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Cognitive dysfunction and prefrontal synaptic abnormalities in a mouse model of fragile X syndrome

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Among the hallmark phenotypes reported in individuals with fragile X syndrome (FXS) are deficits in attentional function, inhibitory control, and cognitive flexibility, a set of cognitive skills thought to be associated with the prefrontal cortex (PFC). However, despite substantial clinical research into these core deficits, the PFC has received surprisingly little attention in preclinical research, particularly in animal models of FXS. In this study, we sought to investigate the molecular, cellular, and behavioral consequences of the loss of the fragile X mental retardation protein in the PFC of Fmr1 KO mice, a mouse model of FXS. We identify a robust cognitive impairment in these mice that may be related to the deficits in cognitive flexibility observed in individuals with FXS. In addition, we report that levels of proteins involved in synaptic function, including the NMDA receptor subunits NR1, NR2A, and NR2B; the scaffolding proteins PSD-95 and SAPAP3; and the plasticity-related gene Arc, are decreased in the prefrontal cortex of Fmr1 KO mice and are partly correlated with behavioral performance. Finally, we report that expression of c-Fos, a marker of neuronal activity, is decreased in the PFC of Fmr1 KO mice. Together, these data suggest that Fmr1 KO mice may represent a valuable animal model for the PFC-associated molecular, cellular, and behavioral abnormalities in FXS and that this model may be useful for testing the efficacy of therapeutic strategies aimed at treating the cognitive impairments in FXS.

Fragile X syndrome (FXS) is the most common form of inherited mental retardation and a leading known cause of autism (1). It is caused by loss of the Fmr1 gene product fragile X mental retardation protein (FMRP), an mRNA-binding protein involved in translational regulation (2, 3). FMRP is thought to repress the synthesis of proteins required for protein synthesis-dependent synaptic plasticity (4, 5). In FXS, the absence of FMRP is hypothesized to result in unrestricted synthesis of plasticity-related proteins (6, 7), impairing the ability of synapses to appropriately undergo plasticity in an activity-dependent and stimulus-specific manner. In support of this hypothesis, mice with a deletion in the Fmr1 gene (Fmr1 KO mice) display aberrant forms of plasticity (4) and an increase in immature dendritic spines that presumably reflects an abnormal synaptic connectivity (8). Together, these synaptic alterations are thought to underlie the cognitive and behavioral phenotypes that are the hallmark features of FXS.

Among the most common symptoms reported in FXS are deficits in attentional function, inhibitory control, and cognitive flexibility (9), cognitive skills that have all been linked to the prefrontal cortex (PFC) and associated fronto-striatal networks (10, 11). Anatomical and imaging studies of individuals with FXS have identified structural alterations in PFC, and numerous fMRI studies have shown aberrant patterns of neural activity in fronto-striatal pathways during cognitive tasks (12). Together, all of these results suggest that the fronto-striatal network is one of the key brain systems impaired in FXS (9).

Results

Fmr1 KO Mice Are Impaired in the Acquisition of a Visuospatial Discrimination Task. A major aim of our study was to identify behavioral deficits in Fmr1 KO mice that represent a model for the core cognitive phenotypes observed in FXS. To this end, we first tested mice in a behavioral task used to assess sustained attention and inhibitory control in rodents, the five-choice serial reaction time task (5CSRTT) (21). This paradigm uses an operant test chamber equipped with five nose-poke holes, i.e., apertures that can detect a nose-poke response by the mouse, and a food magazine into which a food reward can be delivered. In our experiment, Fmr1 KO mice and wild-type (WT) littersmates were first trained to perform a nose-poke response in an illuminated aperture to obtain a food response (see SI Materials and Methods for details of the training phases), and they were then subjected to two final tests, one to measure sustained attention and one to measure inhibitory control (SI Materials and Methods). We found that Fmr1 KO mice did not differ from their WT littersmates in either of the two final tests, suggesting that, under our conditions, these mice do not display deficits in sustained attention or inhibitory control (Fig. S1 A and B). Interestingly, however, we observed a significant and very selective deficit in one of the pretest training phases (Fig. S1C; SI Materials and Methods), and we therefore decided to further pursue this impairment.

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To this end, we trained mice on a shortened paradigm including only the first two training phases of the original 5CSRTT (Fig. 1A). In the first phase, all five apertures were illuminated and active, and a nose-poke response in any of the apertures resulted in the delivery of a food reward. Fmr1 KO mice showed no significant difference in the number of days taken to acquire this task (Fig. 1B; one-way ANOVA for genotype, $F_{1,24} < 1$), suggesting that they are not impaired in the acquisition of an appetitive instrumental response under the current experimental conditions. In the second phase, only one of the five apertures was illuminated and active, and only a response in this aperture was rewarded. Fmr1 KO mice took significantly longer to acquire this phase of the task (Fig. 1B; one-way ANOVA for genotype, $F_{1,22} = 7.11, P < 0.05$), and they made significantly more errors than WT mice (Fig. 1C; one-way ANOVA for genotype, $F_{1,22} = 12.52, P < 0.01$). Further analysis of this phase revealed that Fmr1 KO mice did not differ significantly from WT littermates in the number of trials completed per session, although there was a trend toward a genotype x training day interaction (Fig. 1D; repeated measures ANOVA for genotype, $F_{1,14} = 14.3, P < 0.001$; genotype x day interaction, $F_{3,21} = 8.76, P < 0.001$). These data suggest that Fmr1 KO mice are impaired in the acquisition of a visuospatial discrimination task, but not in the performance of the visuospatial discrimination itself, as indicated by the fact that both groups reach equal levels of performance by the end of a 10-d training period (Fig. 1E; post hoc analysis for day 10, $P = 0.824$).

Fmr1 KO Mice Display a Stronger Spatial Preference in Responding.

To further explore the cause of this impairment, we investigated the pattern of nose-poke responses by spatial location, analyzing each of the five apertures individually. On the basis of the total number of responses made in the last 2 d of phase 1 (i.e., the days in which the mouse was considered to have acquired the task), the apertures were ranked by preference for each individual mouse. Both genotypes showed a spatial preference for certain apertures over others in phase 1 (Fig. 1F; main effect of aperture, $F_{3,72} = 58.82, P < 0.001$). However, Fmr1 KO mice showed a stronger spatial preference; i.e., they made significantly more responses in the most preferred aperture than did the WT mice (Fig. 1F; main effect of genotype, $F_{1,18} = 10.7, P < 0.005$; post hoc analysis for the most preferred aperture, $P < 0.05$). Using the same preference ranking, the number of errors to criterion in phase 2 was assessed for each nose-poke aperture (Fig. 1G; main effect of genotype, $F_{1,18} = 10.7, P < 0.005$; genotype x aperture interaction, $F_{3,72} = 4.06, P < 0.01$). WT mice made similar numbers of errors to criterion in each aperture, suggesting that the slight spatial preference displayed in phase 1 did not influence performance in phase 2. Fmr1 KO mice, on the other hand, made significantly more errors in the previously preferred apertures. These data suggest that a lack of cognitive flexibility in the Fmr1 KO mice may contribute to the delay in acquisition of the visuospatial discrimination task described here.

Behavioral Impairments Are Accompanied by Decreases in Synaptic Markers in Orbitofrontal Cortex and Medial Prefrontal Cortex of Fmr1 KO Mice.

A central question in FXS is how loss of translational regulation by FMRP affects the molecular composition of the synapse, and how this in turn results in the cognitive impairments seen in FXS. To begin to address this issue, we investigated whether expression of synaptic proteins was altered in the PFC of the same Fmr1 KO mice that had undergone behavioral training. We isolated two subregions of PFC (Fig. 2A)—the orbitofrontal cortex (OFC) and the medial prefrontal cortex (mPFC)—both of which are required for various aspects of behavioral flexibility in rodents (22-24). The global profile of protein expression in Fmr1 KO mice was similar to that of WT mice, as assessed by a total protein stain (Fig. 2B). Interestingly,
however, a number of proteins associated with postsynaptic function were significantly decreased in both OFC (Fig. 2 C and D; Table 1) and mPFC (Fig. 2E; Table 1) of Fmr1 KO mice, whereas none of the proteins examined here were significantly increased in the absence of FMRP. The most robust and consistent decreases were those in the NMDA receptor subunits NR1, NR2A, and NR2B, the postsynaptic scaffolding proteins SAPAP3 and PSD-95, and the plasticity-related molecule Arc (Table 1). In contrast, no significant changes were observed in another synaptic marker, SNAP25, or in the cytoplasmic protein actin, suggesting that the decreases may be specific for a subset of components of the postsynaptic apparatus. Together, these data suggest that there is a decrease in either the number or the complexity of postsynaptic terminals in the OFC and mPFC of Fmr1 KO mice, which in turn may be relevant for the cognitive impairment described above.

Behavioral Performance Correlates with Levels of NR2A and NR2B in mPFC. To further investigate which of the above molecular alterations may be most relevant for the cognitive impairment in the Fmr1 KO mice, we analyzed whether expression levels of any of the postsynaptic proteins were directly correlated with behavioral performance (Fig. 3). This analysis revealed that levels of NR2A in mPFC, but not in OFC, were negatively correlated with the number of days taken to reach criterion (Fig. 3A; \( r = -0.59, P < 0.001 \)), indicating that the mice with the lowest levels of these subunits also took the longest to acquire the visuospatial discrimination task. A similar, albeit substantially weaker, correlation was observed for NR2B in mPFC (Fig. 3B; \( r = -0.32, P < 0.05 \)). Interestingly, levels of NR1 were not correlated with behavioral performance in either OFC or mPFC (Fig. 3C; \( r = -0.1, P = 0.5 \)). It therefore appears that the decreases in NR2A and NR2B levels in the mPFC may be particularly important for impairment in the acquisition of the current visuospatial discrimination task.

### Table 1. Expression of proteins associated with postsynaptic function is reduced in both homogenate and synaptoneurosomes of Fmr1 KO mice

<table>
<thead>
<tr>
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<th>Trained (homogenate)</th>
<th>Naive (homogenate)</th>
<th>Naive (SNS)</th>
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<tr>
<td>% WT</td>
<td>n</td>
<td>P value</td>
<td>% WT</td>
</tr>
<tr>
<td>OFC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR1</td>
<td>85.5</td>
<td>22</td>
<td>0.003</td>
</tr>
<tr>
<td>NR2A</td>
<td>82.1</td>
<td>20</td>
<td>&lt;0.001</td>
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<tr>
<td>NR2B</td>
<td>97.0</td>
<td>21</td>
<td>0.647</td>
</tr>
<tr>
<td>SAPAP3</td>
<td>84.5</td>
<td>22</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PSD95</td>
<td>86.2</td>
<td>22</td>
<td>0.017</td>
</tr>
<tr>
<td>Arc</td>
<td>89.2</td>
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<td>SNAP25</td>
<td>97.9</td>
<td>22</td>
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<tr>
<td>Actin</td>
<td>103.1</td>
<td>23</td>
<td>0.494</td>
</tr>
<tr>
<td>mPFC</td>
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<tr>
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For each protein, data are expressed as percentage of WT levels in Fmr1 KO. n represents the number of animals per group. Significance was determined by paired Student’s t test.
receptor composition at individual synapses, we repeated our molecular analysis using a synaptoneurosome (SNS) preparation that is enriched in pre- and postsynaptic structures (25). SNS were prepared from the same set of behaviorally naive WT and Fmr1 KO mice used for the whole-homogenate preparation. We found that the expression pattern of NR1, NR2A, NR2B, PSD-95, SAPAP3, and Arc was virtually the same in SNS and in whole-homogenate samples (Table 1), implying that these changes reflect alterations in the complexity of individual synapses.

**Levels of c-Fos Expression Are Decreased Basally in OFC and mPFC of Fmr1 KO Mice.** In light of our findings that synaptic protein composition is altered basally in OFC and mPFC of Fmr1 KO mice, we investigated whether these changes are accompanied by differences in the basal expression of c-Fos, a marker used to assess neuronal activity (26). Sections were prepared from OFC and mPFC of WT and Fmr1 KO mice, and they were then analyzed for expression of c-Fos and the neuronal marker NeuN by immunohistochemistry (Fig. 4A). Our data revealed that the percentage of c-Fos–expressing neurons was significantly lower in Fmr1 KO mice than in WT mice both in OFC (Fig. 4B; P < 0.05) and in mPFC (Fig. 4C; P < 0.05). These data suggest that neuronal activity is decreased basally in the PFC of Fmr1 KO mice, which is likely to contribute to the other phenotypes described in this study.

**Discussion**

In the present study, we report the identification of a robust cognitive deficit in the Fmr1 KO mouse model of FXS, as well as accompanying changes in synaptic composition and neuronal activity in the PFC of Fmr1 KO mice. Our findings have two important implications. First, the behavioral task developed here provides a tool to screen drug candidates for efficacy in treating the impairments in higher cognitive function that are central to FXS. Second, the observation that postsynaptic proteins, and particularly NMDA receptor subunits, are decreased in the PFC of adult Fmr1 KO mice contributes significantly to our understanding of the molecular mechanisms by which loss of FMRP expression may cause cognitive dysfunction.

The development of reliable behavioral paradigms that can be used to model the symptoms of neurodevelopmental disorders such as FXS and autism in mouse models is important for the validation of new therapeutics (27). A number of behavioral assays have been used to study the efficacy of putative treatment strategies in Fmr1 KO mice, including tests of anxiety, susceptibility to seizures, sensorimotor gating, sociability, and fear memory (7, 13, 28, 29). On the basis of these assays, exciting insights have been gained regarding the potential for developing small molecule therapeutics for FXS. However, none of these studies to date have directly addressed the higher cognitive phenotypes that are the hallmark features of FXS, partly due to a paucity of suitable animal models. Given that these phenotypes are likely to have molecular substrates and developmental trajectories that are very different from those for behavioral traits such as anxiety and sociability, it will be essential to establish whether results obtained from tests of the latter can be generalized. Our study aimed to provide a measure of cognitive dysfunction in Fmr1 KO mice for this purpose. We found that Fmr1 KO mice are not impaired in the acquisition of an appetitive instrumental response, consistent with a previous report (30). In contrast, Fmr1 KO mice were significantly impaired in the subsequent acquisition of a visuospatial discrimination in a manner that is consistent with an inability either to flexibly respond to changing reward contingencies or to shift attention from one perceptual dimension (spatial location) to another (visual cue).

Both of these cognitive skills are impaired in individuals with FXS (31, 32), and both have been linked to the PFC (22, 23). It is therefore likely that the behavioral phenotype identified here reflects a deficit in PFC function in the Fmr1 KO mice, providing a useful paradigm for further research in this area.

In accordance with this assumption, significant decreases were observed in a number of synapse-associated proteins in the OFC and mPFC of Fmr1 KO mice. This finding was initially surprising.
because FMRP is thought to be a translational repressor, the absence of which should cause levels of target proteins to increase rather than decrease (6, 7). Indeed, several of the proteins identified here are known to be directly or indirectly regulated by FMRP, including NR2A (33), SAPAP3 (15, 34), PSD-95 (35), and Arc (36), and previous studies have reported that, under different conditions, these proteins are either increased or unaltered (15, 34, 37, 38). It is likely that the decreases observed in our study reflect an impoverished postsynaptic architecture due to long-term developmental alterations at synapses in the prefrontal cortex, rather than an acute effect of the loss of FMRP-mediated translational regulation. This theory is consistent with reports of an increased density of long filopodial-like spines in the PFC of Fmr1 KO mice (13, 14), which are likely to contain a more immature, and hence less complex, postsynaptic apparatus. Interestingly, the decrease in levels of the NMDA receptor subunits NR2A and, to a lesser extent, NR2B in the mPFC appears to be especially relevant for the cognitive impairments observed here. Although the details of the relationship between NR2A/B levels and behavioral performance have yet to be determined, it is interesting to note that both cognitive flexibility and attentional shifting are dependent on NMDA receptor function and can be impaired by pharmacological antagonism of NMDA receptors (39, 40). Similarly, levels of SAPAP3 were consistently decreased in both regions of PFC. Given that loss of SAPAP3 in mice has previously been linked to aberrations at corticostriatal synapses and repetitive behavioral phenotypes reminiscent of obsessive-compulsive disorder (41), it is possible that the changes in SAPAP3 levels observed here may also partly contribute to the cognitive phenotype in the Fmr1 KO mice.

Parallel to the alterations in synaptic composition, we also observed a decrease in the percentage of c-Fos–positive neurons in both OFC and mPFC of Fmr1 KO mice, indicating a reduction in basal neuronal activity. Interestingly, a recent study showed that spike-timing–dependent plasticity is altered in mPFC of Fmr1 KO mice due to a reduction in L-type calcium channel function and decreased reliability of postsynaptic calcium signaling (14). It is conceivable that the same developmental processes that result in the impoverished synaptic architecture identified above may also affect expression of L-type calcium channels, which in turn may contribute to the decreased c-Fos expression and neuronal activation observed in our study. Overall, it thus appears that loss of FMRP function leads to reduction of synaptic and neuronal function at multiple levels, and that in combination, these alterations may result in substantial cognitive impairments such as the one identified in our study. By inference, similar molecular and cellular abnormalities could potentially underlie human phenotypes believed to be associated with PFC dysfunction—most notably the perseverative behaviors and restricted interests that are a hallmark of FXS and autism.

Materials and Methods

Subjects. Male Fmr1 KO mice (The Jackson Laboratory) and WT littermates were bred on a C57BL/6 background and used for experiments at 2–4 mo (see SI Materials and Methods for details). In all experiments, yoked WT-KO pairs that were processed simultaneously and under identical conditions were used. Experimenters were blind to genotype at all times throughout the experiment. All experiments were approved by the Massachusetts Institute of Technology Committee on Animal Care and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Behavioral Training. Behavioral experiments were conducted in operant conditioning chambers (Med Associates) equipped with five nose-poke apertures and a pellet dispenser and food magazine. Before each experiment, mice were food-restricted to 85% free-feeding body weight and given two daily magazine training sessions, each lasting 10 min, in which each entry into the food magazine was reinforced by one food pellet. Subsequently, mice were trained on one of two behavioral experiments (1): a SCSRTT (see SI Materials and Methods for details) or (2) a visuospatial discrimination task derived from the training phase of the SCSRTT. For the visuospatial discrimination task, mice (n = 14 pairs) were trained in two phases to perform a nose-poke response to a visual cue.

Phase 1. In this phase, all five nose-poke apertures were illuminated and active, and mice were trained on an instrumental response in daily 15-min sessions. Each trial began with the illumination of the apertures, and a response in any aperture resulted in delivery of a food reward (“correct response”). At this time, the lights in the apertures were turned off and the magazine light was turned on. A trial was considered completed when the food pellet was retrieved, at which time the magazine light was turned off and the aperture lights were turned back on. Mice were required to complete at least 15 trials per session for two consecutive days (“criterion”) to advance to the next training phase.

Phase 2. This phase was identical to the previous phase, except that only one nose-poke aperture was illuminated and active at any given time. A response in this aperture resulted in delivery of a food reward (“correct response”), whereas a response in any other aperture had no programmed consequence (“error”). For each trial, a different nose-poke aperture was chosen to be illuminated and active in a pseudorandomized manner. Mice reached criterion for this phase when they completed >15 trials per session and made more correct responses than errors (i.e., >50% accuracy rate) for two consecutive days. Data were recorded as the number of days to criterion, the cumulative number of errors to criterion, the number of trials per day, and the number of errors per trials performed across days of training.

Tissue Preparation for Biochemical Analysis. Behaviorally trained mice (n = 26 pairs) were killed 24 h after the last behavioral training session, and whole-homogenate samples from OFC and mPFC were isolated. Behaviorally naive mice (n = 25–30 pairs) were killed without any prior handling, and tissue homogenates and synaptoneurosomes were prepared using a protocol modified from Hollingsworth et al. (25). See SI Materials and Methods for details.

Immunoblotting. Protein levels in whole homogenate and SNS were determined using standard immunoblotting procedures. Each lane contained 5–10 μg total protein because this amount of total protein was found to yield signals in the linear range for our target proteins (Fig. S2). See SI Materials and Methods for further details.

Immunohistochemistry. Mice (n = 8 pairs) were transcardially perfused with 4% paraformaldehyde, and 50-μm brain sections were stained for c-Fos and NeuN by using standard immunohistochemistry procedures. Images were acquired with an Olympus laser-scanning confocal microscope, and image analysis was conducted on raw images using ImageJ software. See SI Materials and Methods for further details.

Statistical Analysis. Statistical analysis for behavioral experiments was conducted using one-way or two-way repeated measures ANOVA, and post hoc analysis was performed using Scheffé’s test. Group comparisons for the biochemical analysis were conducted using two-tailed paired Student’s t tests. Relationships between behavioral data and protein levels were analyzed using Pearson’s correlation coefficient. See SI Materials and Methods for further details.

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