-based studies estimated the prevalence of the disease as 1 in 4000 males and 1 in 8000 females [8,9] although it has recently been suggested that the best estimate for the frequency of the full mutation (FM) is 1/2500 for both males and females [10].

FXS is associated with an unstable expansion of a CGG trinucleotide repeat within the 5′untranslated region (5′UTR) of the *FMR1* gene, located in the X chromosome [6,11]. In the normal population, the number of CGG triplets in the *FMR1* gene varies from 6 to 54, allowing transcription and translation of the gene. When the number of CGG triplets expands between 55 and 200, the premutation (PM) state occurs, while more than 200 repeats characterize the FM condition. PM alleles are unstable and, upon maternal transmission, tend to expand to FM alleles [12]. The probability of having a FM allele in the offspring born from mothers carrying the PM depends on the size of the maternal CGG repeat tract. Conversely, the presence of AGG interruptions in the CGG repeat locus of the *FMR1* gene increases the stability of PM alleles during prenatal transmission, thus decreasing the risk of expansion of a PM allele to a FM allele [13].

Patients carrying the PM have normal intellectual abilities but some of them show fragile X tremor ataxia syndrome (FXTAS), a late-onset neurodegenerative disorder affecting approximately one in 3000 men and a smaller number of women in the general population [14]. In addition, fragile X–associated primary ovarian insufficiency (POI), which consists in menopause appearance prior to the age of 40, occurs in women carrying the PM [15].

More than 200 CGG repeats cause the FM and the fragile site at Xq27.3 [6]. This expansion triggers hypermethylation and other epigenetic modifications of the CpG island in the promoter region, resulting with the heterocromatinization of the *FMR1* locus and consequently with the loss of the protein product, the fragile X mental

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retardation protein (FMRP) [16].

FMRP is an RNA-binding protein with four major isoforms between 70 and 80 kDa. FMRP is involved in different steps of RNA metabolism ranging from nuclear export to transport of mRNA along neurites and translational control in the soma and at synapses [17,18].

FMRP is expressed in many tissues and it is particularly abundant in the brain, where it is essential for neural development and plasticity [19,20]. FMRP binds a large number of mRNAs, also called the FMRP transcriptome, and many of them code for proteins involved in neuronal excitability and synaptic transmission [18]. In the absence of FMRP, the deregulation of translation/transport/stability of these mRNAs affects multiple neuronal pathways, generating the altered phenotype displayed by FXS patients.

Not only FXS is the most frequent heritable form of intellectual disability, but it is also the most common monogenic cause of syndromic autism spectrum disorders (ASD) [21,22]. Approximately 30% of patients with FXS meet the full diagnostic criteria for ASD [22], while over 90% of individuals with FXS display some ASD symptoms [23].

In addition to severe intellectual disability and autistic-like features, FXS is characterized by seizures, attention deficit and hyperactivity, sleep disturbances, craniofacial abnormalities and macroorchidism. Females typically display milder symptoms than males due to compensation by the second non-affected X chromosome.

Approved therapies for FXS are not yet available: current approaches focus on behavioral therapy and off-label medications that mitigate only a limited set of symptoms, such as hyperactivity, seizure and anxiety. Thus, the search for new treatments able to modify the lifetime course of FXS and to improve its symptoms is urgent. In this context, animal models are excellent research tools for two main reasons: 1. to understand how loss of *FMR1* and FMRP contributes to FXS symptomology; 2. to validate new therapeutic targets and potential drug candidates.

The aim of this review is to outline the importance of animal models in FXS research, by showing how the behavioral characteristics of FXS at the clinical level can be translated into effective, developmentally-specific and clinically meaningful behavioral readouts in the laboratory setting. After introducing the clinical diagnosis and current pharmacotherapy of FXS, we outline how FXS-relevant behavioral features can be modelled in laboratory animals in the course of development: we review the progress to date, discuss how behavioral phenotyping in animal models of FXS is essential to identify potential treatments, and discuss caveats and future directions in this research field.

2. Diagnosis of fragile X syndrome

In general, FXS testing should be considered in: 1. individuals of either sex with mental retardation and developmental delay, especially if they have other physical or behavioral signs of FXS, or a family history of FXS, or relatives with undiagnosed mental retardation; 2. individuals seeking reproductive counseling or women who are experiencing fertility problems, especially if they have a family history of premature ovarian failure, a family history of FXS, or male or female relatives with undiagnosed mental retardation; 3. men and women who are experiencing late onset intention tremor and cerebellar ataxia of unknown origin, in particular if they have a family history of movement disorders, a family history of FXS, or male or female relatives with undiagnosed mental retardation [24]. In all these cases, a family history of intellectual disabilities (ID), ASD and neurological problems such as dementia, ataxia and tremor, assists in the diagnosis of FXS.

2.1. Genetic diagnosis

Prior to the identification of the *FMR1* gene, the diagnosis of FXS was based on the cytogenetic evaluation of the presence of the fragile site at Xq27.3 (FRAXA) in peripheral blood lymphocytes. To improve the detection rate of FRAXA, cytogenetic methods have been replaced by Southern blot analyses and, more recently, by Polymerase Chain Reaction (PCR)-based techniques, which are more sensitive and specific than the karyotype observation and allow the identification of PM carriers [25].

Southern blot analysis detects all *FMR1* alleles including normal, larger-sized PM and FM; furthermore, it allows to determine the methylation status of the *FMR1* promoter region [26]. This technique is time consuming, relatively expensive and difficult to interpret. Standard PCR is based on the direct amplification of the CGG-repeat, it is fast and highly sensitive to detect *FMR1* repeats in the normal and PM range. It can only reveal alleles with up to ~300 repeats in males and up to ~160 repeats in females but it fails to identify larger CGG expansions [27]. Thus, in the past ten years, the combination of traditional PCR plus Southern blot analysis has been considered the best procedure for the molecular diagnosis of the *FMR1* mutation.

The limitations of the PCR plus Southern blot technique lead to the development of a new procedure able to detect all *FMR1* alleles, called Triplet primed PCR (TP-PCR). TP-PCR is the evolution of previous PCR protocols and allows to simultaneously amplify both the full-length *FMR1* alleles and CGG triplets in the same PCR reaction [28].

To detect the normal or mutant *FMR1* gene in the fetus, early prenatal diagnosis tests can be done in pregnant women trough chorionic villus sampling as a predictive test at 10–12 weeks of gestation [24]. However, the methylation status of the *FMR1* gene is often not yet established at this pregnancy stage. For this reason, DNA testing performed on cultured amniocytes obtained by amniocentesis after 15 weeks of gestation is considered the follow-up test to resolve possible ambiguities.

2.2. Physical and behavioral diagnosis

FXS patients usually exhibit long narrow face, prominent ears and forehead, irregular teeth, flat feet, hypotonia and joint hypermobility, and macroorchidism in about 95% of postpuberal males [29].

At the behavioral level, FXS is characterized by mental retardation and by a progressive decline of cognitive abilities [30]. Other common behavioral features displayed by FXS patients involve poor eye contact or gaze aversion, excessive shyness, anxiety, stereotypic movements, unusual speech, self-injury, hand flapping and hand biting, hyperactivity, tactile defensiveness, attention deficits, problems in impulse control, hyperarousal and oversensitivity to tactile, auditory, olfactory or visual stimuli [31–36].

Physical, behavioral and cognitive manifestations of FXS are dissimilar in males and females. The majority of female FXS carriers exhibit normal physical appearance, moderate ID or normal IQ and the severity of cognitive impairment depends on the X activation ratio (the percentage of cells in which the normal X chromosome is the active X chromosome) [37]. Compared to males, girls with FXS display a higher rate of emotional disturbance including mood lability, social withdrawal, inappropriate affect, poor modulation of verbal tone and depression [38,39].

During early infancy, FXS is typically identified through delayed or abnormal development of the infant. Parents report lack of motor coordination, hyperactivity or irritability and delayed developmental milestones as crawling, first speech and walking [40–42]. Male children affected by FXS exhibit severe deficits in motor skills and a significant cognitive delay [43,44]. They often manifest hyperarousal resulting in quick loss of capacity for self-regulation, aggressive outbursts or self-abusive behaviors, gaze avoidance, stereotypies, unusual speech and tactile defensiveness [31,32,34,43–45]. In addition, boys affected by FXS can display hypersensitivity to auditory, tactile, visual and olfactory stimuli [46]. In the social domain, FXS children appear excessively shy and anxious and avoid unfamiliar people [32,47,48]. During the school age, social maladaptive behaviors became more problematic and FXS children demonstrate social avoidance and autistic-like behaviors [35]. The prevalence of attention deficit/hyperactivity disorder