Enriched environment promotes behavioral and morphological recovery in a mouse model for the fragile X syndrome

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Fragile X syndrome, the most frequent form of hereditary mental retardation, is due to a mutation of the fragile X mental retardation 1 (FMR1) gene on the X chromosome. Like fragile X patients, FMR1-knockout (FMR1-KO) mice lack the normal fragile X mental retardation protein (FMRP) and show both cognitive alterations and an immature neuronal morphology. We reared FMR1-KO mice in a C57BL/6 background in enriched environmental conditions to examine the possibility that experience-dependent stimulation alleviates their behavioral and neuronal abnormalities. FMR1-KO mice kept in standard cages were hyperactive, displayed an altered pattern of open field exploration, and did not show habituation. Quantitative morphological analyses revealed a reduction in basal dendrite length and branching together with more immature-appearing spines along apical dendrites of layer five pyramidal neurons in the visual cortex. Enrichment largely rescued these behavioral and neuronal abnormalities while increasing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor subunit 1 (GluR1) levels in both genotypes. Enrichment did not, however, affect FMRP levels in the WT mice. These data suggest that FMRP-independent pathways activating glutamatergic signaling are preserved in FMR1-KO mice and that they can be elicited by environmental stimulation.

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Several genes associated with mental retardation have been mapped on the X chromosome and, among them is the fragile X mental retardation 1 (FMR1) gene. The fragile X mental retardation protein (FMRP) absence or mutation is responsible for the fragile X syndrome (FXS), which is the most common form of inherited mental retardation. Most of the individuals affected carry a trinucleotide repeat that, after methylation, leads to transcriptional silencing of the FMR1 gene (1). Patients with the FXS do not express FMRP and exhibit phenotypic traits ranging from severe (IQ 20) to moderate (IQ 60) mental retardation. FMR1 is highly conserved between human and mouse, with a nucleotide and amino acid identity of 95% and 97%, respectively (2). The expression pattern of mouse FMR1 is similar to its human counterpart in both tissue specificity and timing (3, 4). Interestingly, FMR1-knockout (FMR1-KO) mice, the mouse model for the FXS, lack the normal FMRP and show macroorchidism, hyperactivity, and mild learning deficits (8, 9) reminiscent of the human syndrome.

One common brain feature of fragile X patients and of the mouse model for the syndrome is the presence of long and thin immature dendritic spines indicative of defective pruning during development (10–14). At the molecular level, it has been shown that protein synthesis triggered by the type I metabotropic glutamate receptor (mGluR1) agonist dicyclohexylphosphorylglycine is dramatically reduced in synaptoneurosomes of FMR1-KO mice (15). There is also evidence that FMRP is required for mGluR1-dependent translation of the postsynaptic density protein 95 (16), a scaffolding protein specifically involved in synaptic development and plasticity (17, 18). In addition to this observation, a reduction of cortical α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor subunit 1 (GluR1) expression and long-term potentiation (19) together with an enhancement of hippocampal long-term depression (20) have been reported in this genotype. Altogether, these findings support the view that the neuronal alterations associated with the FXS can be ascribed to a substantial impairment of mechanisms involved in neural plasticity.

Since the early observations of Hebb (21) and Krech and coworkers (22), environmental enrichment has been extensively used to demonstrate behavioral and brain plasticity in response to experience. Rearing animals in a complex environment reduces anxiety (23), accelerates habituation (24), enhances learning (24–26), and deeply affects brain morphology. In particular, an increase in dendrite length and branching (27), spine density and number of mature spines (29, 30, **), synaptogenesis (31), and neurogenesis (32) has been repeatedly found in cortical and subcortical areas of rodents experiencing enriched conditions during development. More recent evidence also indicates that environmental enrichment enhances the brain levels of several synaptic and structural proteins such as neurotrophins (33), CAMP response element-binding protein (34), synaptophysin (35, 36), and postsynaptic density protein 95 (36), thus playing a role in synapse formation and plasticity.

Here we examine the possibility of alleviating the behavioral and neuronal abnormalities of FMR1-KO mice by environmental stimulation. KO and their WT littermates reared in standard or enriched cages were first tested for motor activity then for their propensity to interact with external stimuli, i.e., two behavioral measures of mechanisms involved in neural plasticity. Enrichment also increased the level of expression of the AMPA receptor subunit GluR1 in visual cortex of FMR1-KO mice but it did not affect expression of FMRP in the WT mice. These data suggest that...
neural plasticity mechanisms other than those modulated by the FMR1 gene are preserved in FMR1-KO mice and that they can be elicited by environmental stimulation.

Materials and Methods

Animals. Animal care was conducted according to the institutional guidelines that are in compliance with national and international laws and policies.‡‡Mice used in this study were male C57BL/6 WT (n = 18) and C57BL/6 FMR1-KO (n = 18). The KO mice were backcrossed at least eight times, and their genotype was determined according to Bakker et al. (8). Mice were 21 days old at the beginning of the experiment, and their weights ranged from 12 to 14 g.

Rearing Conditions. Mice were randomly assigned to the enriched or the standard environmental condition. Enriched environment cages consisted of clear Plexiglas cages (35 × 20 × 25 cm) with a horizontal platform (20 × 15 cm) dividing the cage into two floors. On the ground floor, there was a plastic running wheel, nesting material, and an assortment of differently colored and textured plastic toys (balls, tubes, boxes, and bells) that were changed every 3 days. A steel ladder allowed mice to reach the upper floor, where they had access to food and water. Mice were exposed, 2 h/day, to an additional enriched area situated in a different room. This area consisted of a Plexiglas cage (40 × 25 × 20 cm) containing polyurethane foam, cardboard boxes, and objects made of iron. Standard cages were clear Plexiglas laboratory cages (18 × 25 × 13 cm). Mice were caged in groups of three. Enriched and standard cages were placed in a temperature-controlled room (22°C) with a light–dark 12:12 cycle (light on 0700–1900 hours). Food and water were given ad libitum. Mice were housed in each experimental condition for 60 days. The behavioral experiments started when mice were 12 weeks old.

Open-Field Exploration and Habitation to the Objects. The apparatus was a circular arena (60 cm in diameter) with a white-painted floor divided into sectors by black lines. The arena was surrounded by a 20-cm-high wall. Testing consisted of four successive 5-min sessions separated by a 3-min delay during which mice were placed in an empty cage situated on the floor of the experimental room. One exploration session was first given in the empty arena (session 1). Subsequently, five differently colored, textured, and shaped objects (all ~10 cm in height) were placed in the arena, and mice were allowed to explore freely these objects for three sessions (sessions 2–4). The number of central and peripheral sectors crossed during session 1 and the number of contacts with the five objects during sessions 2–4 were recorded. A contact was counted when the subject’s snout was touching the object. In this experiment 18 WT and 12 FMR1-KO mice were used, with half of the mice in each group reared in standard cages and the other half in enriched cages.

Golgí-Cox Impregnation of Brain Tissue. At the completion of the behavioral experiments, mice were anesthetized with chloral hydrate (400 mg/kg) and perfused intracardially with 0.9% saline. The brains were dissected and impregnated by using a standard Golgí-Cox solution (1% potassium dichromate/1% mercuric chloride/0.8% potassium chromate) according to the method described by Glaser and Van der Loos (38). The brains immersed in the Golgí-Cox solution were stored at room temperature for 14 days, immersed in a succrose solution (30%) for 5 days, and then sectioned coronally (200 μm) by using a Vibratome. Sections were mounted on gelatinized slides, stained according to the Gibb and Kolb (39) method, and covered with Permount.

Morphological Analysis. Four brains from mice reared in each experimental condition were processed for morphological analyses. Measurements were performed in fully impregnated pyramidal neurons displaying dendritic tree without obvious truncations (width of the first apical segment, 2.8–3.2 μm; length, >150 μm). Within each hemisphere, three visual cortex neurons with the soma in layer V and apical dendrites reaching layer III were selected. Because no interhemispheric difference was detected, the data were pooled so that six neurons per brain area were considered in each analysis. Measurements were carried out by using an Axioskope microscope (Zeiss) equipped with a camera (Optronics, Chelmsford, MA) and the Neurolucida system (MicroBrightField, Williston, VT). Morphological measurements were made by an experimenter blind to the experimental condition of the animal.

Dendritic Length and Branching. Dendrites from each category (apical, basal, and oblique) were traced along the entire length by means of the Neurolucida system connected to a stage controller, which allowed measurement of dendrites along the z axis. The primary branches of apical dendrites were considered as oblique dendrites. For branching analysis, basal dendrites were classified by using the centrifugal method (40): the branches arising from the soma were numbered as branch order 1 (see Fig. 2 A). Bifurcations on order 1 branches were numbered as branch order 2 (Fig. 2 A). In our Golgí–Cox-impregnated slices, the maximal branch order reached 5. The complexity of basal dendrite branching was estimated by counting the number of branches on each dendrite. Data were expressed as the mean of the number of branches (order ≥2) per dendrite.

Spine Density. Neurons were first identified under low magnification (~200/0.5 numerical aperture). Subsequently, spines were analyzed under a higher magnification (~1,000/1.25 numerical aperture). All protrusions were counted as spines if they were in direct contact with the dendritic shaft. The average spine density (number of spines per 10 μm of dendritic length) was estimated on the focal plane along the entire apical dendrite and along three basal and oblique dendrites. Because this method has proven to produce reliable results (41), no attempt was made to introduce a correction factor for hidden spines.

Spine Morphology. The morphology of up to 35 spines on apical dendrite segments starting 50 μm out from the soma was acquired under high magnification (~1,000/1.25 numerical aperture). The spines were categorized according to ref. 28, along an immature-vs. mature-appearing spine continuum (see Fig. 4 A).

Western Blotting. Five brains from mice reared in each experimental condition were processed for Western blotting. Visual cortex from standard and enriched WT and FMR1-KO mice were dissected and homogenized in lysis buffer (50 mM NaCl/50 mM Tris, pH 7.5/1% Triton X-100/10% glycerol/320 mM sucrose containing 10 μl/ml Sigma protease inhibitor). For each sample, two different amounts of protein extract were separated by SDS/PAGE, blotted, and probed with antibodies against GluR1 (Upstate Biotechnology, Lake Placid, NY), FMRP (42), and β-actin (Sigma), followed by secondary antibodies conjugated to horseradish peroxidase (Promega) and developed with the chemiluminescence reaction (ECL-plus, Amersham Pharmacia). Images were acquired by using a Storm 840 by Amersham Pharmacia, and quantification was performed by using IMAGEQUANT (version 5.0 TL v2003.02). GluR1 and FMRP levels were then normalized to β-actin levels and expressed as arbitrary units. For each animal three independent gels were loaded to reduce experimental variability.

Results

Enrichment Does Not Affect Hyperactivity of FMR1-KO Mice. Hyperactivity is the most robust and reproducible behavioral phenotypic trait of C57BL/6 FMR1-KO mice (8, 9). Therefore, we reexamined motor activity by counting the number of sectors crossed by mice of each group in an empty open field to assess the effect of enrichment on this behavioral parameter. A two-way ANOVA with genotype and rearing condition as main factors revealed only a significant effect of genotype ($F_{1,12} = 18.21; P < 0.01$), indicating higher-order branching in mice experiencing enrichment (Fig. 2B). Pair-wise comparisons, however, revealed that FMR1-KO mice reared in standard cages exhibited fewer branches than their WT counterparts (Fisher’s least significant difference [LSD], $P < 0.05$) and that the effect of enrichment on branching was significant only for the FMR1-KO mice (Fisher’s LSD, $P < 0.01$). In fact, no difference in basal dendrite branching was found between FMR1-KO mice reared in complex cages and WT mice reared in either environmental conditions ($P > 0.1$ for all pair-wise comparisons).

Enrichment Increases Basal Dendrite Length in FMR1-KO Mice. The two-way ANOVA showed a significant main effect of environment ($F_{1,12} = 30.52; P < 0.001$), indicating that, in general, mice reared in standard cages exhibited shorter basal dendrites than their counterparts reared in enriched cages. However, a significant genotype × rearing condition interaction ($F_{1,12} = 9.55; P < 0.01$) revealed that enrichment promoted basal dendrite lengthening in FMR1-KO mice (enriched vs. standard FMR1-KO mice, LSD test, $P < 0.05$) without affecting significantly the length of dendrites in WT mice (Fig. 2C). For the oblique and the apical dendrites, no statistically significant variation in length was detected among the four groups (Fig. 2D and E).

Enrichment Increases Spine Density Along Apical Dendrites in Both Genotypes. The two-way ANOVA revealed a significant main effect of enrichment ($F_{1,12} = 31.41; P < 0.001$). In fact, both FMR1-KO (Fisher’s LSD test, $P < 0.05$) and WT mice (Fisher’s LSD test, $P < 0.01$) reared in enriched conditions showed a higher density of spines along apical dendrites than did their counterparts reared in standard cages (Fig. 3). Spine density along basal or oblique dendrites did not vary significantly according to the genotype or the rearing condition (data not shown).

Enrichment Rescues the Immature-Appearing Spine Morphology in FMR1-KO Mice. The observation that enrichment enhances spine density prompted us to assess whether the dendritic spines could have also undergone a morphological change. As shown in Fig. 4B, FMR1-KO mice reared in standard cages showed more immature and fewer mature-appearing spines than their WT counterparts ($\chi^2 = 85.32, \text{df} = 4, P < 0.0001; 1.478$ spines). Enrichment significantly decreased immature-appearing spines while promoting mature-appearing ones in KO mice (enriched vs. standard FMR1-KO mice, $\chi^2 = 58.55, \text{df} = 4, P < 0.001; 1.664$ spines). In fact, there was no...
difference in the level of maturation of spines between KO and WT mice reared in enriched environmental conditions (Fig. 4C).

The Rescue of the Behavioral and Neuronal Alterations Observed in FMR1-KO Mice Is GluR1-Dependent. To evaluate whether the partial rescue of immature morphology by enrichment was actually due to an increase in mRNA translation and/or stability of key molecules involved in synaptic events, we analyzed the GluR1 levels in the visual cortex of KO mice reared in standard or enriched conditions in parallel to their WT counterpart reared in the same conditions. A two-way ANOVA with genotype and enrichment as main factors showed a significant effect only for the rearing condition (F1,16 = 5.62, P < 0.05). This result indicates that GluR1 levels were significantly increased in mice exposed to enrichment. Subsequent pair-wise comparisons revealed, however, that this increase was significant (Fisher’s LSD test, P < 0.05) only for the KO mice (Fig. 5A).

Enrichment Does Not Affect FMRP Levels in WT Mice. In addition, we analyzed the effect of enrichment on the level of FMRP expression in the visual cortex of the WT mice. As shown in Fig. 5B, FMRP levels did not differ between mice reared in standard and enriched cages (t = 0.024, Fig. 5B).

Discussion

Hyperactivity is a constant feature of the FXS (8, 9). Here we show that independently from the rearing condition experienced during development, FMR1-KO mice crossed more sectors in the empty open field than did WT mice and that enrichment failed to reverse this KO-specific behavioral trait.

Another main feature of human patients is anxiety (2). Interestingly, we observed that FMR1-KO mice reared in standard cages consistently avoided the central sectors of the open field, unlike their counterparts reared in complex cages and unlike WT mice reared in either environmental condition. Thus, enrichment seems to decrease anxiety in FMR1-KO mice. It is worth remembering that an opposite tendency, i.e., a higher rate of permanence in the center of the field, has been reported in FMR1-KO mice in a FVB strain background (43). However, the retinal degeneration identified in the FVB strain as well as differences in testing procedures, namely in the size of the field and the duration of testing (10), may account for this discrepancy. Of importance, our findings show that FMR1-KO mice in a C57BL/6 background exhibit an anxiety-like phenotype, which is consistent with the human syndrome (2) and is fully rescued by enrichment.

Most studies dealing with the characterization of the cognitive profile of the FMR1-KO mice have assessed superior functions (8, 9, 37). How these mice simply explore a set of objects and whether they show habituation when repeatedly exposed to the same situation has, to our knowledge, never been examined. Because autistic behavior is also a feature of the fragile X patients (44) and because FMR1-KO mice show a decreased reactivity to external stimuli (37), we therefore expected abnormal interactions of KO mice with any element situated in their proximal environment. In fact, we observed that standard-reared FMR1-KO mice intensively explored the objects on the first session and failed to show habituation on repeated exposure. Interestingly, enrichment did not attenuate the initial intense object exploration of the objects but fully restored habituation.

In any species, exploration is the result of a complex balance between attention, curiosity, and neophobia, whereas habituation, i.e., a decreased reaction to external stimuli, presupposes a comparison between a current perception and previously stored representations of those stimuli. Exploration and habituation therefore
WT controls. Given that the morphology of pyramidal neurons in branching complexity did not vary between the KO mice and their authors reported that the amount of dendrite arbor and dendrite spines but did not find an increase in spine density. Moreover, these FVB generated in a different background. Using KO mice derived from abnormalities in spine morphology and number in this degeneration made it necessary to confirm the presence of these KO mice was undergoing a mutation-induced retinal degeneration with possible effects on cell morphology in the visual cortex. However, as mentioned above, the FVB strain used to generate them of alterations basically similar to that previously reported in FVB × 129J FMR1-KO mice. That is, gene silencing was not found to affect spine density in any part of the dendritic tree but strongly modified the proportion of mature- vs. immature- appearing spines counted on apical dendrite segments. In particular, using a standard categorization (12), we found fewer multiple head, stubby, and mushroom-structured spines and more thin and elongated ones in KO mice than in their WT littermates. The main finding of our study, however, is that the abnormal morphology of dendrites and dendritic spines observed in the KO mice was fully rescued by enrichment. These data therefore indicate that some aspects of the molecular machinery responsible for experience-dependent neuronal plasticity are preserved in FMR1-KO mice.

As for the morphology and the number of spines, we observed a pattern of alterations basically similar to that previously reported in FVB × 129J FMR1-KO mice (12). That is, gene silencing was not found to affect spine density in any part of the dendritic tree but strongly modified the proportion of mature- vs. immature-appearing spines counted on apical dendite segments. In particular, using a standard categorization (12), we found fewer multiple head, stubby, and mushroom-structured spines and more thin and elongated ones in KO mice than in their WT littermates. The main finding of our study, however, is that the abnormal morphology of dendrites and dendritic spines observed in the KO mice was fully rescued by enrichment. These data therefore indicate that some aspects of the molecular machinery responsible for experience-dependent neuronal plasticity are preserved in FMR1-KO mice.

The fact that, despite the rescue of FMRP deficiency-related neuronal abnormalities, other morphological indexes such as the length of oblique dendrites and the density of spines on apical dendrites were stimulated by enrichment in both genotypes raises
induces a rapid but transient expression of FMRP in the visual cortex. With this hypothesis, it has been shown that visual experience in adult mice already reared in a standard environment but that its expression returns to basal levels if the housing is maintained for a longer period. In agreement with this hypothesis, it has been shown that visual experience induces a rapid but transient expression of FMRP in the visual cortex of rats reared in a dark environment (48).

We can therefore assume that, in the C57BL/6 mouse strain, FMRP is important for normal development of dendrite length and complexity but not for experience-dependent enhancement of these parameters. Thus, possibly, some of the mechanisms whereby enrichment stimulates neuronal plasticity in both the KO mice and their WT controls are FMRP-independent.

In view of their role in the maturation of dendritic spines (49, 50) and the regulation of dendritic architecture (51), glutamatergic receptor expression is likely to be affected by both FMRP deficiency and enrichment. There is evidence that protein synthesis triggered by the type I metabotropic glutamate receptor (mGluR1) in brain synaptosomes (15) and expression of the AMPA receptor subunit 1 in the somatosensory cortex (19) are dramatically reduced in FMR1 KO mice. Enrichment, however, selectively increases AMPA activity (52) and gene expression of GluR1 (53) in the hippocampus. Based on these findings, we investigated the role of FMRP in promoting enrichment-dependent plasticity in children with FXS. Although further investigations are needed to elucidate the molecular mechanisms involved in the enrichment effects, our findings demonstrate that some mechanisms of neuronal plasticity are preserved in FMR1-KO mice and can be triggered by environmental stimulation.

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