Research report

Is fragile X mental retardation protein involved in activity-induced plasticity of dendritic spines?

Menahem Segal\textsuperscript{a}, Ute Kreher\textsuperscript{b,c}, Varda Greenberger\textsuperscript{a}, Katharina Braun\textsuperscript{b,c,*}

\textsuperscript{a}Department of Neurobiology, The Weizmann Institute, Rehovot 76100, Israel
\textsuperscript{b}Department of Zoology/Developmental Neurobiology, Otto von Guericke University Magdeburg, POB 1860, 39008 Magdeburg, Germany
\textsuperscript{c}Leibniz Institute for Neurobiology POB 1860, 39008 Magdeburg, Germany

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Abstract

Dendritic morphology of 2-week-old cultured neurons, taken from postnatal day 1 fragile X mental retardation gene1 knock out (FMR1\textsuperscript{2/2}) mice hippocampus, were compared with cells taken from wild type mice. Under control conditions the FMR1\textsuperscript{2/2} neurons displayed significantly lower spine densities compared to wild type neurons. Pharmacological stimulation of electrical activity, induced by bicuculline, caused a reduction in dendritic spine density in both the FMR1\textsuperscript{2/2} and the wild type cells. In both groups, bicuculline induced a significant shrinkage of spines that were occupied by one or more synaptophysin-immunoreactive presynaptic terminals. The concentration of FMR1 in the wild type cultures was not affected by bicuculline treatment. These experiments indicate that FMR1 is not likely to be an essential factor in activity-modulated morphological plasticity of dendritic spines in cultured hippocampal neurons.

\textsuperscript{1}Corresponding author. Tel.: +49-391-626-3617; fax: +49-391-626-3618.

\textsuperscript{E-mail} addresses: menahem.segal@weizmann.ac.il (M. Segal), katharina.braun@nat.uni-magdeburg.de (K. Braun).

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1. Introduction

The fragile-X mental retardation protein (FMRP), which is absent in Fragile-X mentally retarded patients [28], has been cloned and proposed to be a RNA-binding protein, involved in regulation of protein synthesis [11,30,21,22]. The inability to synthesize FMRP, usually due to an abnormally long CGG repeat in the promoter region leading to hypermethylation and silencing of the FMR1 gene, is the leading cause of genetically inherited mental retardation, which may be mediated by a defect in synaptic circuits within learning-relevant neuronal pathways. Indeed, the protein has been located at the base of dendritic spines, where protein synthesis takes place [36,35]. FMRP-knockout mice lack the gene, and have been shown to express abnormally immature spines on apparently normal cerebral cortical neurons [17]. The physiological and functional correlates of these abnormal spines are not known as yet. Being located near the spine, FMRP is proposed to be involved in processes of spine plasticity. FMRP has been shown to vary as a function of stimulation that can cause morphological plasticity in central neurons [17,16]. Thus, FMRP may affect some of many molecular entities and its absence may result in defective synaptic transmission and plasticity.

The in vivo observations suggest a continuing role of FMRP expression in adult synaptic plasticity and support the hypothesis that synaptic FMRP synthesis is regulated by environmental stimulation and may thus participate in synapse stabilization and elimination in both the developing and adult brain.

Our research on cultured neurons from FMR1\textsuperscript{2/2} mice [19] and corresponding controls is focused on the hippocampus, which shows the highest FMR1 expression among all regions in the human and fetal mouse brain [1],...
cortical regions display somewhat less FMR1 [15]. Thus, neurons in the hippocampus are expected to respond with measurable changes of FMRP during postnatal maturation and in response to experimentally induced neuronal activation. In an earlier study, we found that cultured neurons taken from FMR1−/− mice display delayed formation and maturation of network activity as compared to wild type control neurons [5]. The aim of the present study was to explore the functional role of FMRP in activity-induced plasticity of dendritic spines by comparing reactivity to bicuculline treatment between cultured hippocampal neurons of wild type, and FMR1−/− mice. The alkaloid bicuculline binds to GABA_A-receptors, thereby blocks inhibitory synapses and causes an elevation of network activity [25]. This pharmacologically induced network activation results in modifications of dendritic spine shape and density in cultured rat hippocampal neurons, and it was predicted that the lack of FMRP may impair such activity-driven synaptic plasticity.

2. Materials and methods

2.1. Tissue cultures

FVB strain mice, RD/RD, Fmr1 knockout (FMR1−/−) and their wild type (FMR1+/+) counterparts were used to produce cultured hippocampal neurons (same as in Ref. [5], courtesy of W. Greenough [16]). Mice were genotyped by PCR to confirm the lack of the Fmr1 gene, and housed in a local animal house. The results reported below were collected from five different litters, each experiment was made with FMR1+/+ and FMR1−/− newborn mice of both sexes, born and prepared for culture on the same day. Newborn mice were sacrificed by cervical dislocation and their brains carefully taken out to a chilled (4 °C) dissecting medium, which consisted of oxygenated Leibovitz L15 (Biological Industries, Beit Haemek, Israel) enriched with 0.6% glucose and gentamicin (Sigma, 20 mg/ml). Hippocampi were dissected out and collected in the same medium. Tissue was mechanically dissociated with a fire polished Pasteur pipette and passed to the plating medium, which consisted of oxygenated MEM, and no further changes were made. Medium was then changed to 10% HS in enriched MEM, and applied in a microdrop (diameter 2–4 mm) through a pressure pipette as detailed elsewhere [26]. After 5–8 h of incubation with the dye the neurons were visualized within the following 10 h in a Zeiss 510 confocal laser scanning microscope and 3D images of neuronal somata, dendrites and spines were taken. The cultures were coded, so that all steps, i.e. image acquisition and the quantification of neuronal parameters were performed in a double blind procedure. The neurons that were randomly selected for the analysis typically displayed a large and ramified dendritic tree and high densities of spines.

2.2. Double labeling of neuronal morphology

2.2.1. DiI and presynaptic terminals (synaptophysin)

After fixation in 4% paraformaldehyde for 20 min the cultures were incubated with a mouse antibody to the synaptic vesicle protein synaptophysin (monoclonal, Sigma), a marker for presynaptic terminals, diluted 1:200 in PBS pH 7.3 for 3 h, and then incubated with a secondary anti mouse antibody conjugated to the fluorescent molecule ALEXA 488 (Molecular Probes), diluted 1:200 in PBS for 45 min at room temperature. Single neurons were labeled under visual guidance with the fluorescent marker DiI prepared at saturated solution in oil and applied in a microdrop (diameter 2–4 μm) through a pressure pipette as detailed elsewhere [26]. After 5–8 h of incubation with the dye the neurons were visualized within the following 10 h in a Zeiss 510 confocal laser scanning microscope and 3D images of neuronal somata, dendrites and spines were taken. The cultures were coded, so that all steps, i.e. image acquisition and the quantification of neuronal parameters were performed in a double blind procedure. The neurons that were randomly selected for the analysis typically displayed a large and ramified dendritic tree and high densities of spines.

2.3. Image acquisition and quantitative morphological analysis

Scans of spiny dendrites were taken with a 40×, 1.4 NA objective at a resolution of 512×512 pixels in 0.5-μm z-steps. From each neuron a low power image (Fig. 1A) was initially taken, which, depending on the depth of the culture and extent of the dendritic trees, consisted of stacks of 12–30 images. These low power image stacks were used for the measurement of soma size and dendritic length. From each neuron between three and seven secondary dendrites, starting proximally from the soma, were imaged at higher resolution (100×, 1.4 NA objective, zoomed 5×). These image stacks were used for the quantification of spine densities and detection of presynaptic labeling with synaptophysin antibodies (Fig. 1B,C). In cases where it was difficult to determine a close contact of synaptophysin-labeled puncta with a postsynaptic structure (spine or shaft), orthogonal optical sections through the image stacks revealed a clearer view (see arrows in Fig. 1B). The analysis of spine density and length was conducted in a double-blind procedure.

2.4. Western blot identification of FMRP

Cultures were grown on 12-well plates, to allow for harvesting of more tissue for the western analysis. They were otherwise treated like the other cultures. Cells were
Fig. 1. (A) Composite image of a confocal image stack displaying the morphology of a cultured Dil-labelled hippocampal neuron from wild type (FMR1+/+) mice. (B) Composite image of a confocal image stack illustrating double labeling of dendrites and spines (red) and synaptophysin-immunoreactive presynaptic terminals (green). For better visualization of the contacts between the respective pre- and postsynaptic structures in the $\alpha$-axis we used orthogonal optical planes (blue and purple lines), the image for the blue line is displayed on top of the image and for the purple line on the far right. The majority of spines are occupied by one or more synaptophysin-immunoreactive boutons which are located on their head or neck (white arrows), but 'empty' spines, lacking a synaptophysin-immunoreactive presynaptic structure, are also found. Synaptophysin-immunoreactive structures located directly on the dendrite were defined as shaft synapses (blue arrows).
washed in isotonic Hepes buffer (2×), followed by extraction in SDS sample buffer, boiled, assayed for protein content, and stored frozen. Samples were run on 10% SDS–PAGE gels followed by routine western blotting. The FMRP band was identified using the IC3 mouse monoclonal antibody (1:2000, Courtesy of A.T. Hoogeveen, as in Ref. [31]). Detection was made using chemiluminescence (ECL kit, Amersham). Films were scanned and quantified using NIH Image software.

2.5. Statistical analysis

The results for FMR1+/+ and FMR1−/− neurons were compared for each of the tested parameters (soma size, dendritic length, spine size and density) using two-way ANOVA followed by Tukey’s comparisons and pairwise comparisons by Student’s t-test. The significance level was set at \( P \leq 0.05 \). Means ± S.E.M. are presented except when specified. For spine density \( n \) = the number of cells.

3. Results

Detailed morphometric analysis was conducted with a total of 17 wild type (FMR1+/+) and 12 FMR1−/− untreated neurons, and 13 FMR1+/+ and 9 FMR1−/− cells exposed to bicuculline in four different experiments. Additional cells were analyzed for measurement of soma size as well as total dendritic length per cell. Two-way ANOVA for the spine frequency parameter revealed a significant difference between untreated FMR1+/+ and FMR1−/− neurons. Spine density was significantly lower, by 22%, in the untreated FMR1−/− cells compared to age matched controls FMR1+/+ cells (Fig. 2A). Furthermore the ANOVA revealed an influence of the bicuculline treatment, but no statistically significant inter-
action between mouse type and pharmacological treatment was found. Pairwise comparison using Tukey and Student’s t-test revealed a significant reduction in spine density in the bicuculline-treated FMR1+/+ (−29%) and the FMR1−/− (−39%) cells (Fig. 2A). Two-way ANOVA for the parameters soma size and total dendritic length revealed no significant differences between FMR1+/+ and FMR1−/− neurons under control conditions. A significant increase of soma size after bicuculline treatment (FMR1+/+ (+25%) and FMR1−/− (+32%)) neurons was found, but no effect of bicuculline treatment was seen for the parameter dendritic length (Fig. 2B,C).

The length of spines and their occupancy by presynaptic terminals, labeled by synaptophysin immunoreactivity (Fig. 2D) were analyzed in a sample of 253–776 spines for both FMR1+/+ and FMR1−/−, control conditions and following the bicuculline treatment. Two-way ANOVA revealed no significant difference between FMR1+/+ and the FMR1−/− cells in the length of spines, nor in the proportion of spine occupancy by synaptophysin puncta (Fig. 2D). No interaction between pharmacological treatment and mouse type was found. Taking all spines, empty or occupied by synaptophysin-immunolabeled puncta together, the pair-wise comparison revealed that bicuculline treatment induced a significant shrinkage of the spine length, reaching 12% reduction in the FMR1+/+ cells and 16% in the FMR1−/− cells (Fig. 2D). While for the double and triple occupied spines the bicuculline-induced length reduction was more pronounced in the FMR1−/− cells, the single occupied spines shrank more in the FMR1+/+ cells, whereas the shrinkage of the ‘empty’ spines was evident, but not significant for both mouse types (Fig. 2D).

Under normal control conditions the number of shaft synapses (estimated by their presynaptic immunoreactivity to synaptophysin) did not differ between FMR1+/+ and FMR1−/− neurons (FMR1+/+: 2.0±0.6 shaft synapses/10 μm dendritic length and FMR1−/−: 2.4±0.9 shaft synapses/10 μm dendritic length; ±S.D.). Bicuculline treatment had no effect on the number of shaft synapses in FMR1+/+ (+bic: 2.4±0.7 shaft synapses/10 μm dendritic length) and FMR1−/− neurons (+bic: 2.5±0.9 shaft synapses/10 μm dendritic length).

FMR1 expression in relation to activity changes was studied using either immunocytochemistry or western blotting. The former method did not yield, in our hands, a satisfactorily selective staining of FMRP that is confined to the FMR1+/+ cells (data not shown), thus we resorted to western blotting.

Western blottings were assayed in three separate experiments (Fig. 3). Indeed, cells taken from hippocampal cultures from FMR1−/− mice lacked a specific, ~86 kDa protein which was detected by the monoclonal antibody IC3 in tissue from wild type cultures. In the same gel, though, there were other proteins (e.g. of 52 kDa) reacting with this antibody in both FMR1+/+ and FMR1−/− tissue. These additional immunoreactive bands, which were also observed using other FMRP antibodies (unpublished observations), may explain the non-selectivity of the immunocytochemical staining in our hands, and perhaps in others as well. Exposure to bicuculline for 24 h did not modify the intensity of staining of the FMR1 selective bands in the wild type mice in three experiments tested.

4. Discussion

The present study has failed to demonstrate an involvement of FMR1 protein in activity-dependent synaptic plasticity in cultured hippocampal neurons. Neurons taken from FMR1−/− mice, which under unstimulated control conditions display lower spine densities than neurons from FMR1+/+ mice, reduced their spine densities dramatically in response to bicuculline stimulation, quite similar to the FMR1+/+ neurons. Furthermore, the pharmacologically induced shrinkage of spines was seen in both, FMR1−/− and FMR1+/+ neurons, for the entire spine population at similar magnitudes. Finally, there was no significant change in the concentration of FMR1 protein in the FMR1+/+ cultures following drug stimulation, indicating (i) that its synthesis is not altered by enhanced neuronal activity and (ii) that the protein concentrations in the FMR1+/+ tissue do not reflect the reduced spine density, at least not in a detectable amount for this preparation. Thus, the results in this in vitro network are not consistent with the proposed role of FMR1 in neuronal plasticity related to the formation or pruning of spines [35,7,14]. On one hand, this may indicate that cultured neurons are not an effective model system for the study of
the role of FMR1 in synaptic interaction and plasticity. On the other hand, however, in our previous study [5] we did find a developmental difference in maturation of these in vitro networks of neurons, and our previous observations of decreased densities of dendritic spines in the FMR1−/− neurons was confirmed in the present study. In contrast to these cultured hippocampal neurons, cortical neurons do appear to display elevated spine density in FMR1−/− mice [17,16,24]. However, such differences were not observed in vitro in neocortical organotypic cultures [24].

Even in vivo, the role of FMRP in regulation of neuronal plasticity has not been convincingly documented. Behavioral studies on different strains of FMR1−/− mice cast doubt that FMR1 gene product has a significant role in cognitive functions of the brain. In fact, FMR1−/− mice display only mild [8], if any [27,34] deficits in spatial memory or discrimination tasks [12], and their defects, if any, may be strain-dependent [9], indicating that the lack of FMR1, by itself, may not be the cause of mental retardation. Moreover, the ‘cognitive’ deficits seen in the FMR1−/− mouse strains may, at least to some extent be confounded by sensory defects or other abnormalities observed in these mice [23,6]. MRI studies on FMR1−/− mice revealed no anatomical differences that were comparable to those described for human fragile X patients [18].

Similarly, even in human patients the correlation between cognitive and behavioral capacities, FMRP expression and brain morphology and physiology is not clear and straightforward. For instance, relative to normal individuals, subjects suffering from fragile X syndrome displayed cognitive deficits as well as altered activation patterns in frontal and parietal cortical areas [20]. In subjects with fragile X syndrome, significant correlations were found between FMRP expression and activation during a cognitive task in the right inferior and bilateral middle frontal gyri and the bilateral supramarginal gyri. On the other hand, IQ scores were not significantly correlated with total brain or cortical and subcortical grey matter volumes in fragile X children [10], and autistic behavior in male fragile X boys was not related to FMRP expression, i.e. no autistic behavior×FMRP interaction was found [3]. The correlation between FMRP expression and cognitive, social and motor capacities was highly variable and mostly not significant, suggesting that factors other than FMRP may play a major role [2,4].

Studies on cellular models of plasticity have not yielded conclusive results with respect to the role of FMRP in neuronal plasticity. Thus, FMR1−/− mice do not express impaired LTP, compared to controls [13,27] and there is no clear evidence for an involvement of FMRP in the biochemical consequences of activation of the brain, as in LTP [33]. However, epileptic seizure causes an elevation of FMR1 mRNA at 6 h [33], but not at 2.5 h after the seizure [13]. These results are not consistent with those of another study, showing that sensory stimulation does cause a significant, time dependent increase in FMR1 expression in rat somatosensory cortex [32], but a stronger stimulation (seizures resulting from injection of kainic acid) caused, if anything, a reduction in levels of FMR1 expression. These discrepancies, as well as our lack of effects of FMR1 on bicuculline-induced spine plasticity, may reflect a time-dependent process, in that an initial increase in FMR1 expression may switch to a longer term reduction in its expression.

At the molecular level, an inhibitory role of FMRP on translation [21], which appears to be mediated via interaction with mRNA [30,22] has been found, however no apparent difference in the distribution and dendritic targeting of mRNA in response to stimulation has been detected between control and FMR1−/− mice [29].

Finally, the difference between the cellular response of rat and mouse cultured neurons to bicuculline is not entirely understood. In a previous study [26], we found that bicuculline causes a 25–60% increase in spine density in cultured rat neurons, whereas in the present study on mouse neurons we find a reduction in spine density. Mouse neurons in culture seem to have a higher initial spine density than their rat counterparts [5], which may reflect a higher complexity of the in vitro network and may indicate differences in spontaneous activity or intrinsic properties of the individual neurons. Their reactivity to bicuculline may reflect their initial spine density, such that when spine density is high, excessive stimulation will reduce it, and vice versa. As bicuculline did not affect the density of shaft synapses, the observed spine reduction does not reflect a transition from spines to shaft synapses but may indicate a true loss of synaptic connections located on spines. Since the rules governing spine formation and pruning are only beginning to emerge, further studies are needed to elucidate the role of FMRP and other proteins in synaptic plasticity.

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References


