Fragile X Syndrome/Review

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What Can We Learn about Autism from Studying Fragile X Syndrome?

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Abstract
Despite early controversy, it is now accepted that a substantial proportion of children with fragile X syndrome (FXS) meets diagnostic criteria for autism spectrum disorder (ASD). This change has led to an increased interest in studying the association of FXS and ASD because of the clinical consequences of their co-occurrence and the implications for a better understanding of ASD in the general population. Here, we review the current knowledge on the behavioral, neurobiological (i.e., neuroimaging), and molecular features of ASD in FXS, as well as the insight into ASD gained from mouse models of FXS. This review covers critical issues such as the selectivity of ASD in disorders associated with intellectual disability, differences between autistic features and ASD diagnosis, and the relationship between ASD and anxiety in FXS patients and animal models. While solid evidence supporting ASD in FXS as a distinctive entity is emerging, neurobiological and molecular data are still scarce. Animal model studies have not been particularly revealing about ASD in FXS either. Nevertheless, recent studies provide intriguing new leads and suggest that a better understanding of the bases of ASD will require the integration of multidisciplinary data from FXS and other genetic disorders.

Introduction

Autism is defined by the *Diagnostic and Statistical Manual of Mental Disorders – Fourth Edition* (DSM-IV) as a disorder characterized by a qualitative impairment in social interaction, associated with qualitative impairments in communication, and restricted, repetitive and stereotyped patterns of behavior, interests and activities [1, 2]. During the years since this major conceptual revision in 1994, autism has been shown to be a complex behavioral syndrome rather than a single entity, varying in severity, and with multiple genetic and likely environmental etiologies. Therefore, as proposed in the current revision of the DSM system (DSM-5) [3], the label 'autism spectrum disorder(s)' (ASDs) seems more appropriate for this condition[1]. An important consideration included in

[1] In this review, we use the term 'autism spectrum disorder' (ASD) to describe autistic disorder and its milder clinical forms.

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the DSM-5 proposal is that the cognitive level, better exemplified by language skills, within certain limits modifies but does not preclude the diagnosis of ASD. Thus, the essence of ASD is a core and selective impairment in social interaction that is closely associated with restricted and repetitive patterns of behavior.

Genetic disorders characterized by cognitive delay or impairment (i.e., intellectual disability), and a strong association with ASD, have become valuable windows into the neurobiology of this behavioral syndrome. Fragile X syndrome (FXS) is one such example. FXS is a monogenic disorder linked to the silencing of the *FMR1* gene (i.e., reduced to absent production of the Fragile X Mental Retardation Protein or FMRP), for which there is an abundant, and increasing, body of molecular and neurobiological literature [4].

In the following sections, we critically review how studies of clinical cases and animal models of FXS illuminate key aspects of ASD. We also highlight the gaps in knowledge in many key areas, especially phenotyping of behavioral, neurobiological and molecular features of ASD in FXS, and discuss the complexity of extrapolating data from FXS patients and mouse models to a heterogeneous and behavioral-defined entity such as ASD.

Diagnostic Threshold: Autistic Features versus ASD in FXS

FXS is the most common genetic cause of ASD, accounting for approximately 5% of cases [5, 6]. Although most males with FXS display autistic features, only a small fraction meets all DSM-IV criteria for autistic disorder or pervasive developmental disorder not otherwise specified (i.e., ASD). This high frequency of relatively mild autistic features, and differences in ascertainment strategies and supportive diagnostic methods have led to reported rates ranging widely from 15 to 60% for prevalence of ASD in males with FXS [7–16]. Prevalent but mild autistic or anxious features can complicate the diagnosis of ASD in FXS and other disorders. Indeed, we found that impairments in play-based social interactions are widely distributed, but not diagnostic of ASD in boys with FXS [11]. Similarly, the frequent occurrence of anxiety-like features in affected males and females [17] may lead to ASD diagnostic challenges in FXS, an issue that will be discussed in a following section. Despite these difficulties, careful behavioral and statistical analyses do allow an adequate delineation of ASD as a behavioral syndrome in FXS and, most likely, in other genetic disorders.

Intellectual Disability in FXS: Diagnosis of ASD Reflects Selective Disorder

In the Introduction, we noted that the co-occurrence of intellectual disability and ASD raises significant methodological and clinical issues. These issues are of relevance not only to individuals with FXS, but also to all those with severe cognitive impairment who fulfill DSM-IV criteria for ASD regardless of their etiology. The main concern is whether severe global cognitive delay or selective communication impairment, present in a substantial proportion of males with FXS, precludes a confident diagnosis of ASD. Although this matter is not completely resolved, the selectivity of the social interaction impairment that affects individuals with FXS and ASD has been demonstrated. We have shown, both cross-sectionally [11] and longitudinally [18], that impaired adaptive socialization is by far the greatest contributor to ASD diagnosis and severity when regression models also introduce different communication parameters as well as measures of overall cognition. Hall et al. [19] have recently examined profiles of autistic behaviors in FXS in order to determine whether using the DSM-IV classification is appropriate in this disorder. The authors suggested the need to maintain a conceptual distinction between FXS, as an established biological disease, and idiopathic ASD, as a phenomenologically defined behavioral disorder. The basis for this would be that impairments in social and communicative behaviors occur in general at a lower rate in FXS (i.e., their FXS cohort was not divided according to DSM-IV categories) than in idiopathic ASD [19]. However, several other studies have not found clear behavioral differences between individuals with FXS and ASD and those with idiopathic ASD [10, 12, 20] (as discussed in the next section). Other features supportive of ASD as a distinctive entity in FXS include comorbid conditions, in particular the higher frequency of seizure disorder in individuals with FXS and ASD (i.e., 10–20% higher than in FXS alone) [21, 22]. This association between ASD and seizures has also been reported in other genetic disorders [23] and in the general population [23]. Additional evidence for relative independence between cognitive function and social behavior comes from Williams syndrome. In this disorder, affected individuals are hypersocial despite their cognitive impairment that is comparable to the one observed in many males with FXS [24].

In sum, ASD in FXS is a selective disorder of core socialization skills that is relatively independent of general and verbal cognitive skills. In-depth characterizations of other genetic disorders associated with ASD such as

Down syndrome are in progress [25, 26]. If their findings agree with those of FXS, they will solidify the notion of ASD as a selective disorder in intellectual disability, and should lead to a greater understanding of genetic contributions to ASD.

Profile of ASD in FXS: Deficit in Complex Social Interaction Skills with Frequent Social Withdrawal

Assuming that it is indeed possible to clearly delineate ASD in FXS, as well as in other genetic disorders, its study has implications for (a) addressing the particular diagnostic and therapeutic issues affecting individuals with FXS who have a severe phenotype, and complex medical and educational needs; (b) identifying important genetic and neurobiological mechanisms that may also operate in idiopathic ASD, and (c) expanding the behavioral characterization of idiopathic ASD, with a particular focus on the interaction between autistic and anxious behaviors. The latter effort is in line with the recent interest in subdividing idiopathic ASD into discrete clinical groups or endophenotypes [27, 28]. This section and the next one review key behavioral features of ASD in FXS, while the following sections provide overviews of neurobiological and genetic/molecular issues.

Among the genetic disorders associated with ASD, the behavioral phenotype has been best characterized in FXS [29]. The features of the nonregressive type of ASD observed mainly in males with FXS are similar to those observed in their counterparts with idiopathic ASD: severe social indifference [30], a spectrum of social interaction deficits [11, 31] that is relatively independent of cognitive function [11, 32], greater delay in receptive (understanding) language than expressive (speaking) language [12, 30, 32, 33], persistence of gaze avoidance during continuous social challenge [34], and a fairly stable diagnosis over time [30, 32, 35].

The profile of autistic features in boys with FXS based on the Autism Diagnostic Interview-Revised (ADI-R) [36] indicates that diagnosis and severity of ASD are driven by impairment in complex social interaction behaviors, rather than in simpler nonverbal social behaviors [11]. Specifically, ASD in FXS is mainly characterized by deficits in peer interactions and to a lesser extent by impairments in socio-emotional reciprocity [11], a pattern that has been corroborated by Brock and Hatton [37]. In line with this, impairments in reciprocal conversation with peers are present to a significant degree in individuals with FXS and ASD, whereas impairments in friendship are present in all individuals with FXS regardless of their ASD status [38]. Impairments in friendship may be attributed to anxiety symptoms [39] and communication difficulties, both highly prevalent among individuals with FXS. Emphasizing the core social disturbance in males with FXS and ASD, our statistical models examining measures of communication show that only verbal labeling of emotions is a significant predictor of ASD; other strong predictors of ASD status include recognition and application of rules of social interaction and recognition of emotions [11, 30]. Interestingly, adaptive socialization that includes the latter predictors correlates with verbal reasoning [30], a cognitive domain closely linked to working memory [40–42] and frequently implicated in idiopathic ASD [43, 44].

Our studies indicate that impaired adaptive socialization, probably representing deficits in understanding rules of social interaction linked to neocortical cognitive dysfunction, is one of two major interrelated social behavior abnormalities leading to the ASD phenotype. The second behavioral phenomenon is social withdrawal, which seems to be associated with limbic circuit responses [30]. Social withdrawal includes social behaviors with a strong emotional component, some of them described as avoidance (e.g. shy, seek isolation from others) and others as indifference (e.g. unresponsive to social interactions, difficult to reach or contact). Illustrating the central role of adaptive socialization in ASD in FXS is the fact that, whereas most males with FXS and ASD have impaired socialization skills, only those with severe autistic symptoms also show prominent social withdrawal [30]. Interestingly, we have observed that social avoidance becomes a prominent component of ASD in FXS after age 5, a finding that will be discussed in the next section on ASD and anxiety in FXS. The relevance of the central impairment in adaptive socialization skills in FXS and ASD is underscored by the fact that adaptive socialization is a key reference measure for resolving diagnostic discrepancies in idiopathic ASD, particularly when diagnoses on the two 'gold standard' instruments for ASD, the ADI-R [36] and the Autism Diagnostic Observation Schedule [45], are in disagreement.

Although longitudinal studies of ASD in FXS and other genetic disorders are of great value, considering the complexity of the behavioral syndrome and the lack of diagnostic biomarkers, they are relatively scarce. Nonetheless, the few available studies have demonstrated that ASD diagnosis and autistic behaviors are relatively stable over time in FXS [13, 18, 35]. Furthermore, in a 3-year evaluation of boys with FXS [18], we also corroborated

the stability of selective deficits in peer relationships and adaptive socialization skills (fig. 1). Another complementary approach we have begun to apply is laboratory behavioral paradigms that measure dynamic aspects of social interaction [34, 46]. These paradigms take into consideration familiarity with people and places as contexts for social interaction and evaluate behavior in real time, contrasting with records of abnormal behaviors over days or weeks (i.e., behavioral style) reflected in behavioral rating scales. Furthermore, since laboratory paradigms measure behaviors independent of diagnostic labels or preconceptions, they can provide insight into the relationship between ASD and anxiety as well as other aberrant behaviors.

In conclusion, the distinctive profile of complex social interaction deficits in ASD in FXS further supports the selectivity of the behavioral disorder. The central impairment of adaptive socialization and the presence of social withdrawal also suggest that ASD in FXS involves a wide network of cortical and limbic regions [47–49] rather than discrete or lower-level brain areas (e.g. superior temporal sulcus, fusiform gyrus). A more detailed discussion in the subject is provided in the section on neurobiological correlates below.

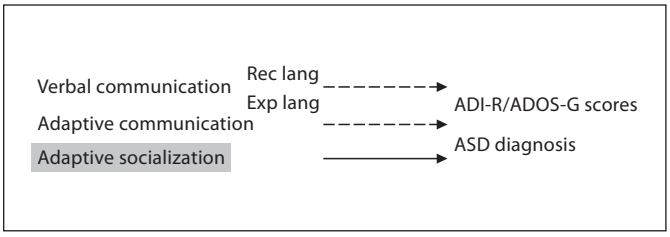

Fig. 1. Diagram of the relationship between skills and ASD in FXS. Note that the delay in socialization skills, and not in overall cognitive or language skills, is a selective contributor to the diagnosis and severity (measured as ADI-R/ADOS-G scores) of ASD in FXS. Rec = Receptive; Exp = expressive; lang = language skills; ADI-R = Autism Diagnostic Interview-Revised [36]; ADOS-G = Autism Diagnostic Observation Schedule-Generic [45] (see fig. 4.1, p. 87, in Kaufmann et al. [57]). Reprinted with kind permission of Springer Science + Business Media.

Anxiety in FXS: Challenging Differentiation from ASD in FXS

Anxiety is another behavioral disorder affecting social interaction. One of its variants, social anxiety, is a disorder characterized by avoidance in social situations. A unique relationship between ASD and anxiety in FXS has been postulated, in part, because of the high prevalence of anxiety in individuals with the genetic disorder. For instance, according to the National Parent Survey, anxiety is the second most common behavioral abnormality in FXS individuals older than 6 years (the most common being attention deficit hyperactivity disorder) [9]. Extending these survey-based findings, a recent study by Cordeiro et al. [50] characterized the association between ASD and anxiety in a large cohort of subjects with FXS. The study found a greater percentage of individuals with FXS meeting DSM-IV criteria for a variety of anxiety disorders than in other intellectual disability groups or in the general population [51]. Although limited systematic research has been conducted in this area, its relevance to ASD in the general population is highlighted by a recent report of social anxiety as the most common comorbidity in idiopathic ASD [52]. The relationship between ASD and anxiety is also relevant to animal models of ASD, since anxiety-like behaviors are commonly reported in these mice and, sometimes, interpreted as evidence of autistic behavior (see section on animal models).

In the present review, we focus on social anxiety and its related traits in FXS. Tranfaglia et al. in this issue address the general features of anxiety in FXS. Widely accepted features of the FXS neurobehavioral phenotype include the closely related excessive shyness, anxious behavior, tactile defensiveness, and sensory hyperarousal [4, 53]. However, their distinction as traits versus clinically relevant problems is unclear. Merenstein et al. [54] reported that 75% of young males with FXS display excessive shyness and social anxiety and 50% have panic attacks, whereas Freund et al. [55] reported that females with FXS also have excessive shyness, social anxiety, and avoidance personality. Other studies emphasize that although individuals with FXS are interested in social interaction, they often display anxiety- and withdrawal-like behaviors in response to unfamiliar people and novel situations [8, 56]. Although these publications describe general features of individuals with FXS who meet DSM-IV criteria for different anxiety disorders, they are not specific regarding social anxiety and its delineation from the diagnosis of ASD.

Our in-depth study of social withdrawal in boys with FXS has led to an initial understanding of the relationship between ASD and social anxiety in this genetic disorder [30, 57]. We classified items of the social withdrawal scales of the Aberrant Behavior Checklist [58] and the Child Be-

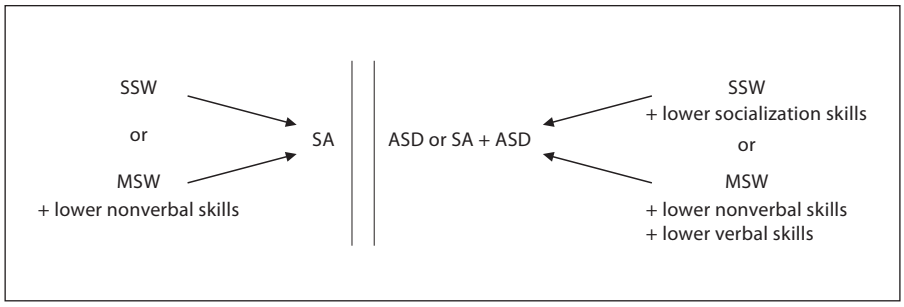

Fig. 2. Model of the relationships between social withdrawal, cognitive impairment, social anxiety, and ASD in FXS. Note that either severe social withdrawal (SSW: SW-I or SW-S) per se or mild social withdrawal (MSW) in conjunction with lower nonverbal skills would lead to social anxiety (SA). A more complex combination of deficits, specifically the addition of lower socialization or verbal skills, is required for ASD alone or comorbid with social anxiety. SSW = Severe social withdrawal; SW-I = social withdrawal-intermediate; SW-S = social withdrawal-severe; MSW = mild social withdrawal; SA = social anxiety (see fig. 4.2, p. 88, in Kaufmann et al. [57]). Reprinted with kind permission of Springer Science + Business Media.

havioral Checklist [59] as either avoidance or indifference [30]. Initially, we found that social avoidance and not, as expected, social indifference was a predictor of ASD diagnosis in boys with FXS, particularly in those older than 5 years [30]. In order to understand this finding, we examined the distribution of social avoidance and social indifference in boys with FXS and found a continuum of severity. We then identified two groups of boys with FXS and marked social withdrawal [57] using clinically relevant cutoffs of the social withdrawal scales [59–64] and confirmatory factor analyses [65]. Boys with intermediate social withdrawal display high scores on a wide range of behaviors with a predominance of avoidance items. In contrast, boys with severe social withdrawal show high scores on both avoidance and indifference items. Moreover, intermediate social withdrawal status was linked to the diagnosis of social anxiety while severe social withdrawal was predominantly associated with severe ASD [57]. The remaining boys with FXS, with mild social withdrawal, tend to display shyness without apparent functional or clinical consequences [57]. These clinical observations suggest that ASD and social anxiety have a common behavioral root in FXS, namely the phenomenon of social withdrawal. As mentioned in the preceding section, the interaction between social withdrawal and impaired adaptive socialization, and its cognitive correlates, ultimately determines the type of social interaction disorder in FXS: ASD and/or social anxiety [30, 57] (fig. 2). We postulate that these behavioral studies in FXS may be particularly informative for understanding the relationship between ASD and social anxiety in the general population.

Neurobiological Correlates of ASD in FXS: Anomalies of the Cerebellar Vermis and Limbic Dysfunction

Although the obvious implication of studying ASD in a genetic disorder such as FXS is the identification of the molecular mechanisms underlying the behavioral syndrome, such studies can also identify neuroanatomical and other neurobiological correlates of ASD. The behavioral profiles discussed in the two preceding sections suggest an obligatory cortical component (i.e., adaptive socialization), probably involving prefrontal and temporal regions [47] that, when combined with limbic dysfunction (i.e., social withdrawal) [49], leads to a severe ASD phenotype. Because of limited access to tissues of affected individuals, these and other hypotheses related to the pathogenesis of ASD in FXS have been tested through neuroimaging [66] and animal models. The next section reviews the literature on mouse models of FXS of significance to ASD.

Virtually all relevant FXS neuroimaging data are derived from structural neuroimaging; the marked cognitive impairment in individuals with FXS and ASD precludes their participation in paradigm-driven functional neuroimaging studies. Several studies have shown that the brain in FXS is slightly larger than in unaffected individuals, as is the case in idiopathic ASD [67]. Selective size changes affecting in general children with FXS include: (a) enlargement of the caudate nucleus [68, 69] and the parietal lobe, the latter being the most consistently reported cortical enlargement in FXS affecting either gray matter [69] or white matter [67, 70]; (b) reductions

in posterior cerebellar vermis, amygdala, and superior temporal gyrus [68, 69], and, particularly in very young males, the hypothalamus, insula, and medial and lateral prefrontal cortices [71, 72]. Limited data are available on differences between boys with FXS and ASD, and their nonautistic counterparts. In a recent study [73], we confirmed our previous finding that the posterior-superior vermis is significantly larger in boys with FXS and autism than in boys with FXS but no ASD [68]. These changes appear to be specific to the individuals with ASD (i.e., autistic disorder) since boys with FXS and social anxiety show increases in anterior, but not posterior, vermis size [73]. Gothelf et al. [69] also reported positive correlations between size of the posterior vermis and caudate and several scales of the Autism Behavior Checklist. In a preliminary study, we also found that in boys with FXS and autism the frontal white matter is also enlarged when compared with nonautistic counterparts, an increase mainly driven by the prefrontal region [73]. Altogether, these findings seem to be significant since the increased posterior-superior cerebellar vermis size affects the same region (i.e., lobules VI–VII) that is smaller in individuals with idiopathic ASD [74–76]. Also, the enlarged frontal white matter in boys with FXS and autism is in line with similar findings in idiopathic ASD [77]. Nonetheless, underscoring the neurobiological heterogeneity of ASD, there are differences between ASD in FXS and ASD in the general population. Hoeft et al. [78] and Meguid et al. [79] have reported differences in the direction of morphologic abnormalities in the two types of ASD. Individuals with FXS, with or without ASD, have a larger caudate nucleus and smaller amygdala than their counterparts with idiopathic ASD [80]. Although these distinctions could be accounted for by methodological issues, they support the notion that ASD is a neurobiologically heterogeneous behavioral syndrome [81]. Overall, the neuroimaging data reviewed above support the concept that ASD in FXS shares brain circuitry abnormalities with ASD in the general population. Interconnected cerebellar and prefrontal regions develop in parallel during the late prenatal and early postnatal period [82], which makes them particularly susceptible to environmental influences. To what extent this ontogenetic profile affects social development [83, 84] and ASD pathogenesis in FXS and the general population is still unknown.

Another window into the neurobiology of ASD in FXS is the study of limbic-hypothalamic function, approached through measuring cortisol levels and other related parameters. Children with FXS typically show a slower return to baseline of cortisol levels after cognitive or social challenges than typically developing children [85]. Furthermore, in children with FXS and severe autistic behavior, the variability of the cortisol response to a social challenge is decreased [46, 86]. In contrast, in children with FXS with prominent social avoidance, the variability of the cortisol response to a social challenge is increased [85]. We also showed that in boys with FXS and ASD, reduced social approach behavior, despite increased social familiarity, correlated with elevated baseline and regulation (i.e., a few hours after a social challenge) cortisol levels [46]. This pattern distinguished children with FXS and ASD from those with FXS but no ASD and from controls. Thus, cortisol regulation appears to be abnormal in FXS, with individuals with FXS and ASD having blunted neuroendocrine and behavioral responses to social stimuli. Although the mechanisms of this abnormal cortisol regulation are unknown, mouse and human studies have provided some insight. We have reported a higher frequency of acetylation of the glucocorticoid-negative regulator annexin-1 in males with FXS than in normal controls [87], particularly in those with severe social withdrawal [88]. Since annexin-1 is involved in the acute phase of cortisol modulation [89], it is unclear whether our finding is related to the abnormal response to cognitive and social challenges (late phase) [85]. Another candidate for abnormal cortisol regulation in FXS is the glucocorticoid receptor alpha. The synthesis of this low-affinity cortisol receptor is directly regulated by the deficient FMRP [90], and in a mouse model of FXS, its levels are decreased in dendrites of hippocampal neurons [91]. These mice also displayed increased cortisol levels and slow return to baseline after a stressful situation [92]. The relevance of these cortisol anomalies to ASD is still unclear.

In conclusion, the best-characterized neurobiological correlate of ASD in FXS is a relative enlargement of the posterior vermis. Neuroendocrine data suggest that limbic regions may also be affected. Integration of these findings with the behavioral data reviewed in preceding sections indicate that severe autistic behavior in FXS is correlated with abnormalities in multiple brain regions and their circuits. Refinement of these data will require careful differentiation of ASD – from social anxiety-related abnormalities, application of multiple behavioral and neurologic approaches (e.g. transcranial magnetic stimulation) [93] to FXS cohorts, and integration of human and animal model data. The latter will be reviewed in the following section.

Mouse Models of FXS and ASD: Challenges in Portraying ASD-Relevant Behaviors

The study of animal models of FXS has already been valuable for understanding the neurobiology of the disorder. Three major abnormalities have been reported in mouse models of FXS: (a) aberrant configuration of dendritic spines (long, tortuous, immature appearance) [94], in correspondence with postmortem findings in individuals with FXS [95, 96]; (b) enhanced activity of class I metabotropic glutamate receptors, leading to increased long-term depression [97], and (c) reduced GABAergic transmission [98]. All these findings have been linked directly or indirectly to the postulated negative regulatory role of FMRP in protein synthesis [97, 98]. Although the aforementioned anatomical and neurotransmitter abnormalities involve brain regions implicated in FXS with and without ASD, their ubiquitous nature and lack of corresponding data on individuals with FXS and ASD (e.g. no PET imaging studies in humans with FXS) preclude the establishment of meaningful relationships with autistic symptomatology. Some of the experimental findings seem nonetheless relevant to ASD in FXS, as the decreased inhibitory/GABAergic transmission [99] we have reported in the lateral amygdala, a brain region linked to both anxiety and ASD [48, 100]. Evidence in support of GABAergic deficit in ASD in FXS also comes from an initial clinical trial using a GABA-B agonist (arbaclofen), which demonstrated selective improvement of social withdrawal behaviors [101]. Despite these intriguing neurochemical findings, and considering that ASD is a behavioral syndrome, the most important contribution of experimental data to ASD in FXS should come from behavioral studies of animal models.

FMR1 is a highly conserved gene, with a nucleotide and amino acid human-mouse identity of 95 and 97%, respectively [102, 103]. Although the *Fmr1* knockout (KO) mouse does not share the gene silencing mechanism observed in individuals with FXS, the net result is the same with undetectable levels of *Fmr1* mRNA and protein [104]. The *Fmr1* KO mouse manifests several of the behavioral abnormalities observed in FXS (i.e., hyperactivity, anxiety-like behaviors) [97]; however, there is a significant variability in their frequency and severity which leads to mixed overall results. In contrast with behavioral abnormalities, cognitive deficits in the *Fmr1* KO mouse have been surprisingly mild [105] and apparent only in some background strains [104, 106].

In terms of social interaction, tables 1 and 2 illustrate the different behavioral assays employed to test ASD-like behaviors and associated symptoms (e.g. general anxiety-like behaviors). These paradigms have been applied to *Fmr1* KO mice and other animal models of relevance to ASD [107–109]. The most widely applied assay for testing social interaction (i.e., frequently termed sociability) is social approach (table 1), typically an integral part of the social choice paradigm along with social novelty [110–116]. Table 2 depicts the most common measures of general anxiety-like behaviors applied to *Fmr1* KO mice. In general, there is considerable variability in the outcome of social interaction studies (table 1), with a wide distribution of social approach performance among different mouse backgrounds and models [108, 112, 117]. This contrasts with a greater consistency for specific measures of general anxiety (table 2). Complicating the situation is the fact that the design of many studies of social interaction is confounded by anxiety-like behavior assessments, which are sometimes interpreted as evidence of ASD-like behaviors.

Table 1. Behavioral tests in *Fmr1* KO versus WT mice: social interaction behaviors

Test	Background	Age months	Result	Stimulus	Ref. No.
Tube test of social dominance	B6	3–4	↓ =	– +	122
Partition (social recognition)	B6 B6	3–4 2–3	= =	+ +	122 109
Social exploration	B6	3–4	↓	–	126
Direct social interaction	(a) B6 (b) B6 (c) B6 $B6D_2$ (d) B6	3–4 3–4 2–3 2–3 3–4	=/↑ ↑ ↑ ↓ ↓	+ + + + –	122 134 109 126
Social approach test[1]	(a) B6 × FVB B6 (b) FVB/NJ (c) B6 and FVB B6 and FVB	∼3 ∼3 2–3 3–4 2	= = ↓/= = =	– – – – –	119 125 121 117

WT = Wild type; ↑ = KO > WT, $p \leq 0.05$; = indicates no difference between KO and WT mice, $p > 0.10$; ↓ = KO < WT, $p \leq 0.05$; – = unfamiliar stimulus-WT mouse; + = familiar stimulus-WT mouse; C57Bl/6J × FVB/NJ background; FVB = FVB/NJ background; $B6D_2$ = C57B1/6J × DBA/2J background.

[1] The Social Approach Test scores time spent by *Fmr1* KO mouse in a side chamber containing a restrained novel mouse (stimulus mouse, S1 WT) vs. time spent in a opposite side chamber within inanimate, nonsocial object (i.e., an inverted wire cup).

Table 2. Behavioral tests in *Fmr1* KO versus WT mice: general anxiety-like behaviors

Test	Background	Age months	Result	Ref. No.
Elevated plus maze	B6	3–4	↓	122
	B6	3–4	↓	132
	B6	~2	=	117
	FVB	~2	=	
	B6	3–4	=	128
	B6	3–4	=	129
	B6 × FVB	3–4	=	
	B6	~1	↑	130
	FVB	~1	↑	131
Elevated zero maze	FVB	2–3	↓	121
Mirrored chamber	B6	3–4	↓	122
Light/dark exploration	B6	3–4	↓	132
	B6	3–4	↑	134
	B6	2–3	=	109
Open field (center distance)	B6	3–4	↓	132
	B6	3–4	=	134
	B6	2–3	=	109

WT = Wild type; ↑ = KO > WT, $p \leq 0.05$; = indicates no difference between KO and WT mice, $p > 0.10$; ↓ = KO < WT, $p \leq 0.05$; B6 = C57Bl/6J background; B6/FVB hybrid = C57Bl/6J × FVB/NJ background.

Studies of social approach have, in general, shown mild differences between *Fmr1* KO and wild-type mice. In addition, as shown in table 1, results have been inconsistent because of the use of different behavioral assays and considerable variability for a given measure. While some studies have shown that genetic background has a significant impact on the presentation of the *Fmr1* phenotype, including autistic-like behaviors [109, 117], additional important methodological issues (e.g. usage of only adult mice of variable age) [109, 117, 118], which are discussed in several publications [109, 119–121], further complicate the picture. Consequently, there is no clear consensus on the level and pattern of social approach impairment in *Fmr1* KO mice. Analyses of social interaction data, after controlling for general anxiety-like behaviors, have drawn parallels between abnormal sociability in *Fmr1* KO mice [117–119, 121, 122] and social anxiety in individuals with FXS [53, 123, 124]. Findings from some of these studies also suggest impairment in other aspects of social cognition [119, 125] and atypical social behaviors (e.g. blunted negative reaction to a more aggressive 'non-preferred' unfamiliar mouse) [126]. Finally, the usefulness of social interaction paradigms in the *Fmr1* KO mouse is underscored by the recent study by Mines et al. [125] who applied them as outcome measures for a lithium carbonate trial. Although only a mild improvement in social behavior was observed, these animal data support the targeting of glycogen synthase kinase (GSK3β) for treating social behavior abnormalities in FXS. The article by Tranfaglia and colleagues in this issue addresses the relationship between abnormal activation of GSK3β and FXS neurobehavioral phenotype.

A number of studies suggest that anxiety-like behaviors can be a significant challenge for interpreting findings relevant to ASD symptoms in mouse models, especially over time [127]. Yet, other studies suggest that these difficulties may be behavioral assay-dependent [117, 128, 129]. As illustrated in table 2, the majority of studies of *Fmr1* KO mice showed decreased general anxiety-like behaviors, including Spencer et al. [109], which used mice of six genetic backgrounds, and Liu and Smith [121] who applied more advanced paradigms such as an elevated zero maze (table 2). Along the same line, Moy et al. [117] found no change in general anxiety-like behavior in *Fmr1* KO mice on either background strain (i.e., C57 and FVB) when using an elevated plus maze performance. Only studies of very young animals (i.e., 2–3 weeks) have reported increased anxiety-like behaviors [130, 131], although some methodological issues limited generalization of these results. Supporting the positive findings, restoring functional *Fmr1* has rescued some of the anxiety-like phenotypes in KO mice [132–134].

In sum, the *Fmr1* KO mouse social interaction findings are inconclusive, including the ASD versus social and other types of anxiety dilemma. Moreover, the anxiety-related data are in contrast to expectations based on the FXS phenotype. Refinements in methodological approaches could help in addressing these matters and determining whether the *Fmr1* KO mouse is a useful model of ASD and/or anxiety in FXS. For example, studies of juvenile (prepubescent) mice can help by better understanding the effect of *Fmr1* on social interaction and anxiety-like behaviors [135] at the developmental stages when the problem is clinically recognized. Although not prominent components of the ASD phenotype in FXS, restricted and repetitive behaviors could also be tested in available models [136, 137] in the context of Fmrp deficit. Another example is to measure the effect of anxiolytic drugs on both social and anxiety-related behaviors [138]. Nevertheless, ASD behaviors and anxiety are complex phenomena that cannot be described by a single behavioral

task or by the action of a single pharmacological compound [139, 140]. In spite of all these difficulties, animal behavioral studies emphasize that genetic background is a critical factor [109, 117], supporting the notion of an interaction between *FMR1* and 'modifier' background genes in the pathogenesis of ASD in FXS. In this regard, additional approaches such as the use of C58/J male prepubescent mice that model multiple components of the autism phenotype [136, 141], B6D2 hybrid strain that displays even less anxiety-like behaviors than congenic B6 mice [109], *Fmr1/RGS4* double KO mice that rescue abnormal sociability and other phenotypes [118], and conditional KO technology [142] could produce significant advances in this important area.

Genetic and Molecular Bases of ASD in FXS: Initial Insights into Common Pathways

There have been major advances in the understanding of the genetic and molecular bases of ASD in the general population. However, progress in understanding the molecular basis of ASD in FXS (i.e., differentiation between FXS with and without ASD) has been modest. This is due to major methodological obstacles. Even if analyses of tissue samples and cell lines of affected patients lead to straightforward results, the links between in vitro measures in peripheral cells and ASD neurobiology are tenuous. Integration of peripheral sample and postmortem brain tissue data would be informative; however, brain samples of individuals with FXS and ASD are largely unavailable and, if existing, they could reflect cumulative processes (i.e., end stage) and not the pathology of a dynamic syndrome such as ASD. Here we review the basic molecular biology of FXS and a few studies of relevance to ASD in FXS.

FXS is defined on a genetic basis: a full mutation level expansion of a CGG polymorphism within the 5′-untranslated region of *FMR1,* which leads to hypermethylation, silencing of the gene, and the resulting reduction in its product (FMRP) [143]. In contrast with consistent reports on correlations between magnitude of FMRP decrease and severity of physical and cognitive phenotype [53, 144], lymphocytic FMRP levels do not seem to predict behavioral abnormalities in FXS [145]. Initial studies demonstrated a modest relationship between FMRP deficit and severity of autistic behavior [13, 146]; however, subsequent studies showed that after controlling for IQ the relationship vanishes [41, 147]. Negative findings are not surprising considering that FMRP is an RNA-binding protein that regulates the synthesis, particularly at synaptic sites, of a relatively large number of proteins (5–8% total mRNA) [143, 148, 149]. Consequently, specific neurobehavioral features of FXS are more likely to depend on a relatively greater involvement of certain FMRP targets and neuronal circuits that are not reflected in general measures of FMRP in the periphery.

To our knowledge, only one study has compared molecular or biochemical profiles in FXS individuals with and without ASD. Ashwood et al. [150] studied plasma cytokine/chemokine profiles, comparing individuals with FXS with and without ASD and normal controls. They found that levels of IL-6, eotaxin, and MCP-1 were increased in individuals with FXS and ASD when compared with their nonautistic counterparts. These differences seemed specific since differences between FXS and normal controls involved a different set of cytokines (e.g. IL-1α).

Another study by Nishimura et al. [151] examined gene expression profiles in lymphoblasts from boys with FXS and ASD, comparing them with typically developing controls and boys with duplication of chromosome 15 and ASD (dup15q; a recognized genetic abnormality associated with ASD) [29]. Of 120 differentially expressed genes, including 15 previously identified in neuronal [91] and 'phenotypically generic' lymphoblast [90] FXS/FMRP-deficient samples, 68 were also dysregulated in the dup15q group (table 3). Among them there was G-protein-coupled receptor 155 (GPR155), a gene regulated by the cytoplasmic FMR1-interacting protein 1 (CYFIP1), an antagonist and binding partner of FMRP that is a member of the Rac GTPase system involved in neurite development [94, 153]. Since CYFIP1 and another one of its targets [the janus kinase and microtubule-interacting protein 1 (JAKMIP1 or MARLIN-1)] were also dysregulated in patients with dup15q, Jakmip1 was reduced in brains of *Fmr1* KO mice, and JAKMIP1 and GPR155 were differentially expressed in male sibling pairs discordant for idiopathic ASD; it can be concluded that the CYFP1 signaling pathway is implicated in FXS and one other major genetic form of ASD. In the Prader-Willi (PW) phenotype of FXS, a subgroup associated with obesity and hyperphagia similar to PW syndrome but without cytogenetic or methylation abnormalities at 15q11–13, Nowicki et al. [154] have demonstrated CYFIP levels two- to fourfold lower than in individuals with FXS without the PW phenotype. It is interesting that the PW-like subgroup displays even higher rates of ASD (10/13, 77%) than individuals with FXS and ASD without the PW phenotype. Although the study by Nishimura et al. [151] did not formally compare subjects with FXS with

Table 3. Genes dysregulated in lymphoblasts from patients with FXS and ASD

Gene name	Gene abbreviation	Levels
Hairy and enhancer of split 1	HES1[a, b]	upregulated
Iduronate 2-sulfatase	IDS[a]	upregulated
Immunoglobulin superfamily member 3	IGSF3[a]	upregulated
Nuclear receptor subfamily 3 group C member	NR3C1[c]	upregulated
CDK2-associated protein 2	CDK2AP2[a]	downregulated
CD44 antigen	CD44[a]	downregulated
C-terminal binding protein 1	CTBP1[a]	downregulated
F-box protein 6	FBXO6[a]	downregulated
G protein-coupled receptor 155	GPR155[d]	downregulated
MAX-like protein X	MLX[a]	downregulated
Mitogen-activated protein kinase kinase	MAP3K11[a]	downregulated
Ribosomal protein S5	RPS5[a]	downregulated
Sorting nexin 15	SNX15[a]	downregulated
Spleen tyrosine kinase	SYK[a]	downregulated
Ubiquitin-specific peptidase 8	USP8[a]	downregulated
Vimentin	VIM[c]	downregulated

From Kaufmann et al. [57, table 4.8, p. 98]. Reprinted and slightly modified with kind permission of Springer Science + Business Media.

[a] Reported by Brown et al. [90].

[b] Associated with attention deficit hyperactivity disorder, described by Brookes et al. [152].

[c] Reported by Miyashiro et al. [91].

[d] Also found in patients with chromosome 15 duplication and ASD, described by Nishimura et al. [151].

and without ASD, the comprehensive and comparative nature of the assays suggests that the study of peripheral cells from individuals with FXS and ASD may lead to the identification of biomarkers and could be highly informative for understanding mechanisms underlying ASD in general.

Another intriguing signaling pathway abnormality linking FXS with other genetic disorders associated with ASD is the recent demonstration of elevated mammalian target of rapamycin (mTOR) pathway phosphorylation and activity in the hippocampus of *Fmr1* KO mice [155]. The mTOR signaling cascade controls initiation of cap-dependent translation under control of mGluRs and, consequently, of FMRP. Furthermore, activity-dependent phosphorylation of FMRP in hippocampal neurons by the S6 kinase (S6K1) requires signaling inputs from different pathways including mTOR [156]. Two upstream components of the mTOR pathway, PTEN and Akt, are implicated in other genetic disorders associated with ASD. Mutations in PTEN, an inhibitor of mTOR signaling, have been described in individuals with ASD who are frequently macrocephalic [6]. Akt is a kinase that phosphorylates tuberin, the product of *TSC2*. A large proportion of individuals with loss-of-function mutation of *TSC1* and *TSC2* (i.e., tuberous sclerosis) meet DSM-IV criteria for ASD, constituting the second most common genetic etiology of ASD [6]. Thus, mTOR abnormalities seem to link PTEN, tuberous sclerosis, and FXS. Nevertheless, some publications have shown unaltered basal mTOR signaling in the *Fmr1* KO mouse [157, 158]. Although these discrepancies could be attributed to factors such as differences in mouse background strains, the fact that mTOR signaling abnormalities have only been shown in animal models and have not been directly linked to ASD-like behaviors (PTEN, tuberous sclerosis, and FXS also share intellectual disability) suggest caution about these exciting findings.

In support of secondary genes modulation of the neurobehavioral phenotype of FXS, including ASD, Hessl et al. [159] reported that polymorphisms of the serotonin transporter gene, but not of the monoamine oxidase A gene, influenced aberrant behavior in males with FXS. Individuals who were homozygous for the high-transcribing long genotype exhibited a more aggressive and destructive behavior and higher levels of stereotypic behavior [159].

In conclusion, very limited data support a molecular differentiation between FXS individuals with and without ASD. Some studies support common molecular abnormalities to FXS and other genetic disorders associated with ASD (i.e., CYFIP1, mTOR). However, data are still fragmentary and, for the most part, not directly linked to subjects with FXS and ASD. On the other hand, approaches like those adopted by Nishimura et al. [151] combining lymphoid samples from affected patients with brains of mouse models and, possibly other sources, are quite promising. Continuous survey of molecular and neurobiological similarities between FXS and other ASD-associated disorders seem to also be critical.

FMR1 Premutation and ASD: Another Form of ASD?

The relationship between *FMR1* premutation (i.e., intermediate level expansion of the CGG polymorphism), usually a carrier status that is not associated with atypical methylation or gene silencing, and clinical manifes-

tations has been one of the most controversial ones in the FXS literature. At present, it is well accepted that two disorders, fragile X-associated tremor/ataxia syndrome (FXTAS) and primary ovarian insufficiency, are linked to *FMR1* premutation [4]. However, there is less clarity about the diverse neurobehavioral manifestations, with most publications arising from small series of cases and with few structured phenotypical evaluations. The recent National Parent Survey confirmed the presence of neurobehavioral abnormalities in a significant fraction of individuals with premutation older than 6; however, the proportion of affected subjects seems lower than that reported for individuals with FXS [9]. The most frequent diagnoses in individuals with premutation are intellectual disability, attention deficit hyperactivity disorder, and ASD [160–162]. Although most of these publications report on boys, girls with premutation can also present with cognitive impairment or ASD (e.g. autism in 19% of males and 1% of females) [9]. Of relevance to ASD in FXS is the fact that individuals with premutation also have a notably high lifetime risk for mood and anxiety disorders [163, 164]. While the risk is particularly high in premutation carriers with FXTAS, individuals without FXTAS are also significantly affected (i.e., increased lifetime rates of social phobia) [163]. In line with previous concerns that affective and anxiety disorders are secondary in premutation, Roberts et al. [164] found in females with premutation an association between diagnosis of anxiety and the number of children with FXS and problematic behaviors these women have. In contrast to FXS, the relationship between ASD and anxiety and other behavioral abnormalities has not been systematically examined in *FMR1* premutation.

Emerging data on the neurobiology of the premutation might have general implications for future studies of ASD in this *FMR1* abnormality. Studies of CGG knock-in mice indicate that cognitive deficits are CGG repeat length-dependent [165] and that, despite marked decreases in anxiety-like behaviors, they show subtle deficits in social interaction [166]. Furthermore, murine neuronal cultures with increased CGG repeats display abnormal development and decreased viability [167]. Interestingly, findings like the latter link the type of developmental abnormalities seen in FXS with degenerative processes found in FXTAS. Whether these are common substrates of ASD in FXS and *FMR1* premutation is unknown.

Overall, at this point, it is premature to determine the significance of the reports on ASD in premutation. Is the involvement of molecular mechanisms related to the *FMR1* premutation the cause of ASD and other neurobehavioral disorders or a secondary ('risk') contributing factor? If *FMR1* premutation is involved in ASD pathogenesis, a different set of mechanisms, most likely *FMR1* mRNA accumulation-toxicity and intracellular inclusions that have been implicated in FXTAS would play a role [168].

Concluding Remarks

We have presented published and preliminary data, as well as some hypothetical models, supporting the notion that ASD in FXS is a well-delineated syndrome with distinctive (a) behavioral, (b) neuroimaging, and perhaps also (c) molecular profiles. We have also postulated that the study of ASD in FXS has important implications not only for affected individuals, but also for ASD in the general population. (a) The behavioral features of ASD in FXS are informative of the core social interaction impairment in idiopathic ASD, the relationship between ASD and social anxiety, and contribute to a better understanding of the relationship between cognitive impairment and autistic features. (b) The emerging knowledge on neuroimaging of ASD in FXS emphasizes the involvement of brain areas already implicated in idiopathic ASD, in particular the cerebellum. These MRI morphometric approaches may eventually identify additional neural circuits involved in ASD of multiple etiologies. (c) While virtually no data are available on molecular distinctions between FXS individuals with and without ASD, recent studies are beginning to reveal signaling pathways common to FXS and other ASD-associated disorders. Because of the limited and inconsistent data on abnormal social interaction, it is not yet clear to what extent animal models of FXS and other genetic disorders will provide valuable data for idiopathic ASD. Methodological issues affecting this area include lack of optimal social interaction behavioral paradigms, difficulties distinguishing ASD from anxiety-like behaviors, and selection of adequate background strains.

Integration of all these heterogeneous pieces of data is a major challenge. Although the priority in the field is to acquire more data on ASD in FXS, it is also necessary to introduce new approaches. In terms of (a) behavioral studies, experimental paradigms in patients and mouse models should complement findings derived from clinical measures. Our recent work on identifying dynamic behavioral features of ASD in FXS with the Social Approach Scale [34, 46] is a good example of such a strategy. Naturalistic observations may also be informative, as re-

vealed by our studies of the neurobehavioral phenotype of Rett syndrome through video recordings by parents [169]. Novel mouse background strains that will allow a distinction between ASD- and anxiety-like behaviors are also promising [108, 109, 117]. In terms of (b) neuroimaging, there is the need for applying the entire spectrum of MRI techniques. Given the close association of severe cognitive impairment and ASD in FXS, paradigm-related functional MRI will probably remain an elusive approach; however, resting state functional MRI [170] and MR spectroscopy [171] are distinctive possibilities. Most likely, the study of (c) gene expression profiles in lymphoid and postmortem samples from affected individuals will continue to provide promising leads. The challenge here is the integration of molecular and neurobiological data; the comprehensive evaluation of CYFIP1 and its targets in ASD associated with FXS and chromosome 15 duplication by Nishimura et al. [151] illustrates that such work is feasible.

The present review, though intended to cover most of the pertinent literature, was centered on our work and on discussing major issues affecting ASD in FXS and its neurobiology. It is our opinion that despite our limited knowledge on the molecular, neurobiological, and behavioral correlates of ASD in FXS and other genetic conditions, these disorders are valuable models for ASD research.

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